Title

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Publication Date

2023

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

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A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Alisa Victoria Voll

2023

ABSTRACT OF THE THESIS

The Role of Kappa Opioid Receptors on the Serotonergic Neurons in the Dorsal Raphe Nucleus in the Dysphoric Component of Chronic Pain

by

Alisa Victoria Voll

Master of Science in Physiological Science

University of California, Los Angeles, 2023

Professor David L. Glanzman, Chair

Chronic pain has both a sensory and an affective component. The links between chronic pain and affective disorders such as depression have been widely shown, with up to 85% of those with chronic pain also suffering from severe depression. When this comorbidity exists, the prognosis is worse, chronic pain is increased, and it is less responsive to treatment. Between 2003 and 2014, the percentage of people who committed suicide who also had chronic pain rose

by almost 3 percentage points. The kappa opioid receptor, KOR, has been implicated in the link between chronic pain and depression. The expression and function of KOR and its ligand, dynorphin, are both enhanced in chronic pain. Conditioned place aversion (CPA), a measure of aversion to a paired stimulus, is enhanced in chronic pain states compared to non-pathological states, and is driven by KORs. It has been shown that ablating or inhibiting KORs on all serotonergic neurons throughout the central nervous system is sufficient to block this KOR mediated place aversion. We hypothesized that KOR on serotonergic neurons projecting from the dorsal raphe nucleus (DRN) is responsible for the enhanced KOR agonist-induced aversion in chronic pain states. To test this, we ablated KOR from serotonergic neurons in the DRN and assessed the effect on the development of aversion using the conditioned place preference/aversion (CPP/A) paradigm. We confirmed that U50,488, a small molecule KOR agonist, produces enhanced CPA in chronic pain mice. The chronic constriction injury used in our chronic pain model increased mechanical responsiveness, suggesting the presence of mechanical allodynia. Ablating KOR on the serotonergic neurons in the DRN blocked CPA, suggesting that KOR on serotonergic neurons in the DRN may indeed be responsible for agonist-induced CPA. We also found that U50,488-mediated analgesia, originating from KOR in the spine, is intact in animals that lack KOR in serotonergic neurons in the DRN, showing that KOR analgesia derived from spinal KOR remains intact.

The thesis of Alisa Victoria Voll is approved.

Gregory S. Payne

Gina Rochelle Poe

David L. Glanzman, Committee Chair

University of California, Los Angeles

2023

For my family

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Introduction

The percentage of people who committed suicide who also had chronic pain rose by almost three percentage points between 2003 and 2014 (Petrosky, 2018). Major depressive disorder, linked to suicide, is the leading cause of disability in the U.S. for those ages 15 to 44. Both depression and chronic pain are quite destructive on their own, and in conjunction that is further exacerbated. More than 8% of the US population has depression at any given time, and those suffering from depression caused by chronic pain have a worse prognosis than those with chronic pain alone. They also have more chronic pain than those without depression, and the chronic pain is more difficult to treat. Given the prevalence of depression in chronic pain patients, it is important to understand mechanisms driving negative affect in chronic pain.

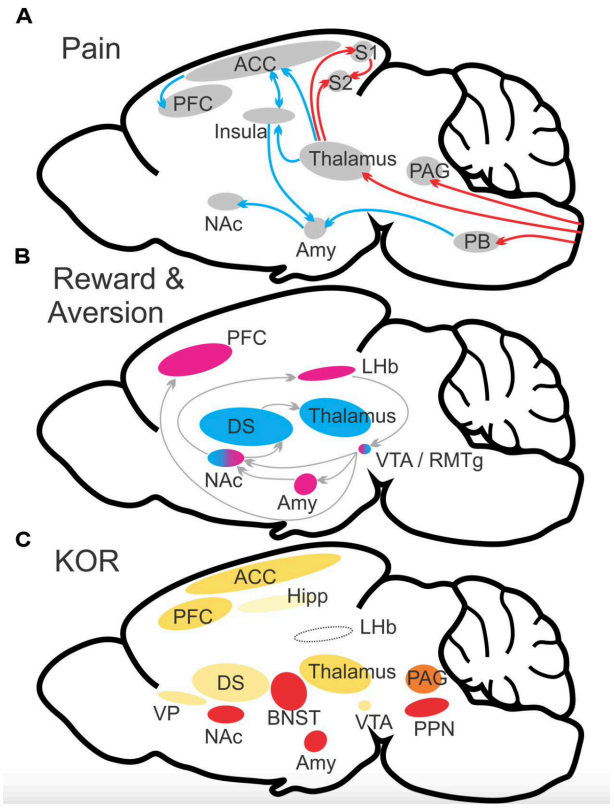
The transition of acute pain into chronic pain can have some important outcomes. In a 2019 study by the NIH, chronic pain was strongly associated with an increased risk of disability after controlling for other chronic health conditions. Additionally, it has been shown that chronic pain can frequently induce depression in someone who was not depressed before. These 2 illnesses—chronic pain and depression—often cooccur. In fact, up to 85% of those with chronic pain also have severe depression.

Pain has two components - the sensory component, and the emotional, or affective, component. The affective component of pain is considered to be the part of pain that makes it feel unpleasant or bothersome, and is also the part that is thought to define quality of life for those with chronic pain. In fact, there are some pain medications with which patients report they can still feel some of the pain, but it no longer bothers them, effectively reducing the experience of pain. This implies that in chronic pain, unless there is total eradication of nociception, what is actually being treated is the emotional component of pain, rather than the sensory.

Depression has many components, including affect, and it has been shown that reward and aversion play a role in affective states. There is extensive overlap in the reward/aversion, and pain circuits. The thalamus, for example, is implicated in both components of pain, as well as reward. The nucleus accumbens is involved in reward, aversion, and the affective component of pain.

In the recent decades, the kappa opioid receptor, KOR, has immerged as a common and key element and in both the reward/aversion and pain circuits (Figure 1), drawing increasing interest of the scientific community, including investigation into possible applications for treatments of chronic pain, depression, and opioid use disorder (OUD).

Figure 1



(Cahill, 2014)

Figure 1: Overlapping Circuitry of Pain, Reward and Aversion, and KOR

Mouse brain schematics of pain (A), and reward/aversion circuits (B) showing extensive overlap. (A) Red arrows indicate sensory pathways, blue indicating affective ones. (B) Pink ovals indicate parts of the brain involved in aversion, and blue the parts involved in reward. (C) KOR is implicated in both circuits. A heat map shows the locations of KOR, with red being higher concentrations and yellow lower concentrations.

Chronic Pain Causes Physiological Changes

The Cahill lab confirmed previous studies that showed that pain has to persist for some time for KOR and dynorphin expression and function to increase. In other words, chronic pain causes these physiological changes, but acute pain does not. They also found that when a kappa agonist is administered, it causes an aversion, and most importantly, that aversion is enhanced in chronic pain animals over non-pain animals. This enhanced aversion in chronic pain was only seen in males, but not in females.

The Search for the Origination of KOR Mediated Aversion

It has been assumed in the field that kappa agonist mediated conditioned place aversion is due to a decrease in dopamine release in the nucleus accumbens due to the receptor's inhibitory effect on dopaminergic neurons (Shippenberg et al., 1993). However, a 2015 Chavkin lab paper showed that kappa aversion can be mediated by both serotonergic and dopaminergic neurons. They found that ablation of KOR within all serotonergic neurons was sufficient to block kappa agonist mediated place aversion. While these findings put into question the dopamine hypothesis, and point to serotonergic neurons as possible mediators, these studies were not done in chronic pain animals. Thus, it is unclear whether and how the increase in KOR and dynorphin that are present in chronic pain states would impact these results.

There is enhanced KOR expression and function in chronic pain, enhanced aversion in chronic pain driven by KOR, and ablating KOR on serotonergic neurons being sufficient to

block KOR agonist mediated place aversion. Given this, three questions arise: Where are the kappa receptors in the brain that are causing the enhanced kappa induced place aversion in chronic pain states? Which part of the brain is that signal originating from? What other circuits does it engage, if any?

The research described here investigated serotonergic neurons in the dorsal raphe nucleus (DRN) as a source of KOR agonist induced conditioned place preference. The DRN has the highest concentration of serotonergic neurons in the brain. There are dopaminergic neurons that have only recently been found to exist in the DRN, as well. In 2020, the Kaneko lab found that stimulation of serotonergic neurons in the DRN and their projections to the ventral tegmental area (VTA) increased conditioned place preference, and that inhibition of DRN 5-HT neurons and their projections to VTA decreased conditioned place preference. This, taken with that it has also been shown that either ablating KOR or inhibiting it with an antagonist blocks the aversion that kappa agonist usually causes, could mean that KOR's inhibition of the serotonergic neurons in the DRN is what is responsible for the enhanced conditioned place aversion in chronic pain states.

My master's research project tested the hypothesis that KOR on serotonergic neurons projecting from DRN is responsible for the enhanced KOR agonist-induced aversion in chronic pain states. This was done by ablating KOR from serotonergic neurons in DRN, known to be implicated in KOR induced place aversion, and measuring the aversion with the conditioned place aversion/preference (CPA/P) behavioral test on male and female mice in a chronic pain state.

Materials and Methods

Animals

Male and female mice, C57BL/6J wild-type, were either purchased from Jackson Laboratories and received, or bred and transferred to the lab's vivarium at 8 weeks of age. Different male and female mice engineered to be able to have KOR knocked out on serotonergic neurons were also purchased or bred, and transferred to the lab's vivarium at 8 weeks of age. The vivarium was kept on a 12 h reverse light/dark cycle. Mice were habituated to the vivarium for at least a week after arrival, then handled for at least 3 days before beginning any procedure.

Surgeries

Prior to all surgeries, all animals received 100 ul of liquid children's acetaminophen (160 mg/ml) via mouth. All surgeries were performed under anesthesia, using gaseous isoflurane, 2-4% in oxygen depending on the mouse. Acetaminophen in peanut butter was given in the cage after the surgery and again the day after.

Pain Model - Chronic Constriction Injury

To study the KOR's role in negative affective states in chronic pain, I used the chronic constriction injury (CCI) pain model. Here, I performed cuffing of the sciatic nerve, placing a 2 mm polyethylene tube on the sciatic nerve of the left hind paw. This induced a chronic pain state in the mice after approximately a week.

During cuffing, a 1 cm incision was made in the upper left thigh, cutting through the muscle. Then, carefully separating the tissue with surgical tools, the sciatic nerve was isolated. A slit was cut along the side of the polyethylene cuff and the two sides of the cuff were slightly pulled apart so they could by placed around the nerve. Once the cuff was in place, the tubbing was pinched back together slightly with tweezers. To ensure that the cuff was not pinching the nerve, I checked that it could be slid back and forth along the nerve without obstruction. The nerve was then pushed back in place and the incision sutured.

Von Frey

One effect of chronic pain is the development of allodynia, the experience of pain in response to normally non-pain inducing stimuli. The von Frey test was used to measure the onset of, and changes in, chronic pain as represented by mechanical allodynia.

Mice were poked 10 times in the center of their left hind paw with a 2-g von Frey filament, usually a non-painful stimulus, and the number of times the mouse withdrew their paw was recorded. The von Frey test was performed before the CCI surgery to get a baseline response, and post-healing after the surgery, to assess the development and extent of allodynia.

KOR Knockout

KOR knockout mice were used to test the affect of the absence of KORs on the serotonergic neurons of the DRN. To knock out the KOR on serotonergic neurons, we used the Cre-lox system. In this approach, specific sequences, called lox P sites, are inserted on either side of the KOR gene, which ultimately allows that gene to be deleted. The two lox P sites are recognized by a Cre recombinase, introduced to the mouse on an adenovirus plasmid (AAV),

expressed under the control of the tryptophan hydroxylase promotor. This enzyme is responsible for the initial steps of the catalysis of serotonin, and is only present in neurons that secrete serotonin. Once the virus is introduced, the Cre is expressed in serotonergic neurons, and excises the lox P flanked KOR gene.

Virus injection surgeries were performed on transgenic male and female mice. The surgeries began with anesthetizing the mouse and securing it in a stereotaxic set up, making a small incision in the skin over the skull, and finding the coordinates of the DRN in relation to bregma. Because the DRN is directly along the midline, it was necessary to drill only one small hole through the skull. The needle containing the virus, a 2/9 AAV rAAV TPH2 Cre WPRE hGHpA, was inserted into the brain to the necessary depth, the virus injected at 100 nl/min for 4 min into the DRN, after which the needle was left stationary for an additional 5 min. The needle was extracted slowly, and the edges of the surgical incision in the scalp sutured. It takes at least 4 weeks for the KORs to be eliminated post-surgery. Further experiments were done from 2-4 months after the virus injection, depending on the cohort. Sham surgeries were performed in the same way except for drilling and virus injection.

Behavioral tests

All tests were run separately for male and female mice.

Conditioned Place Aversion/Preference

Dr. Cahill's previous work showed that the kappa agonist, U50,488, causes place aversion in mice, and that this effect is enhanced in animals with chronic pain (Liu, 2019). To test whether knocking out KORs from serotonergic neurons in the DRN prevents place aversion,

the conditioned place preference/aversion (CPP/A) behavioral test was performed. For this study, KOR knockout mice were split into 2 groups, one with chronic pain (CCI surgery), and one without (CCI sham).

Both groups were first habituated to the two chambers of the CPP box, being allowed free access to both sides for 15 min. The next day, the mice were again given access to both chambers this time for 30 min; during this period the mice were filmed to be able to determine how long they spent in each chamber before and after conditioning. After the initial post-habituation test of the length of time the mice spent in the experimental chamber, the chronic pain induction surgeries were performed and the mice allowed to recover for 6 d. After this recovery period, the mice were given 6 d of conditioning during which each mouse learned to associate one of the two chambers in the CPP box with the kappa agonist U50,488. Each mouse received alternating days of an intraperitoneal injection of either U50,488 or saline; after each injection the mouse was placed in the closed-off corresponding assigned chamber.

The day after the last day of conditioning, a post-conditioning test was run; during this test a mouse did not receive an injection of either drug or saline, and was allowed free access to both chambers of the box for 30 min while being filmed. The day or few days after the post-conditioning test, the state-dependent test was run. During this test, all mice received an injection of U50,488 and were then given free access to both chambers for 30 min, while being filmed. This test is termed state-dependent because we are observing behavior (the dependent variable), that is determined by the state caused by the independent variable, in this case, the kappa agonist U50,488. The recordings were analyzed by EthoVision, a behavior tracking software, for the number of seconds the mice spent in each chamber. CPP scores were calculated using the following formula:

CPP score = [Time in Test(paired) - Time in Test(unpaired)] - [Time at baseline- (paired) - time at baseline(unpaired)]

post-conditioning pre-conditioning

Tail Flick Test

Because there were negative data in the conditioned place preference tests, we wanted to conduct a test that could be used as a positive control. To ensure that the KOR knock out in the serotonergic neurons in the DRN was not altering other kappa-mediated effects, we ran the tail flick test. This tests kappa-induced analgesia through its binding to spinal receptors.

A baseline tail flick response was determined by holding a mouse's tail in 51 C° water and timing how long it took for the mouse to flick its tail up. U50,488 was then injected intraperitonially into the mouse and tail flick response times were tested at 20 and 40 min post-injection.

Results and Discussion

Proof of Principle

Figure 2

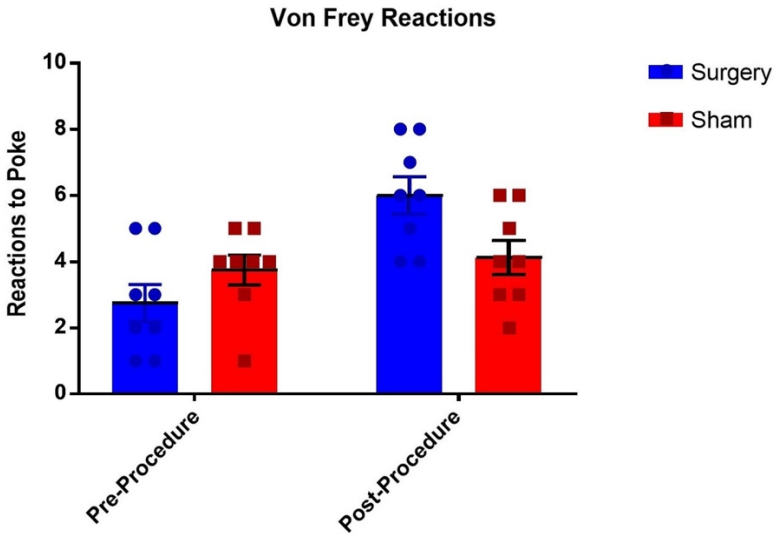


Figure 2: Von Frey tests demonstrate CCI surgery induces allodynia. A cohort of 16 mice were subjected to the von Frey pre-surgery (either CCI or sham) and again at least 6 days after. The bar height indicates the mean and the error bars indicate standard error from the mean (SEM).

In order to test our CCI chronic pain model, von Frey was performed on wild type mice about 7 days after the CCI surgery. Figure 1 illustrates that for the pain free mice (sham), in red, the responses post-surgery did not increase, while for the chronic pain mice, in blue, the responses increased significantly after the cuff surgery. These von Frey data confirm that the cuff surgery did induce allodynia, and chronic pain, and thus this pain model can be used for our studies.

Figure 3

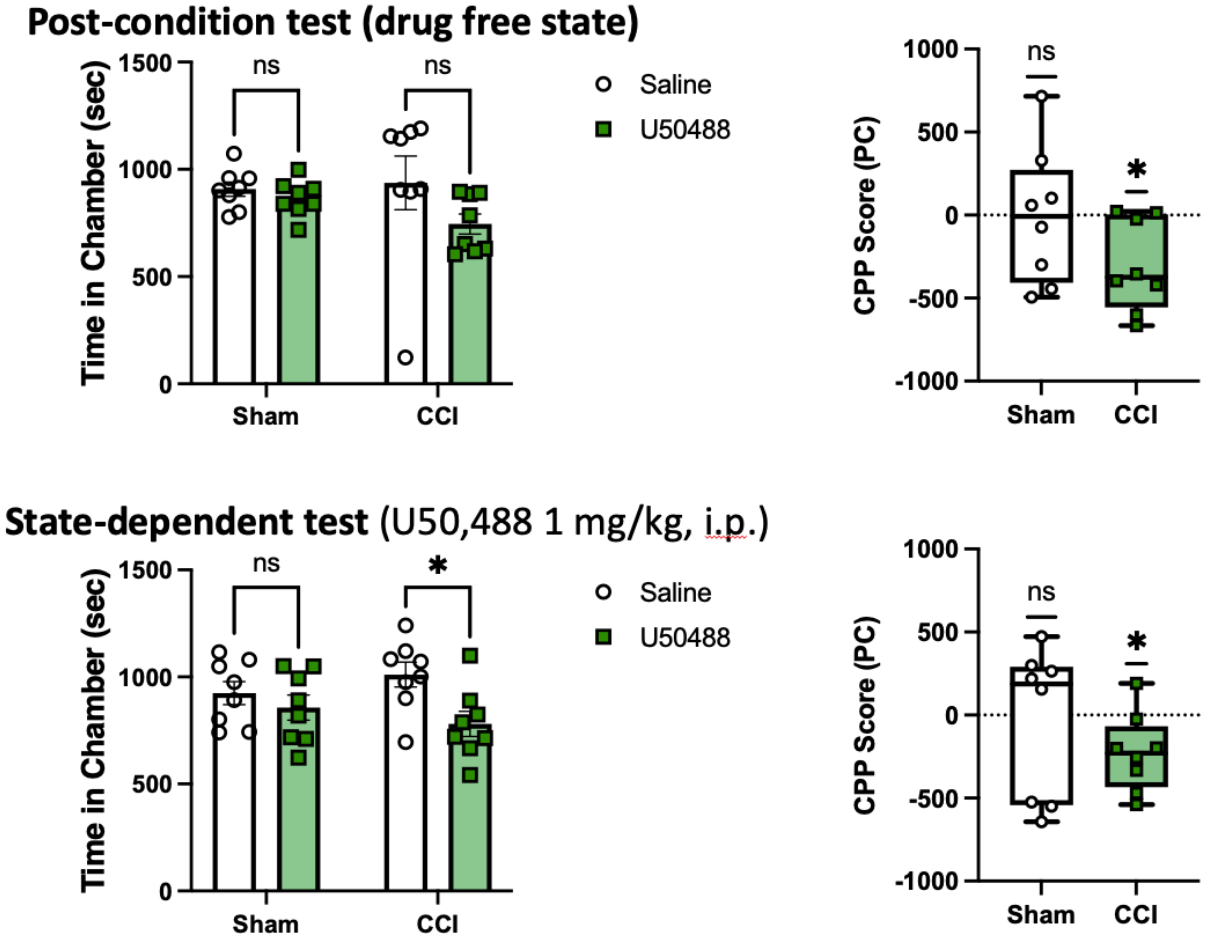


Figure 3: Conditioned Place Preference shows enhanced kappa agonist aversion in wild type chronic pain animals. CPP was performed with 16 mice (8 pain, 8 sham), using a low concentration of U50,488 (1mg/kg) for conditioning to more easily be able to see the enhanced effect in the chronic pain animals. The chronic pain animals developed an aversion to the drug chamber, while the non-pain animals did not. The error bars indicate standard error from the mean (SEM).

After von Frey, we performed the conditioned place preference behavioral test. From work previously done in the Cahill lab, it is known that kappa agonist concentration of 1mg/kl does not produce aversion in non-pain animals. Thus, we performed the chronic constriction surgery on half of a cohort of wildtype mice (without the KOR knockout), and ran them through the CPP protocol, using that low concentration of U50,488 for conditioning to more easily be

able to see the enhanced effect in the chronic pain animals. There were 16 mice total, 8 pain and 8 sham, or control, mice.

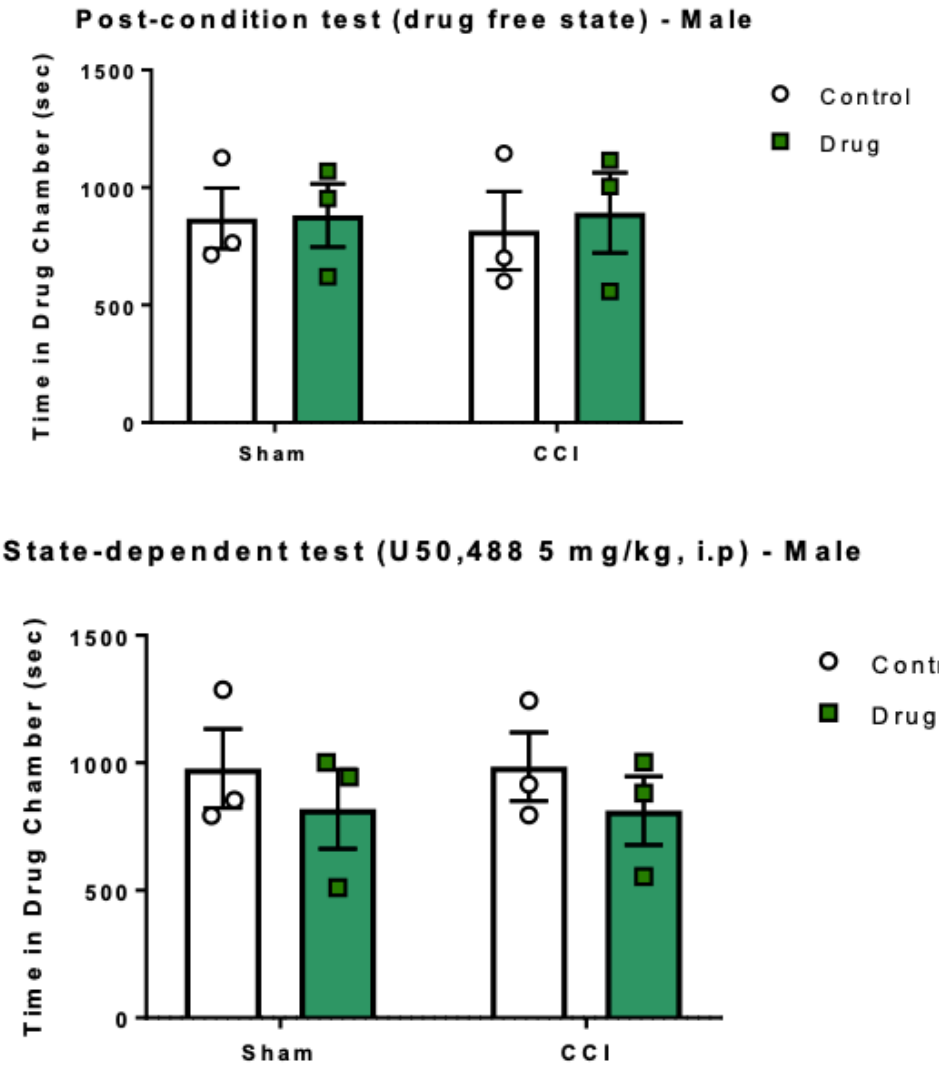
Figure 2 are the results of the post-condition and state dependent tests, with the results plotted against either time in either the agonist or vehicle chamber, or against the CPP score.

$$\text{CPP score} = \underbrace{[\text{Time in Test(paired)} - \text{Time in Test(unpaired)}]}_{\text{post-conditioning}} - \underbrace{[\text{Time at baseline- (paired)} - \text{time at baseline(unpaired)}]}_{\text{pre-conditioning}}$$

The CPP score incorporates the pre-conditioning time spent in both chambers, and compares it to the post. CPP scores are therefore more nuanced representations of the organism's learned aversion or preference. Post conditioning data shows that the chronic pain animals spent less time in the agonist chamber than the vehicle chamber, and the sham, spent an equal amount of time in either chamber both with and without the agonist. Thus, the chronic pain animals developed an aversion to the drug chamber, while the non-pain animals were unaffected. The CPP score confirms this. The pain animals had significant negative scores, indicating they developed an aversion, while the control animals' scores overall remained close to 0. The state dependent test, in which the agonist is administered to all mice, shows the same result as above. However, the difference in the time spent in the agonist vs. the saline chamber was significant for the pain animals, as was the CPP score. Overall, the pain mice responded to the agonist as expected with this pain model.

Figure 4:

A


Post-condition test (drug free state) - Male
Time in Drug Chamber (sec)
1500
1000
500
0
Sham
CCI
Control
Drug
State-dependent test (U50,488 5 mg/kg, i.p) - Male
Time in Drug Chamber (sec)
1500
1000
500
0
Sham
CCI
Control
Drug

B

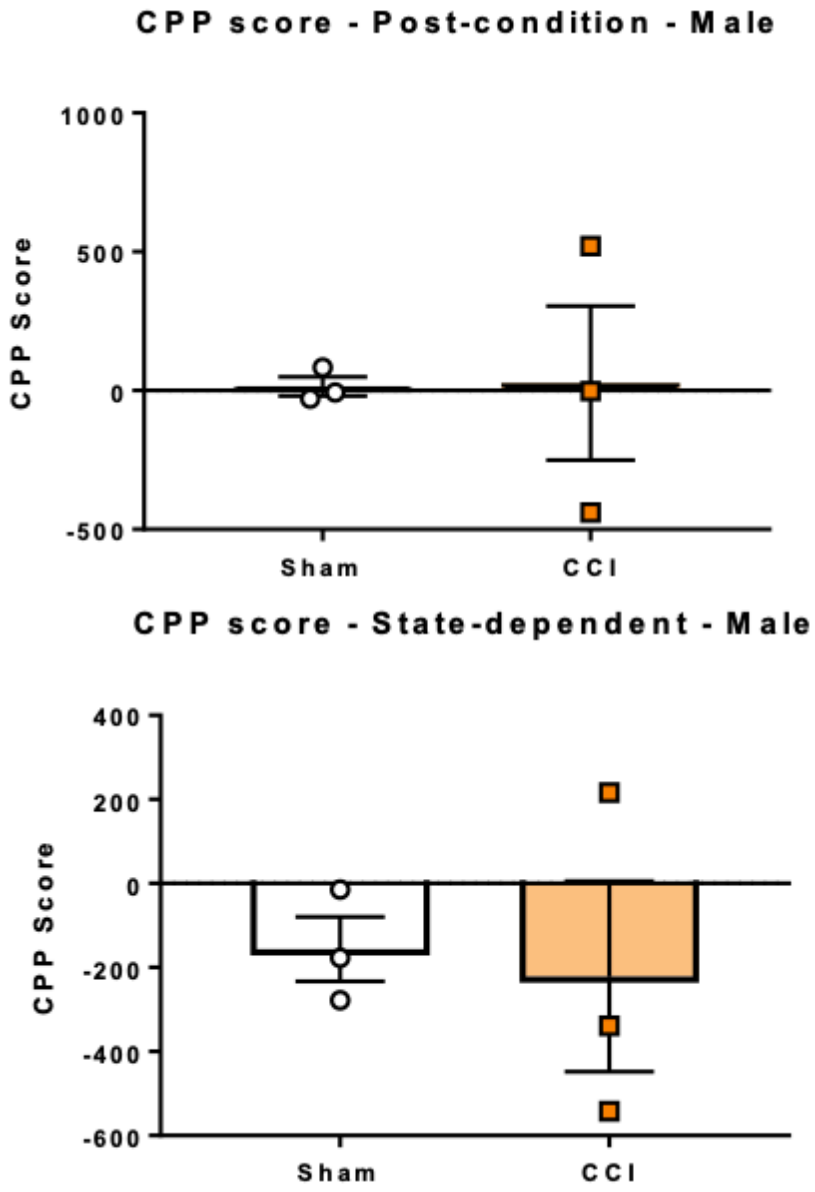


Figure 4: Kappa agonist aversion in male KOR knockout mice present in state-dependent test, but not in post-condition test, with increased agonist concentration. CPP was performed with 3 pain and 3 sham mice, using 5mg/kg of kappa agonist, a dose that should induce kappa mediated aversion in both chronic pain and non-pain animals. Time (A) and CPP scores (B) indicated aversion in the state-dependent test. This suggested that knocking out KOR in the serotonergic neurons in the DRN did not prevent the aversion. The bar height indicates the mean and the error bars indicate standard error from the mean (SEM).

Once it was confirmed that the pain model worked, I performed surgeries to inject the adenovirus to knockout KOR on the serotonergic neurons in the DRN in the first cohort of mice, male and female. Both sexes were tested to see if the results would match previously observed sex differences (Liu, 2019). The consequent CPP was performed using an increased dose of kappa agonist of 5mg/kg. This dose was expected to induce kappa mediated aversion in non-pain animals, as well as chronic pain animals (Liu, 2019).

Figure 3 shows that in both post-condition and state-dependent tests, sham mice spent about equal time in the drug and vehicle chambers, a little less in the drug for state dependent, but the difference was not significant. For the pain mice in post conditioning, it also looked like they spent about equal time in the drug and vehicle chambers, a little less in the drug for state dependent, but the difference was not significant. Figure 4 shows that the CPP score also did not show an aversion.

These first CPP results were encouraging as they showed that there was no conditioned place aversion in the pain, CCI group. This implies that knocking out KOR on serotonergic neurons in the DRN does block the aversion, per our hypothesis, which could also mean that blocking the kappa opioid receptor in the dorsal raphe nucleus could diminish or prevent the emotional component of pain that chronic pain patients struggle with.

However, in this preliminary study, there was no positive control—there were no mice without the knockout. These control mice would have also gotten virus surgeries, but the control virus would not knockout KOR, or have a circuit-altering effect. Time and CPP scores seemed to indicate that aversion was not inhibited in the state-dependent test, which would seem to suggest that knocking out KOR in the serotonergic neurons in the DRN did not prevent the aversion.

However, in addition to not having the positive control, the sample size was quite small and the results were not significant.

Figure 5

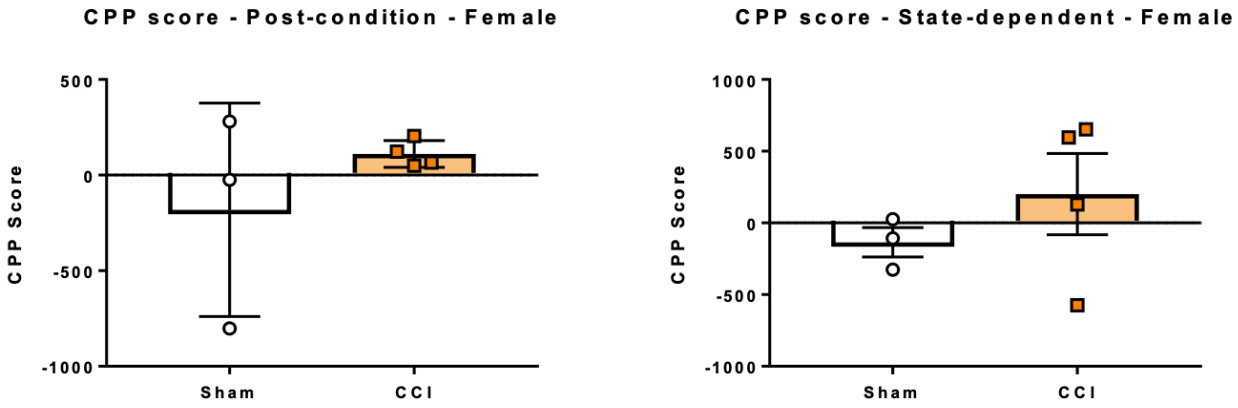


Figure 5: Female KOR knockout mice did not exhibit aversion or preference to kappa agonist. CPP was performed with 3 sham and 4 pain female mice, using 5mg/kg kappa agonist. The bar height indicates the mean and the error bars indicate standard error from the mean (SEM).

For the KOR knockout females (Figure 5), the chronic pain females developed a slight preference for the drug chamber and non-pain knockouts possibly developed a slight aversion. However, the data is not statistically significant and the sample size is only 3 for sham and 4 for CCI.

Figure 6

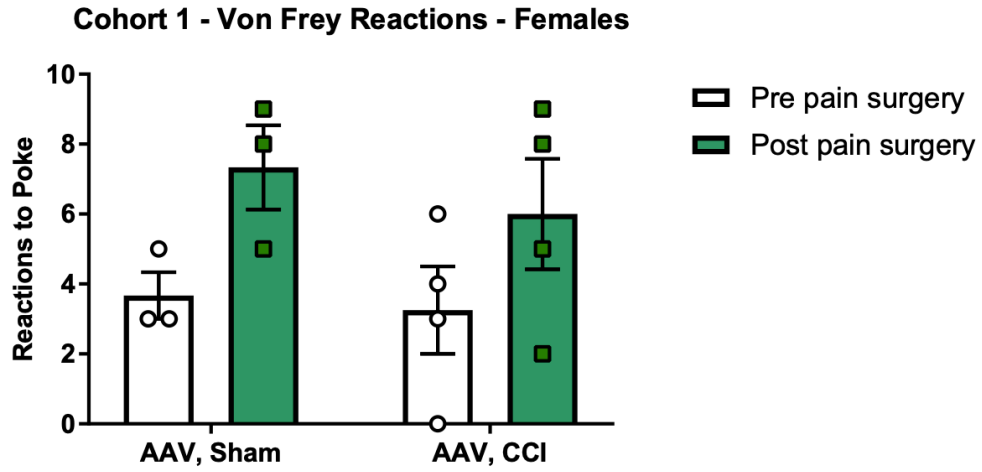


Figure 6: No difference in increase in von Frey responses between chronic pain and control mice post CCI surgery in female KOR knockout mice. Von Frey data for the 3 sham and 4 CCI female mice shows increase responses from before to after cuff surgery for KOR knockout sham and pain mice. The bar height indicates the mean and the error bars indicate standard error from the mean (SEM).

Von Frey data for the females showed increase responses from before to after cuff surgery for both the KOR knockout sham and pain mice. As is expected with females, there was no difference in response increase between the pain and sham groups. Importantly, this confirmed that the introduction of the adenovirus that causes KOR knock out did not interfere with the development of allodynia post constriction cuff injury. Unfortunately, we do not have von Frey data for the males of this first knockout cohort.

Figure 7

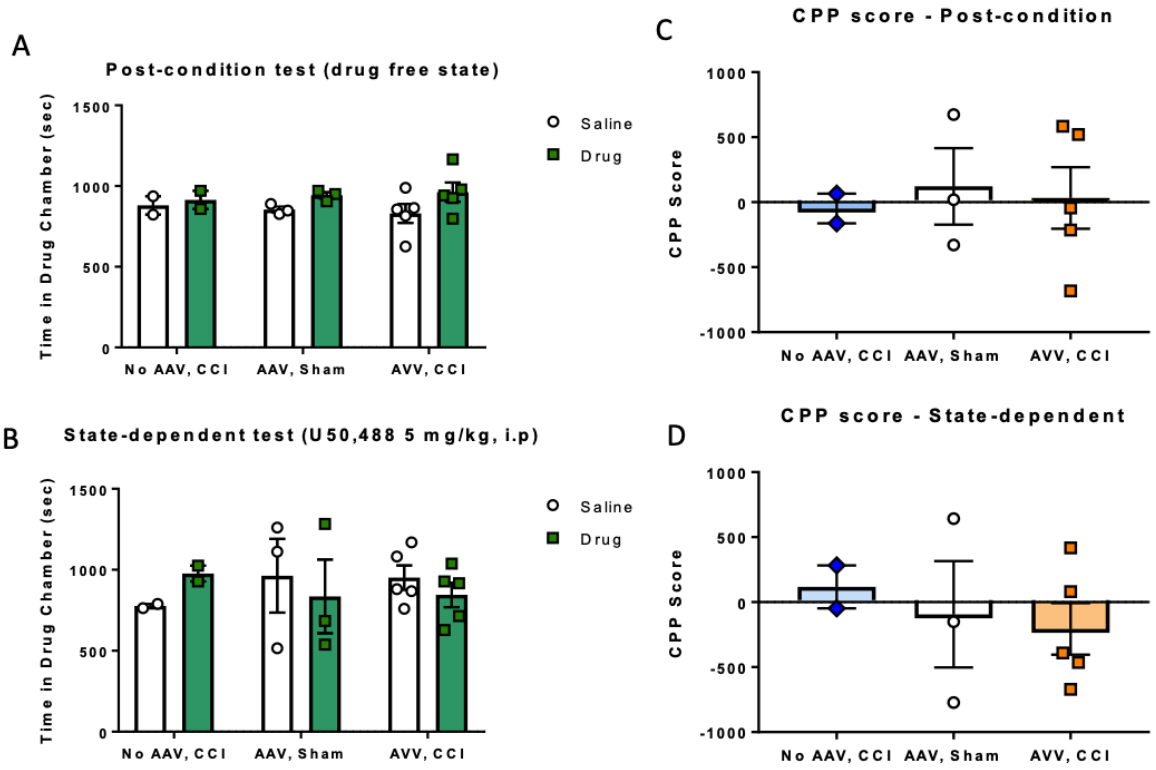


Figure 7: Kappa agonist aversion in male KOR knockout mice present in state-dependent test, but not in post-condition test. CPP was performed with 8 KOR knockout mice (5 pain and 3 sham), and 2 virus-free, non-KOR transgenic pain mice. Post-conditioning showed no preference or aversion to kappa agonist in all groups. State dependent showed aversion in the KOR knockout animals, with more pronounced effect in the pain group. The bar height indicates the mean and the error bars indicate standard error from the mean (SEM).

As females do not develop allodynia from chronic pain caused by the CCI, the following sets of experiments were done only on male mice. I performed the KOR knockout on 8 additional male mice. They were split into 5 pain, and 3 sham. Two additional mice did not receive the virus, but were given the pain surgery. The CPP post-conditioning test results (Figure 7) again did not show a clear trend towards aversion to the drug chamber in any group. Time in chamber and CPP scores seemed to show that aversion was not inhibited for the knockout mice in the state-dependent test (Figure 8), however. This suggests that knocking out

KOR in the serotonergic neurons in the DRN did not prevent the aversion to U50,488, nor the memory of which chamber the agonist was administered in, but the aversion itself was only present in the presence of the agonist. With additional investigation it would be interesting to discern mechanisms and circuits involved in this differential effect, and what this means about memory associated with pain and affect. Additionally, the KOR knockout sham sample size was small, and the wild type pain (positive) control animals did not show the expected agonist aversion in either the post-conditioning and state-dependent tests, which could indicate that the experimental results as a whole are not reliable and thus cannot be definitively interpreted.

Figure 8

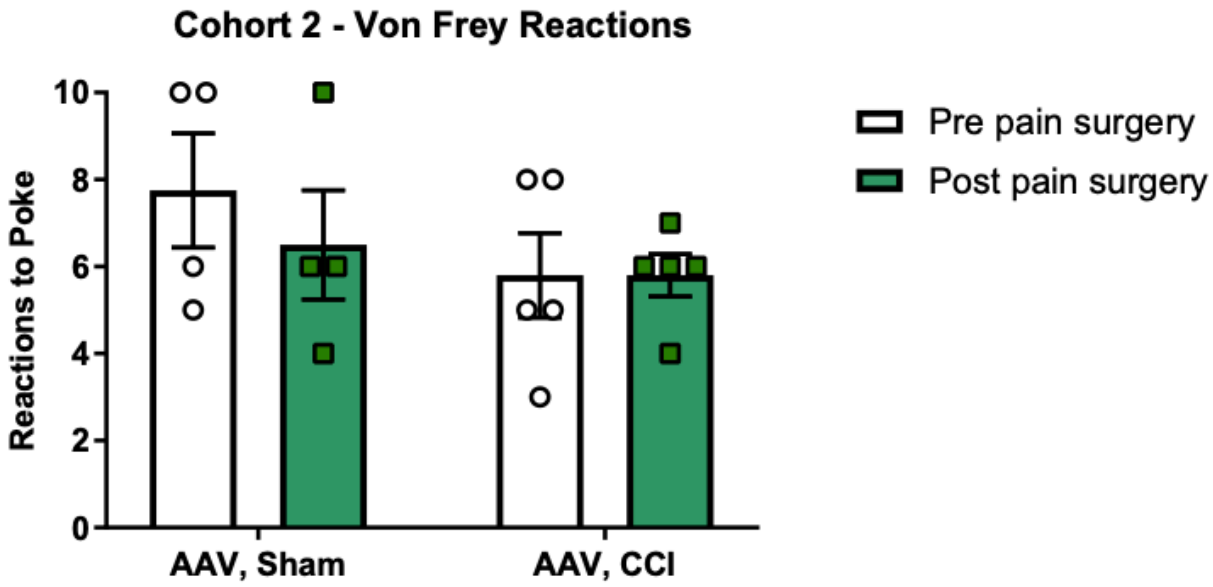


Figure 8: No increase in responses to von Frey in either pain or sham groups post CCI surgery. Von Frey was performed on 8 KOR knockout mice (4 sham, and 4 pain) before and after CCI surgery. Pre-surgery responses were higher than normal, with the average for sham being 8 and for CCI, 6. The bar height indicates the mean and the error bars indicate standard error from the mean (SEM).

For the second cohort of knockout mice, the von Frey test results (Figure 8) were not significant. The pre-surgery baseline responses are much higher than usual to begin with. Two of the sham pain surgery animals started at 10 out of 10 responses, and the average for sham was 8 and for CCI 6. The common pre-surgery average is no higher than 5. Therefore, it is possible that there was a ceiling effect in some of the sham animals, and the post surgery increase would not be visible. There was no increase in responses post surgery in the pain group. There were technical challenges in the performance of this test that are likely to explain the high starting numbers and the slight overall decrease in sham, and no change in CCI group responses after constriction cuff surgery.

Figure 9

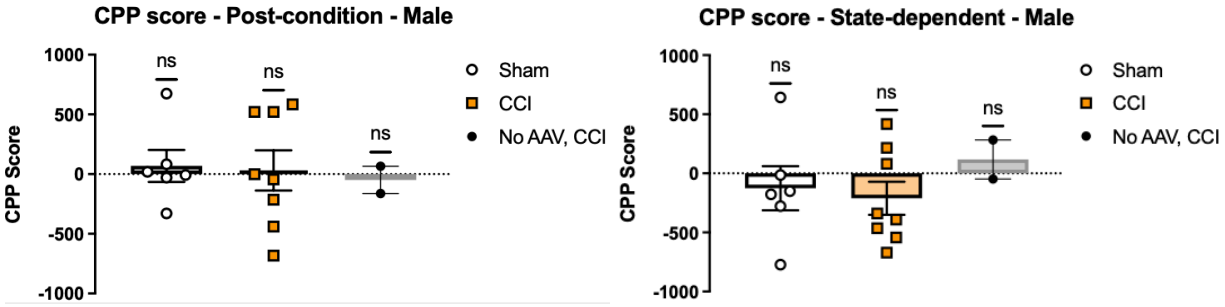


Figure 9: Average of CPP scores for all KOR knockout males shows no significant aversion, but positive control also does not exhibit aversion. Data from all KOR knockout male cohorts combined (8 pain and 6 sham) and 2 virus-free, non-KOR transgenic pain mice, showed no significant aversion caused by the kappa agonist for either the pain or non-pain animals. However, the positive control did not exhibit the expected kappa mediated aversion. The bar height indicates the mean and the error bars indicate standard error from the mean (SEM).

When the data from all the male cohorts is combined (Figure 9), it shows that there was no aversion caused by the kappa agonist for either the pain or non-pain animals. This implies that the hypothesis is true—that KOR on serotonergic neurons projecting from DRN is responsible

for the enhanced KOR agonist-induced aversion in chronic pain states. However, the positive control group was very small (2 animals) and more importantly does not exhibit the expected kappa mediated aversion.

Figure 10

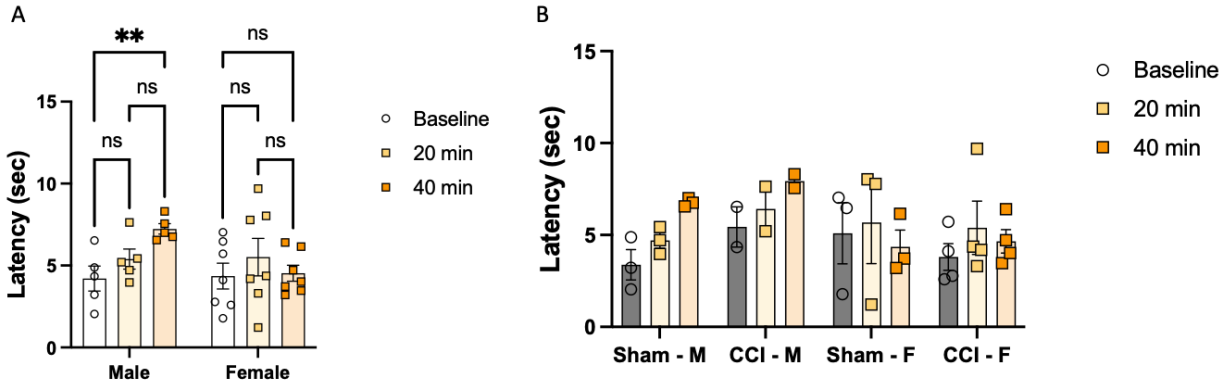


Figure 10: Kappa agonist had no effect on KOR knockout females, but increased reaction time in males in tail flick test. Tail flick test was performed on 12 KOR knock mice (7 females and 5 males) at baseline, and 20min and 40min post administration of kappa agonist. Tail flick response times for KOR knockout females did not increase, but increase for KOR knockout males, with strongest effect at 40 min after administration.

Because the KOR mediated aversion was not enhanced in pain animals (the positive control) we performed the tail flick test to ensure that the KOR knockout did not spread beyond the DRN and to the spinal cord. KOR activation in the spinal cord has a pain relieving effect. As is expected for females, the agonist did not increase the tail flick response times. For males though, KOR agonist increased reaction times to the hot water with the strongest effect being at 40 minutes after administration. This suggested that kappa induced analgesia was still intact in the mice that had KOR knocked out from their serotonergic neurons in the DRN.

Conclusions and Future Experiments

In summary, my data confirms that the KOR agonist, U50,488, produces an enhanced CPA in wild type chronic pain animals compared to pain free animals. Additionally, the constriction cuff surgery increases mechanical responsiveness, suggesting development of mechanical allodynia, and U50,488 mediated analgesia is intact in animals post injection of the KOR knockout virus.

Finally, the data also suggest that KOR on serotonergic neurons in the DRN may play a role in agonist induced CPA. As many like Ehrich, *et al*, and Nagai, *et al*, have shown, 5-HT plays a role in aversive place conditioning (Ehrich, 2015, Nagai, 2020) in the VTA, DRN, and others. It was interesting, in particular, that my data showed that there was a CPA in the state-dependent tests for males, while neither a CPA nor CPP was present in the post-conditioning tests (Figures 4, 7, and 9). In other words, while there was no preference or aversion observed without the presence of the agonist, the presence of aversion to the drug paired chamber with the agonist indicates the formation of memory of which chamber the drug was paired with. This means that if the KOR on the serotonergic neurons in the DRN was successfully knocked out, that it did not prevent the memory formation of where the drug was administered, nor the association of its aversive quality.

Confirmation of the KOR knockout on serotonergic neurons via immunohistochemistry should be done to confirm the level of knockout. More control experiments need to be performed to confirm the above effect, especially including KOR transgenic, pain-naïve mice. While Liu, *et al.* showed that 5mg/kg was sufficient to induce kappa agonist aversion (Liu, 2019), it would be useful to repeat the CPP experiments on mice with KOR knockout on serotonergic neurons in

the DRN using higher dosing to confirm whether the dosing of agonist was too low to produce an aversion in post-conditioning specifically in serotonergic KOR KO transgenic mice.

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