Pain relief of cannabinoids in the KATP pathway and its connections to the opioid pathway in mice

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Introduction

In the United States from 1999 to 2017, nearly 218,000 people died from overdosing on prescription opioids. The number of overdose deaths in 2017 was five times higher than in 1999 (CDC, 2016). Opioids are known to be addictive drugs, and long-term use may lead to overdose or death. To combat the opioid epidemic, different therapies should be used for pain management. One potential alternative to prescription opioids is cannabis. The science of cannabinoids, the neurotransmitters released in the intake of cannabis, is relatively new research. There are proposed pathways from cannabinoids binding to cannabinoid receptors, but none of them have been confirmed. Furthermore, there is even less research into the relationship between cannabinoids and opioids.

There are two primary cannabinoid receptors: CB1 and CB2, both being G-protein coupled receptors (Pertwee, 2006). The receptors are especially sensitive to the THC-component in cannabis, resulting in pain relief. CB1 is found in higher concentrations in the brain, specifically the cerebellum, while CB2 is found within the immune system, especially in the spleen (Pertwee, 2006). The hypothesized pathway begins with a cannabinoid binding to its receptor, which inhibits adenylyl cyclase. Adenylyl cyclase produces cyclic adenylyl phosphate (cAMP) from ATP, so inhibiting adenylyl cyclase decreases the concentration of cAMP, resulting in the potassium channel opening (Pertwee, 2006). The influx of potassium rapidly depolarizes the cell, which influences the rate of action potential firing. This aligns with the accepted pathway for opioids (Figure 2), and it is possible cannabinoids and opioids affect the same potassium channels.

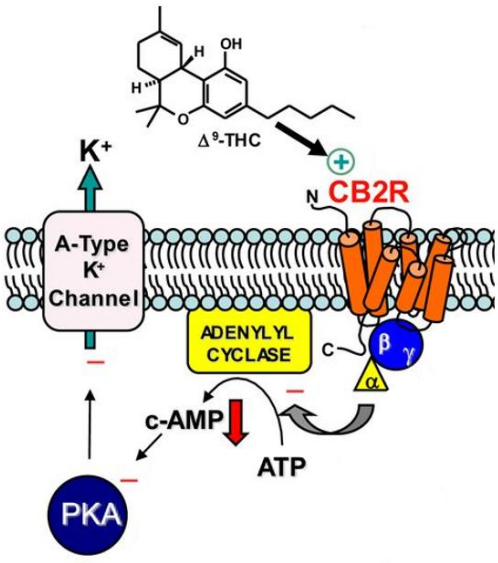

Figure 1. The proposed cannabinoid pathway. When the THC-component binds to the CB receptor, adenylyl cyclase is inhibited, decreasing the concentration of cyclic-AMP. Ultimately, this opens the potassium channel. When adenylyl cyclase is activated, the concentration of cyclic-AMP increases and the potassium channel is inhibited (Dhopeshwarkar et al., 2014).

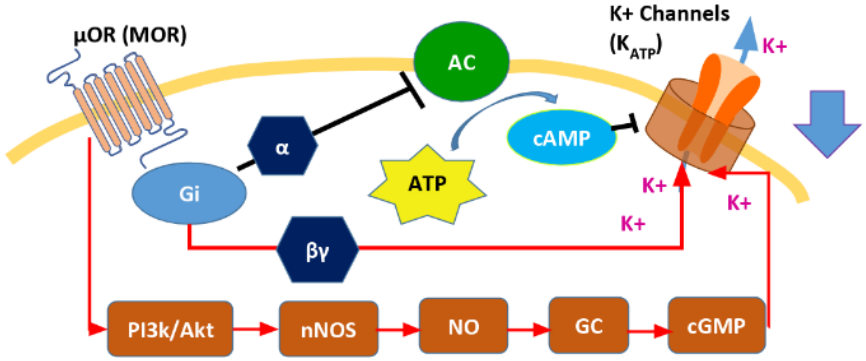

Figure 2. The proposed and accepted morphine pathway. When the inhibitory G-coupled protein receptor binds morphine, adenylyl cyclase is inhibited, decreasing the formation of cyclic-AMP. This opens the potassium channels, allowing an influx of potassium out of the cell (Cunha et al., 2010).

Similar to opioids, there is some evidence of cannabinoid tolerance and dependence. When the CB receptor is used repeatedly, the concentration of cAMP actually increases from an increase in the concentration of adenylyl cyclase, down-regulating the potassium channel (Bushlin, et al., 2009). The dependence of cannabinoids is still being researched, however, as the pathway needs to be defined first. In this research, experimentation of mice was used to observe the potassium channel connections between the opioid and cannabinoid pathways. This was the starting point in finding a potentially safer therapy than opioids but with similar pain relief efficacy.

Materials and Methods

Mice and breeding

C57BI6 mice were acquired from Charles River in Raleigh, North Carolina. SUR1 global knock out mice were acquired from Dr. Joseph Bryan at the Pacific Northwest Research Institute in Seattle, Washington. The "Hot Shot" method was used to verify genotypes of DNA extracted from mouse tail biopsies. The presence of the SUR1 knock out allele was confirmed using PCR with the following primer set: forward 5'- CTG TCC ATC TGC ACG AGA CT-3' and reverse 5'-AGG TTG TTG GTG GAG GTC AG -3'. The resulting knock out band was 350 bp and the wild-type band was 524 bp. The PCR temperature and cycling protocol were repeated for 40 cycles as follows: 95°C for 10 minutes, 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 45 seconds. All experimental procedures were performed and approved in accordance with the University of Minnesota Institutional Animal Care and Use Committee guidelines.

Tissue collection and RNA extraction

Mice were anaesthetized with 5% isoflurane in oxygen then decapitated. In uninjured and sciatic nerve ligated C57BI6 mice, the cerebral cortex, brainstem, trigeminal ganglia, sciatic nerves, and lumbar dorsal root ganglia and spinal cords (L3-L6 segments) were dissected then snap frozen in liquid nitrogen. The tissues were placed in long term storage at -80°C. Trizol reagent (Sigma-Aldrich) was used to purify RNA from the frozen tissue samples. The RNA was then isolated using the RNeasy Mini Spin Columns (Qiagen) with a DNase I treatment step. The quantity and quality of the RNAs were analyzed using Nanodrop spectrophotometry (Thermo Fisher Scientific) and Qubit fluorometric quantification (Thermo Fisher Scientific).

Quantitative PCR

cDNA was synthesized from 50 nanograms of each purified RNA sample at the same thermal cycling conditions using the Omniscript RT Kit and protocol from Qiagen. Primers were designed using sequences from NCBI primer design (Table 1). Quantitative PCR carried out with SYBR Green I dye (Roche) using LightCycler 480 technology (Roche). The temperature and cycling protocol was repeated for 45 cycles as follows: 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 20 seconds. The internal control was the expression of 18s, a housekeeping gene. The presence of genomic DNA was analyzed using negative RT-PCR controls. The relative expression levels of each gene of interest was calculated using LightCycler 480 Software by the equation: $\frac{Mean\ Concentration}{Mean\ Concentration\ of\ 18s}$. Three experimental replicates were performed.

Table 1. NCBI primer design for Cnr1, Cnr2v1, and Cnr2v2.

Gene Symbol	NCBI Gene ID	mRNA accession	Forward primer	Reverse primer
Cnr1	12801	NM 007726.3	CGT TGA GCC TGG CCT AAT CA	GGT CTG TGG TGA TGG TAC GG
Cnr2v2	12802	NM 009924.4	CAC TCA TCT GCG AAA GTG TGA G	TCT TCT ACT GGA GCT GTC CCA
Cnr2v2	12802	NM 001305278.1	TGC ATG CAT TTC CTT TCC TAA C	ATG GAT GGG CTT TGG CTT CT

Mechanical Paw Thresholds

Mice were placed in a transparent plastic box and acclimatized to the test chamber for twenty minutes. Withdrawal latencies were assessed at baseline and over a duration of sixty minutes after injection. The mice were injected subcutaneously in the loose skin on the neck with one hundred microliters of WIN 55212-2 (5 mg/kg, CB1 and CB2 agonist), GW 405833 (30 mg/kg, CB2 agonist), or vehicle (5% dimethylsulfoxide (DMSO) in saline (0.9% NaCl)). Paw withdrawal thresholds were measured on the left and right hind paws with a handheld electronic Von Frey hair (2392, IITC Life Sciences, Woodland Hills, California) on a wire mesh floor. Positive responses included a rapid flinching or withdrawal of the hind paw from the stimulus. All behavioral tests were conducted in a blinded manner.

Results

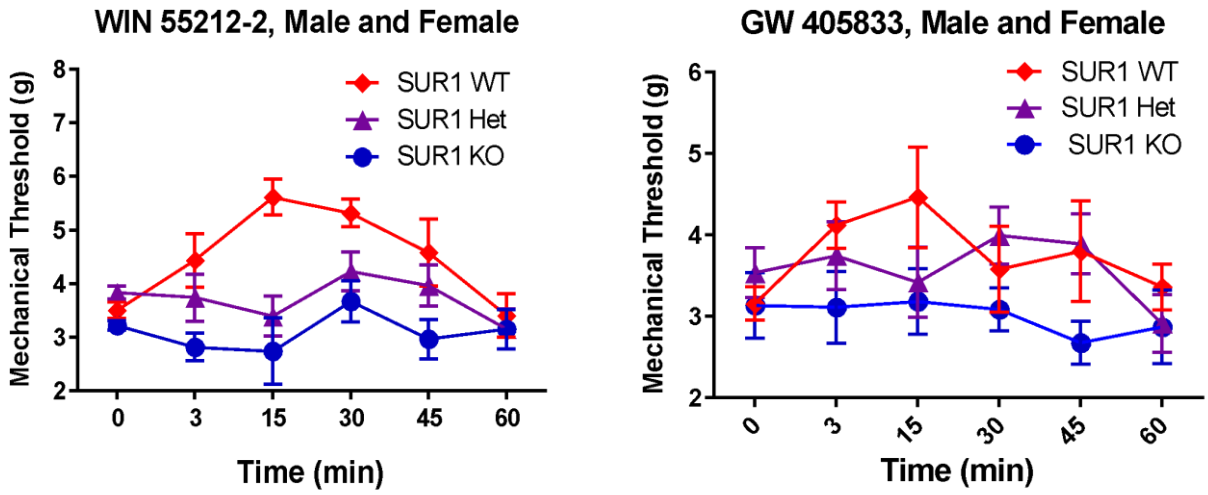

Figure 3. Mechanical threshold results after injection of WIN 55212-2 (CB1 and CB2 agonist) and GW 405833 (CB2 agonist) compounds. After injection of one hundred microliters of the compound at time=0, male and female SUR1 mice in wild-type (WT), heterozygous (Het), and knockout (KO) genotype groupings were measured with mechanical paw threshold every fifteen minutes for sixty minutes.

Mice were grouped according to sex and genotype, with each group consisting of two to five mice, then subjected to mechanical paw threshold testing. The knockout mice lacked the SUR1-subtype of ATP-sensitive potassium channels (KATP channels), wild-type mice had fully functional KATP channels, and heterozygous mice were an intermediate of the SUR1 wild-type and knockout mice. The mice were injected with one hundred microliters of WIN 55212-2 or GW 405833 then measured every fifteen minutes for sixty minutes. Both left and right hind paws were measured then averaged for each time point. WIN 55212-2 injections increased mechanical threshold for wild-type and heterozygous mice, and there was a significant difference between wild-type, heterozygous, and knockout mice over time (repeated measures ANOVA, $F(10, 100) = 2.312$, $p = 0.0172$) (Figure 3). GW 405833 injections also increased the mechanical threshold for wild-type and heterozygous mice, and there was a significant difference comparing wild-type, heterozygous, and knockout mice, but not over time (repeated measures ANOVA, $F(2,19) = 4.614$, $p = 0.0233$) (Figure 3).

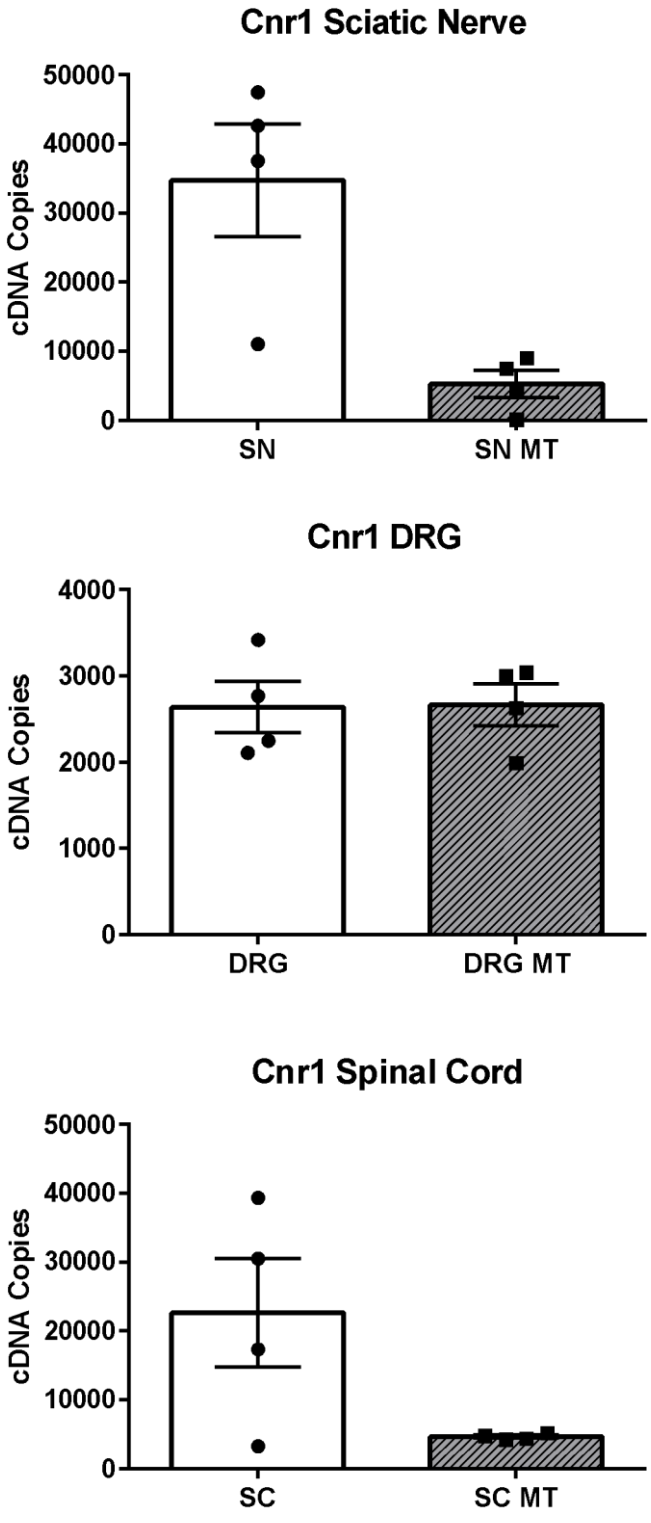

Figure 4. Results of qPCR for the Cnr1 gene. cDNA was synthesized from RNA samples in sciatic nerve (SN), dorsal root ganglia (DRG), and spinal cord (SC) of mice and compared to expression in the same tissues in morphine tolerant (MT) mice. Four samples were used for each tissue.

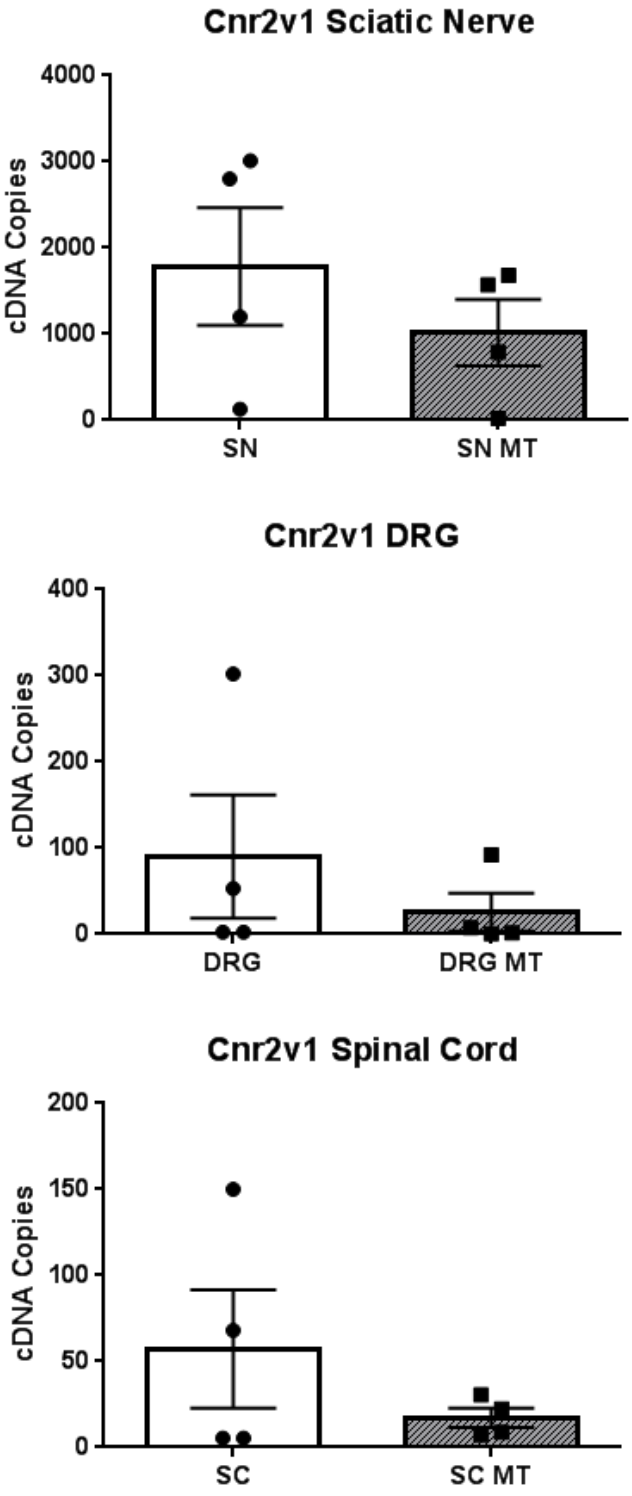

Figure 5. Results of qPCR for the Cnr2v1 gene. cDNA was synthesized from RNA samples in sciatic nerve (SN), dorsal root ganglia (DRG), and spinal cord (SC) of mice and compared to expression in the same tissues in morphine tolerant (MT) mice. Four samples were used for each tissue.

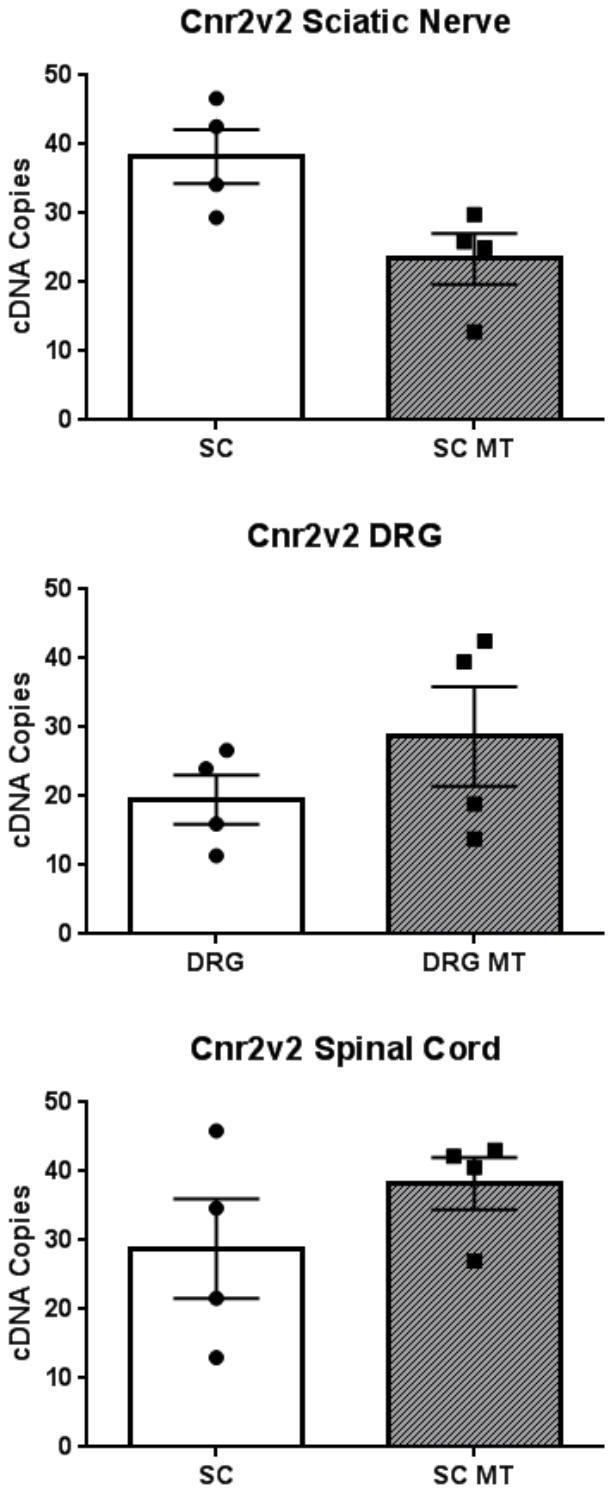

Figure 6. Results of qPCR for the Cnr2v2 gene. cDNA was synthesized from RNA samples in sciatic nerve (SN), dorsal root ganglia (DRG), and spinal cord (SC) of mice and compared to expression in the same tissues in morphine tolerant (MT) mice. Four samples were used for each tissue.

Quantitative PCR was carried out for the genes Cnr1, Cnr2v1, and Cnr2v2. cDNA expression of each gene was measured in four mouse samples of sciatic nerve, dorsal root ganglia, and spinal cord, which was compared to the expression of cDNA in the same tissues in morphine tolerant mouse samples. The results showed gene expression was typically lower in morphine tolerant tissues compared to the control tissues (Figure 4, 5, 6), with significant differences in Cnr1 and Cnr2v2 sciatic nerve tissues (unpaired t-test, $p = 0.0125$ and $p = 0.0328$, respectively). Cnr2v2 had very low expression in all three tissues (Figure 6), while Cnr1 showed very high expression in the tissues (Figure 4). Cnr2v1 had high expression in the sciatic nerve but low expression in the dorsal root ganglia and the spinal cord (Figure 5). Cnr2v2 had higher expression in morphine tolerant dorsal root ganglia and spinal cord (Figure 6), but Cnr1 and Cnr2v1 had overall lower expression in the morphine tolerant tissues (Figure 4, 5).

Discussion

The results of the mechanical paw threshold experiment support the hypothesis that cannabinoids open the same potassium channels that morphine act on. Both the wildtype and heterozygous SUR1 mice, possessing functional potassium channels, experienced an increase in mechanical threshold after administration of the CB1 and CB2 agonist compounds. An increase in mechanical threshold correlates with an increased pain tolerance in the mice. In the knockout mice, lacking the SUR1 subtype of the KATP channel, there was no change in mechanical threshold. The differences in thresholds between genotypes can be attributed to the functionality of potassium channels. The results also support cannabinoids as an effective pain relief. When the mice were injected with the CB1 and CB2 agonist or the CB2 agonist, wild-type and heterozygous mice experienced pain relief as indicated by the increase in mechanical threshold. Though the CB2 receptor is primarily found in the immune system, the research suggests the receptors are also involved along the pain pathway with CB1 receptors.

Quantitative PCR supported the presence of CB1 and CB2 receptors in the pain pathway, specifically in the sciatic nerve, dorsal root ganglia, and spinal cord of mice. The Cnr1 gene, coding for the CB1 receptor, was highly expressed in all three tissues, more so in the sciatic nerve and spinal cord than the dorsal root ganglia. This was expected as this receptor is accepted as found primarily in the central nervous system. Cnr2v1 and Cnr2v2 are variants of genes that code for the CB2 receptor, and they had significantly lower expression in the tissues as compared to Cnr1. Cnr2v1 had the highest expression in the sciatic nerve while the dorsal root ganglia and spinal cord had less than 300 cDNA copies each. Cnr2v2 had especially low expression of cDNA, with fifty or less cDNA copies per tissue. This supports CB1 as the primary receptor responsible for pain relief with CB2 as a less essential receptor. Compared to morphine-tolerant tissues, the control tissues had typically more expression of the genes, seen in the results for Cnr1 and Cnr2v1. This could be attributed to the down regulation of receptors as a safety mechanism. Down regulation provides protection from overuse of receptors to the cell by internalizing the receptor or potentially triggering apoptosis. The results are specific to the time point of tissue collection from the morphine tolerant mice. Collecting tissues at any other time point would reflect the expression of cannabinoid receptors at the mouse's exact degree of

tolerance to morphine. This could alter the data greatly depending on the expression of cannabinoids of a mouse more tolerant to morphine versus one that is less tolerant.

The results of qPCR were variable with large error bars regardless of the gene or tissue. Only three experimental replicates were performed per tissue, so performing more replicates would decrease the standard error. Going forward, more experimental replicates would be performed for each tissue. Also, more tissues along the pain pathway such as spinal nerves can be assessed in qPCR. More behavioral tests can be done to further research in the cannabinoid pathway such as open field testing and rotarod. Cellular and protein testing can also be done with procedures such as FLIPR and Western Blotting. Further testing will focus on proposed intermediate products in the cannabinoid pathway to confirm or reject the hypothesized pathway.

Conclusion

This research was merely a starting point in determining the pain efficacy of cannabinoids. The data supported the hypothesis of cannabinoids opening potassium channels to provide pain relief, similar to the mechanism of morphine, but the intermediates of the proposed pathway are yet to be confirmed. Further experimentation will focus on determining the cannabinoid pathway in comparison to the opioid pathway to explore a potential solution to the opioid epidemic.

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