

TARGETING TRAFFICKING OF VOLTAGE GATED CALCIUM CHANNELS: A
NOVEL APPROACH IN THE TREATMENT OF PAIN

by

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DEDICATION

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"Talent is God given. Be humble. Fame is man-given. Be grateful. Conceit is self-given. Be careful."

-John Wooden

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ABSTRACT

Pain is the most common and debilitating medical problem for which patients seek medical care. Opioids remain the gold standard in the treatment of pain but are limited by poor side effect profiles such as emesis, constipation, dependence/addiction and respiratory depression. Despite a myriad of analgesic compounds on the market, tri-cyclic antidepressants, opioids, anticonvulsants, non-steroidal anti-inflammatory agents and combinations thereof, nearly two thirds of the chronic pain patients report inadequate pain relief; therefore, a new approach in the development of pain management is necessary.

In recent years, the N-Type voltage gated calcium channel (CaV2.2) has become an attractive target in the treatment of chronic pain. Ziconotide, a selective CaV2.2 blocker, has been FDA approved in the United States for the treatment of severe chronic pain that is refractory to other treatments, but due to its profound side effect profile (nausea/vomiting, somnolence, vertigo, muscle spasms, myalgia, insomnia, anxiety, tremor, memory impairment and induced psychiatric disorders), the use of Ziconotide is severely limited.

Mapping of the CaV2.2 interactome led to the identification of novel regulatory proteins, including collapsin response mediator protein 2 (CRMP2). Initially identified as an intracellular protein in the specification of axon/dendrite fate and axonal outgrowth, it is now known that this protein can regulate the activity of CaV2.2 and hence may be a critical regulatory node in pain modulation. Here, I describe a novel peptide aptamer derived from CRMP2, designated CaV2.2 binding domain 3 (CBD3), which when fused with the HIV transactivator of transcription protein (TAT), created tat-CBD3, which was able to significantly reverse thermal and mechanical hypersensitivity induced by the surgical incision on the plantar surface of the left hind paw in rats, a pre-clinical model of post-operative pain. Additionally, tat-CBD3 significantly attenuated thermal hypersensitivity induced via intraplantar injection of carrageenan, a model of acute inflammatory pain. Furthermore, the administration of tat-CBD3 did not produce any rewarding behaviors as measured by the conditioned placed preference (CPP) paradigm, nor did the administration of tat-CBD3 produced any motor coordination deficits measured using the rotarod

performance test. Moreover, the addition of a 14-carbon myristate (myr) group to the parent peptide, myr-tat-CBD3, had increased efficacy in the attenuation of paw incision and carrageenan induced thermal/mechanical hypersensitivities when compared to the parent peptide (tat-CBD3). These types of novel compounds that lack unwanted side effects and addiction propensities are urgently needed to relieve individuals suffering from chronic pain.

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Pain

Pain is the most common reason people seek medical care [1]. It is estimated that in 2008 there were approximately 100 million chronic pain patients costing the United States an estimated \$635 billion a year [2]. Not only does pain contribute to a significant social-economic burden on society, the current therapies in the management of pain: narcotic analgesics, nonsteroidal anti-inflammatory agents, tri-cyclic antidepressants, and anticonvulsants, produce undesired side effects in which many patients report rather dealing with the pain than the side effects.. Additionally, despite the myriad of pain alleviating therapies, a significant proportion of patients do not achieve adequate pain relief, leaving a significant unmet medical need for the development of novel, safe and effective treatment of acute and chronic pain [3]. To develop novel therapies in combatting pain, the mechanisms contributing to the pathophysiology of pain must be better understood.

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage [4]. Noxious stimuli are detected in the periphery by specific sensory receptors called nociceptors [5]. These neurons have free nerve endings in the periphery with cell bodies in the dorsal root ganglia or trigeminal ganglia and terminate in the superficial layers of the dorsal horn of the spinal cord or brainstem, respectively. At the level of the spinal cord, the first central synapse, pro-nociceptive neurotransmitters such as glutamate, substance P and calcitonin gene related peptide (CGRP) are released; facilitated primarily by the actions of various voltage gated ion channels. The release of these pro-nociceptive neurotransmitters at the ipsilateral dorsal horn of the spinal cord activates second-order neurons. The corresponding second-order neurons then cross the spinal cord at the level of entry via the anterior white commissure, enter the spinothalamic tract and travel towards the brainstem and thalamus where they relay pain information to third order neurons. At the level of the thalamus, third order neurons transmit the pain sensation to the somatosensory cortex and the insula for the location and perception of pain. While each of these physiological sites in the

neural transmission of pain signals offer an unique target in the treatment of pain, the focus of this thesis will emphasize targeting the first central synapse, terminating peripheral pain signals at the level of the spinal cord [6].

Another key facet in developing safer and more effective treatments for pain is understanding the neural processing of pain; which can be roughly divided into three parts: transduction, transmission and modulation[6]. Transduction can be described as the process in which noxious stimuli such as heat, mechanical and thermal insult, results in the activation of nociceptors. Nociceptor afferent fibers are considered pseudounipolar neurons, i.e. with a peripheral and central terminal. As such, after a noxious insult, neurotransmitters are release at both ends, contributing to both the peripheral sensation of pain and the central perception of pain. Transmission of noxious signals is relayed by mainly two types of primary afferent nociceptors: C-fiber and A-delta fibers. C-fibers are non-myelinated fibers that conduct in the range of 0.5 to 2 meters per second with a polymodal nature of processing mechanical, thermal and chemical stimuli. A-delta fibers are thinly myelinated fibers that conduct in the range of 2 to 20 meters per seconds with all fibers responding to high-intensity mechanical stimulation with some responding to thermal stimuli as well. Modulation of pain signals, an important aspect of neural processing of pain, is where noxious signals received at the dorsal horn of the spinal cord are selectively inhibited by interneurons at the spinal cord and/or descending inhibitory neurons through the dorsolateral funiculus, so that the transmission of the pain signal to higher centers is modified [7]. The focus of the work presented here is modulating the transduction step of the neural processing of pain, specifically, blocking the conversion of electrical signal to chemical signal at the level of the spinal cord by modulating the trafficking of voltage gated calcium channels.

1.2 Voltage Gated Calcium Channels: Distribution and Function

The relative clinical successes of Ziconotide, gabapentin and pregabalin in the treatment of chronic pain established the N-type voltage-gated calcium channel (CaV2.2) as a *bona fide* pain target. Currently, 10 members of the voltage gated calcium channels have been identified; each

serves as the key transducer of cell surface membrane depolarization events into local intracellular calcium currents that facilitate diverse physiological changes. Calcium channels are complex protein channels comprising four to five subunits. The $\alpha 1$ subunit is the largest protein subunit composed of four homologous domains (I-IV), each with six transmembrane segments (S1-S6), and makes up the conduction pore. The S4 segment serves as the voltage sensor while the pore loop between S5-S6 determines ion conduction and selectivity [8]. An intracellular β subunit also exists, which forms a disulfide bridge with $\alpha 1$ subunit and serves modulatory functions of the channel.

In addition to the arrays of subunits associated with voltage gated calcium channels, ten different sub types of the channels have been identified in mammals, each present in distinct cell types and hosts different physiological and pharmacological properties. A lettered nomenclature was devised to classify each of these channel subtypes according to the calcium current [9]. The family can be broadly divided into 5 families: L (CaV1.1-CaV1.4), P/Q (CaV2.1), N (CaV2.2), R (CaV2.3) and T (CaV3.1-CaV3.3). L-type calcium channels requires a strong depolarization for activation and is long lasting, found predominately in skeletal muscles, cardiac myocytes and endocrine cells. P/Q and N type calcium channels are predominantly located on nerve terminal and dendrites and are involved in neurotransmitter release and dendritic calcium transients. T type calcium channels can be found on neuronal cell bodies and dendrites and cardiac muscle myocytes and are involved in repetitive firing, giving them the pacemaking ability. Preferential expression of CaV2.2 at the nerve terminals, and its involvement in neurotransmitter release are key aspects targeted by of Ziconotide's efficacy in the treatment of pain via this channel. Modulation of CaV2.2 at the first central synapse will be the focus of the work presented here.

1.3 Collapsin response mediator protein 2 (CRMP2): A Novel Target in Pain?

CaV2.2 are key players in the increase of neuronal excitability and neurotransmission following injury-induced pain [10]. Predominantly expressed at the primary afferent nociceptive terminals in the spinal cord [11], these channels are critical for the release of pronociceptive neurotransmitters in the transduction of pain. The therapeutic potential of targeting CaV2.2 has been demonstrated by the clinical success of ziconotide, gabapentin and pregabalin. Each of the

drugs target different elements of CaV2.2 to alleviate pain experienced by patient that is refractory to current mainstay therapies [12]. The efficacy of these drugs is further supported through preclinical investigations through intrathecal administration in rats [13] and CaV2.2 knock out mice [14]. However, targeting CaV2.2 directly produces problematic side effects such as nausea/vomiting, somnolence, vertigo, muscle spasms, myalgia, insomnia, anxiety, tremor and memory impairment, resulting in narrow therapeutic window and challenging dosing regimen. These drugs that directly target the CaV2.2 at higher doses begin to interact at other calcium channels to produce some of their side effects. The present work describes a novel strategy targeting protein interaction modulating CaV2.2 in order to circumvent these problems.

Collapsin response mediator protein 2 (CRMP2), an axonal growth/guidance protein, was recently identified to interact with CaV2.2 [15]. Further work in the Khanna laboratory demonstrated that the interaction between CRMP2 and CaV2.2 upregulates surface expression of CaV2.2, thus promoting increased neurotransmitter release from both central and peripheral synapses [16]. Here, CaV2.2 binding domain 3 (CBD3), a novel 15 amino acid peptide derived from CRMP2, which has been demonstrated to uncouple the protein-protein interaction between CRMP2 and CaV2.2, is hypothesized to decrease thermal and/or mechanical hypersensitivity induced in the paw incision model of post-operative pain and carrageenan induced acute inflammatory pain.

1.4 CaV2.2 Binding Domain 3 (CBD3): Novel Peptide in Treatment of Pain

A recent proteomic screen revealed a novel CaV2.2 binding partner: CRMP2 [17]. In order to determine the specific domains critical for the interaction between CaV2.2 and CRMP2, peptides covering the entire length of CRMP2 were synthesized and tested. CRMP2 was found to have three CaV2.2 binding domains (CBD1-CBD3), while all three domains were able to disrupt the interaction between CaV2.2 and CRMP2, immunoprecipitation studies from rat spinal cord lysates revealed that CBD3 was able to attenuate the interaction between CaV2.2 and CRMP2 but did not affect an unrelated interaction between CRMP2 and tubulin [18].

CaV2.2 membrane trafficking was effectively disrupted by CBD3 (amino acid sequence: ARSRLAELRGVPRGL) [15]. In order to achieve effective cell permeation, CBD3 was fused to

the transduction domain of the HIV-1 TAT protein to generate tat-CBD3. Tat-CBD3 has been demonstrated to inhibit evoked CGRP release [18], AIDS induced painful peripheral neuropathy [19], a rodent model of migraine [20] as well as tibial nerve injury related painful peripheral neuropathy [21]. However, the efficacy of tat-CBD3 on the thermal and/or mechanical hypersensitivity following surgical operation or acute inflammatory insult (i.e. carrageenan) have not been investigated and are the focus of my studies here. Additionally, undesired side effects seen with current pain therapies such as rewarding potential and motor coordination deficits are explored after tat-CBD3 administration to rats.

A variant of the CBD3 peptide will be tested in my studies. Adding a myristate motif, which tethered the peptide to the cellular membrane, generated this novel peptide, myr-tat-CBD3. By remaining membrane bound, the myr-tat-CBD3 peptide would be expected to have a more pronounced effect on membrane-expressed, functional CaV2.2 channels than tat-CBD3, which would also work within the intracellular milieu. Consequently, in comparison to tat-CBD3, the myr-tat-CBD3 peptide is expected to be more efficacious in attenuation of thermal and/or mechanical hypersensitivity in rats subjected to either a paw incision surgery or acute carrageenan injection. Side-effect profiles of both peptides will be compared using the conditioned place preference paradigm and rotarod performance test

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Male, Sprague-Dawley rats (275-300 g; Harlan, Indianapolis, IN), were maintained on a 12-hr light/dark cycle (lights on 7am / lights off 7pm) with food and water available *ad libitum*. When possible, animals were housed socially. All procedures were performed in accordance with the policies and recommendations of the International Association for the Study of Pain, the National Institutes of Health guidelines for the handling and use of laboratory animals, and by the Animal Care and Use Committees of the University of Arizona.

2.2 Surgical procedures

Aseptic technique: All surgeries were performed aseptically. Specifically, stereotaxic devices were sanitized before use by wiping off any visible debris with Versaclean, wiped with 70% ethanol, then flat areas are covered with a blue underpad. Instruments were autoclaved and guide cannulas were gas sterilized. The instruments were cleaned between surgeries with 70% ethanol and placed in bead sterilizer. The animals are monitored closely until euthanasia while recording in the notebook their weight, appearance, and pain behaviors. Animals' condition was monitored twice daily for the first three days post-surgery and recorded in lab notebook maintained in laboratory.

Intracerebroventricular Cannulation (i.c.v.): Direct injection of solution into the lateral ventricles allowed study of any supraspinal effect, therefore, indwelling cannulation in rats was performed. Rats were anesthetized using ketamine/xylazine 100 mg/ kg i.p. (vol/vol: 80/20, Sigma-Aldrich; St. Louis, Missouri USA) and placed in a stereotaxic head holder with the nose bar set at -3.5 mm. The skull was exposed approximately 2 cm. Two 1 mm holes were then drilled by hand (DH-0 Pin Vise, Plastics One Inc., Roanoke, VA) on either side of midline, at least 3 mm back from bregma, and stainless steel screws (#MPX-080-3F-1M, Small Parts Inc., Miami Lakes, FL) attached, keeping the screws 2mm from the midline to avoid large blood vessels in the dura. The guide cannula (C131, PlasticsOne) was inserted using the following

coordinates from bregma, according to the rat brain atlas (Paxinos and Watson, 1998): Anteroposterior: -1.5 mm; mediolateral: -2 mm; dorsoventral: -3 mm from the skull. The cannula was inserted with a slow, smooth motion and superglued carefully to the skull without clogging the inner diameter. Dental cement was applied around the screws and cannula to further secure the placement to the skull and allowed to dry for 5 - 10 min. A dummy cannula was inserted to prevent clogging of the inner diameter prior to experimental test days. Rats were housed individually and allowed 5-7 days recovery before any manipulation was performed.

Intrathecal Cannula Implantation (i.th.): Rats were anesthetized using ketamine/xylazine 100 mg/ kg i.p. (vol/vol: 80/20, Sigma-Aldrich; St. Louis, Missouri USA) and placed in a stereotaxic head holder. Rats were placed on a heating pad and head placed in stereotax with the nose pointed down at a 120 degree angle. A 1.5cm longitudinal incision was made from the back ridge of the skull to C1 (feel for the separation of the muscles, “butt crack” of the head). Exposing the atlanto-occipital membrane by using the retractor to spread the muscles, a very shallow (< 0.5mm), T shaped, 1-2 mm incision was made using the #11 blade into membrane (diamond shaped, surrounded by bone) in order to remove clear CNS fluid from the incision site. Lifting or pulling rat by tail (to straighten the spine) with right hand, catheter was inserted flat to the skull and slowly advanced to the lumbar enlargement of the spinal cord. The anesthesia plane was deep enough to allow advancement of the catheter without “twitching” of the animal. The catheter knot was secured into the muscle mass above the membrane with 3-0 silk suture and the skin incision is closed with wound clips. Each rat was given an injection of gentamicin (8 mg/ml, 1ml/kg, s.c.) and 5 cc of prophylactic saline s.c. Catheters were checked for leaking or blood in the tube by flushing with 50ul heparin/saline and resealed with a cautery tool.

Paw Incision (P.I.): Paw incision injury produces signs of post-operative pain including mechanical allodynia and thermal hypersensitivity [22]. All incision surgical operations occurred 5 -7 days after intrathecal catheter implantation. Rats were anesthetized with 2.5% isoflurane in O₂ anesthesia delivered at 2 L/ min. The plantar aspect of the left hind paw was scrubbed with betadine and 70% alcohol three times. A 1-cm long incision, starting 0.5cm from the heel and extending toward the toes, was made with a number 11-blade, through skin and fascia of the plantar aspect of the left hind paw including the underlying muscle. The plantaris muscle was

then elevated and longitudinally incised, leaving the muscle origin and insertion intact. After hemostasis with gentle pressure, the skin was closed with two mattress sutures of 5-0 nylon on a curved needle. Rat were given an injection of gentamicin (1ml/kg of 8 mg/ml, s.c.) and allowed to recover from the anesthesia before returning it to the housing pan. Sham animals were anesthetized and the left hind paw scrubbed with betadine three times, then 70% ethanol, but no incision was made. All animals were allowed twenty four hours to recover prior to any behavioral testing. Any animals exhibiting signs of motor deficiency, infection, or greater than 10% loss in total body weight were euthanized.

2.3 Injection Procedures

Intracerebroventricular (i.c.v.) Compound Administration: For rats with indwelling i.c.v. cannula, tubing connected the injection cannula (C313I) to a 10 μ l Hamilton syringe that was filled with 5 μ L of experimental or control compound followed by 0.5 μ L air and a 4 μ l saline flush. The saline flush monitored the movement of the fluid through the tubing. After waiting 30 seconds, the injector was slowly removed and the dummy cannula inserted to prevent any back-flow of drug.

Intrathecal (i.th.) Compound Administration: Rats with indwelling i.th. cannulas to the lumbar level were injected while moving freely in their home cages. Briefly, tubing connected the injection cannula (C313I) to a 25 μ L Hamilton syringe filled first with 9 μ L saline followed by 0.5 μ L air and 5 μ L of experimental compound. The saline flush monitored the movement of the fluid through the tubing. Tubing was disconnected from the indwelling cannula after 15 seconds to prevent any back-flow of drug.

Intraperitoneal (i.p.) Compound Administration: Injected anesthesia was given intraperitoneally, though no experimental compounds were evaluated after this route of administration. Briefly, animals were lightly restrained by hand and inverted allowing for exposing of the ventral lower quadrants of the body. A 25 gauge needle was inserted at a 45° angle and positioned so the tip pointed toward midline. Volume of injection was 1ml/kg.

Subcutaneous Compound Administration: Subcutaneous (s.c.) injections were performed by manually holding the animal and inserting a 25 gauge disposable needle on a disposable 1 mL

syringe dorsally, assuring that the needle remained between the muscle and the skin of the animal. Injections of compounds or vehicles were performed over a 5 sec period and were noted as positive by the development of an out-pocketing of the skin at the site of injection. All compounds were dosed in mg/ kg as determined by total body weight in a 1 ml/kg volume.

Intraplantar Compound Administration: Intraplantar (i.pl.) injections were performed by lightly restraining the animal in a plastic tube with ventilation with its left hind paw exposed. A 25 gauge needle was inserted parallel to its dorsal surface. Volume of injection of carrageenan was 50 μ l of 2% carrageenan in MilliQ water and the volume of injection of experimental compounds varied by the weight of the animal.

2.4 Compounds and Reagents

Anesthesia: Induction of anesthesia was performed using both injected and inhaled general anesthetics. The majority of procedures were performed under ketamine/xylazine (vol/vol: 80/20, respectively) purchased from Sigma-Aldrich (cat # K-113). The ketamine/xylazine mixture was given in a dose of Ketamine (80 mg/ml):Xylazine (12 mg/ml, 1 mL/ kg injection volume, i.p.). Paw incision and sham-operations were induced at 5% and maintained under inhaled anesthesia with 2.5 % isoflurane (Fisher Scientific, cat # NC9171659; Tustin, CA, USA) in air delivered at 2 L/ min.

Control Compounds: A random control peptide (YGRKKRRQRRRWEAKEMLYFEALVIE); two scramble CBD3 peptides myr-tat-CBD3scr1 (sequence: YGRKKRRQRRRAALRLLPRERGRGSV, the underlined sequence is a scramble version the CBD3 peptide), and myr-tat-CBD3scr2 (sequence: YGRKKRRQRRRLPLVRRLEARGRGAS) were provided and synthesized and HPLC-purified by Genscript USA Inc. (Piscataway, NJ). The control peptides were dissolved in saline for intrathecal administration (20 μ g in 5 μ L injection volume).

Experimental compounds: tat-CBD3 (YGRKKRRQRRRARSLAELRGVPRGL) and myr-tat-CBD3 (N-myristoyl- YGRKKRRQRRRARSLAELRGVPRGL), with tat sequence denoted in underlined text were provided and synthesized and HPLC-purified by Genscript USA Inc.

(Piscataway, NJ). The experimental peptides were dissolved in saline for intrathecal and intracerebroventricular administration (20 µg in 5 µL injection volume).

Carrageenan: Carrageenan (Sigma, cat # C1867) was purchased from Sigma. The compound was dissolved in MilliQ water with final carrageenan concentration of 2%.

2.5 Behavioral Testing Protocols

Antinociception: The efficacy of experimental compounds against normally painful stimuli was assessed in the absence of injury as an initial screening process by measuring paw withdrawal latency in rats.

Paw Withdrawal latency: Rats were allowed to acclimate to the testing room in Plexiglas holders on a glass table (Ugo Basile, Comerio, Italy) for 30 min prior to testing. Baseline paw withdrawal latencies (PWL) to an infrared radiant heat source [23] were measured and typically ranged between 17.0 and 23.0 s. The heat intensity was set to 40, a cut off of 33.0 s was established to prevent tissue damage. Observed values were obtained at time points appropriate for the chosen route of administration.

Carrageenan: A 2% carrageenan solution was used to induce inflammation in the hind paw (50 µL, i.pl.). Baseline paw withdrawal latencies to a radiant heat source were obtained as described above pre- and 3 hours post-carrageenan. Testing was at appropriate time points for the chosen compound of interest. The infrared, noxious stimuli was aimed at the inflamed paw (left hind), until the animal moved its foot and the timer stopped. Results were quantified into averages, and standard error determined. In a separate experiment, hind paw edema was assessed by measuring paw thickness. Measurements of paw thickness were made before carrageenan administration and 3 hours after carrageenan administration.

Rotarod Performance Test: Following placement of the intrathecal catheters, the rats were trained to walk on a rotating rod (10 rev/min; Rotamex 4/8 device) with a maximal cutoff time of 180 seconds [24]. Training was initiated by placing the rats on a rotating rod and allowing them to walk on the rotating rod until they either fell off or 180 seconds was reached. This process was repeated 6 times and the rats were allowed to recover for 24 hours before beginning the treatment session. Prior to treatment, the rats were run once on a moving rod in order to establish a baseline

value. Saline or peptides were administered spinally via the intrathecal catheter. Assessment consisted of placing the rats on the moving rod and timing until either they fell off or reached a maximum of 180 seconds. This was repeated every 15 minutes for the first hour after drug administration and 30 minutes for the 4 hours after for a maximum time course of 5 hours.

Mechanical Hypersensitivity: The assessment of mechanical hypersensitivity consisted of measuring the withdrawal threshold of the paw ipsilateral to the site of injury in response to probing with a series of calibrated von Frey filaments (innocuous stimuli). Animals were acclimated to the suspended wire-mesh cages for 30 min before testing. Each von Frey filament was applied perpendicularly to the plantar surface of the ligated paw of animals for 5 sec. A positive response was indicated by a sharp withdrawal of the paw. For rats, the first testing filament is 4.31g. Measurements were taken before and after administration of test articles. The paw withdrawal threshold was determined by the non-parametric method of Dixon, in which the stimulus was incrementally increased until a positive response was obtained, then decreased until a negative result was observed. The protocol was repeated until three changes in behavior are determined (“up and down” method [25]). The 50% paw withdrawal threshold was determined as $(10[X_f + k\delta])/10,000$, where X_f = the value of the last von Frey filament employed, k = Dixon value for the positive/negative pattern, and δ = the logarithmic difference between stimuli. The cut-off values for animals were no less than 0.2 g (3.61 filament) and no higher than 15 g (5.18 filament); A significant drop of the paw withdrawal threshold compared to the pre-treatment baseline is considered mechanical hypersensitivity. Paw withdrawal thresholds were calculated in grams using the Dixon non-parametric test and expressed as the mean paw withdrawal threshold \pm SEM in Prism by GraphPad. Contralateral paw was not tested, as injured animals placed more body weight on the uninjured paw than on the injured side.

Thermal Hypersensitivity: Rats were allowed to acclimate in Plexiglas holders for baseline testing (pre- and post-nerve ligation/ exposure) for 30 minutes (Ugo Basile, Comerio Italy). A mobile radiant heat source was used to direct heat to the plantar surface of the left hind paw as reported [23]. Paw withdrawal latencies (PWLs) were measured in seconds with an automatic shutoff of the heat source at 33.0 s. Baselines and pre-nerve injury PWLs were established between 17.0 - 23.0 s for antinociception experiments. Post-injury baselines were obtained at

appreciate time point dictated by the experimental protocol. On test days, animals were dosed and paw withdrawal latencies were recorded for each rat; paw withdrawal latencies were expressed as the mean withdrawal latency \pm SEM in Prism by GraphPad. The contralateral paw was not evaluated for hypersensitivity as injured animals placed more body weight on the uninjured paw when compared to the injured side.

Conditioned Place Preference/ Conditioned Place Aversion (CPP/CPA): The ability of a drug to be reinforcing (potentially rewarding) can be measured using conditioned place preferences [26]. The amount of time a rat spends in the putative side of conditioning is recorded before and after drug conditioning occurs. The animal is exposed to both drug and vehicle, each treatment paired with the separate visual and mechanical cues of a two chamber box, over the course of five drug exposures. Rat CPP boxes from San Diego Instruments (San Diego, CA) have end chambers customized as follows: horizontal black and white striped walls with a smooth floor or grey walls with a rough floor. The center transition chamber was always set to have bright light and a grated floor. Sixteen y-axis sensors and four x-axis sensors allow for record fine motor, ambulatory, and exploratory movements. Each automated recording session was programmed to start all boxes in unison for a total of 20 min, recording at 5 min intervals. Animals were allowed to habituate to the testing room for 60 min prior to baseline recordings. Two baseline recordings were obtained on days 1 and 4 and the average used as the overall baseline and preconditioning time per chamber. Any animal showing a greater than 80% total time preference prior to conditioning was excluded.

At random, animals were separated into treatment groups and conditioned to associate one of the isolated end chambers with treatment and the opposite end chamber with vehicle. Although previous literature suggests penetration of tat-CBD3 into the brain following an intraperitoneal injection [18], here the intracerebroventricular route was chosen so as to allow for assessment of direct actions of the peptides on CNS regions associated with reward pathways. The time course of conditioning was as follows: On days 5, 7, 9, 11, and 13, tat-CBD3 or myrtat- CBD3 (10 μ g in 5 μ L, i.c.v.) were paired with one of the end-chambers, while rats were confined to that chamber for 20 min. On days 6, 8, 10 and 12, animals received an injection of vehicle (saline 5 μ L, i.c.v.) paired with the opposite chamber. On day 14, in a peptide-free state, the total time spent by the

rat was recorded and compared to baseline values for each chamber (paired t-test). Preference was instated if there was a significant increase in the total time spent in the putative conditioning chamber; likewise, aversion was noted by a significant decrease in total time.

2.6 Data Analysis

GraphPad Software (LaJolla, CA) was used to determine the statistical significance. The statistical significance of differences between means was determined by Student's t-test or a one- or two-way ANOVA followed by post hoc comparisons (Dunnett's or Tukey's test). Groups were compared using ANOVA followed by pair-wise comparisons using the Student's t test with Bonferroni correction. Repeated-measures ANOVA was used when repeated measurements were made on the same animals. Significance was defined as $P < 0.05$.

CHAPTER 3: RESULTS

3.1 tat-CBD3 and myr-tat-CBD3 peptides reverse carrageenan-induced inflammatory pain

Administration of carrageenan (50 μ L of 2%) significantly decreased paw withdrawal latencies (PWLs) by ~76% three hours post carrageenan injection (Fig. 1A-C). Intrathecal administration of tat-CBD3 (20 μ g/5 μ L, i.th.) significantly attenuated carrageenan-induced thermal hypersensitivity at 90, 120, 210, 240, and 300 min following tat-CBD3 administration compared to vehicle group (Fig. 1A, 1D). Administration of myr-tat-CBD3 (20 μ g/5 μ L i.th.) significantly attenuated carrageenan-induced thermal hypersensitivity at 15, 60, 90, 120 and 180 min following myr-tat-CBD3 administration compared to vehicle group (Fig. 1B, 1D). Data are plotted as area under the curve (AUC) using the trapezoid method, for PWLs for studies in figure 1. The administration of control peptides myr-tat or myr-tat-CBD3 scr1 or my-tat-SBD3 scr2 did not decrease carrageenan-induced thermal hypersensitivity (Figure 1C-1D). Administration of carrageenan (50 μ L of 2%) produced a significant increase in paw thickness 3 hours post carrageenan injection (Fig. 1E). The carrageenan-induced edema was not blunted by either peptide suggesting that the peptides do not reduce inflammatory factors, edema, and injury-induced vasodilation but are active against inflammatory pain by inhibiting nociceptive fibers.

Thermal Latency

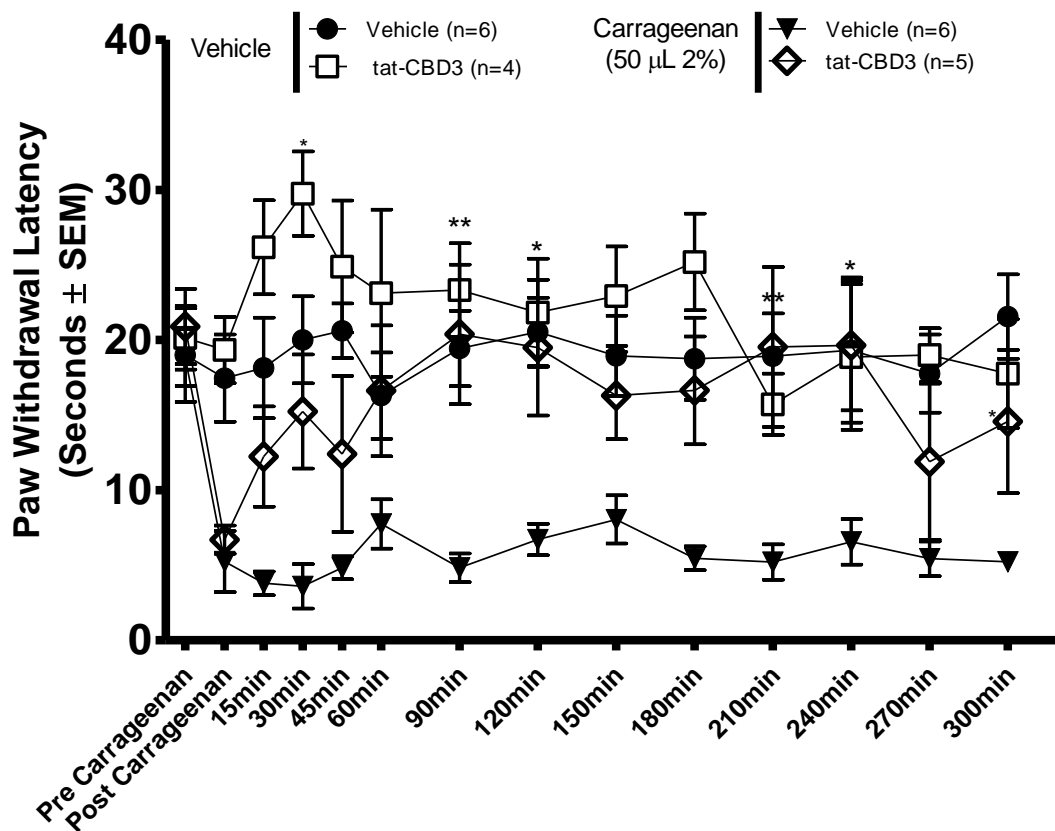


Figure 1A. Spinal administration of tat-CBD3 (20 µg/5 µL i.th.) significantly attenuate carrageenan (50 µL 2% i.pl.) induced thermal hypersensitivities in rats. Paw withdrawal latencies resulted in a significant decrease 3 hours post-carrageenan injection. Administration of tat-CBD3 (20 µg/5 µL i.th.) significantly attenuated carrageenan induced thermal hypersensitivity 90, 120, 210 and 240 minutes following tat-CBD3 administration compared to vehicle treated group (n=(5-6), *p < 0.05, **p < 0.01, ***p < 0.001). Data analyzed using two way ANOVA followed by Bonferroni post-tests where time was treated as "within subjects" factor, while treatment was treated as "between" subjects factor.

Thermal Latency

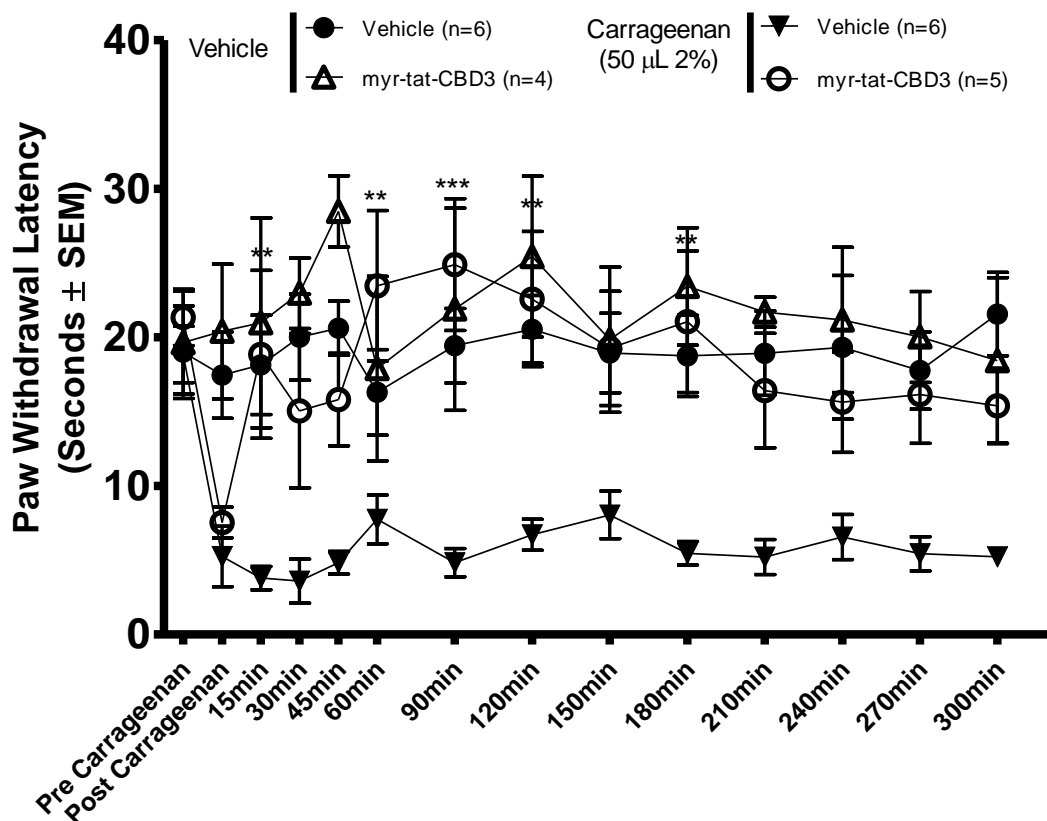


Figure 1B. Spinal administration of myr-tat-CBD3 (20 µg/5 µL i.th.) significantly attenuate carrageenan (50 µL 2% i.pl.) induced thermal hypersensitivities in rats. Paw withdrawal latencies resulted in a significant decrease 3 hours post-carrageenan injection. Administration of myr-tat-CBD3 (20 µg/5 µL i.th.) significantly attenuated carrageenan induced thermal hypersensitivity 15, 60, 90, 120 and 180 minutes following myr-tat-CBD3 administration compared to vehicle treated group (n=(5-6), *p < 0.05, **p < 0.01, ***p < 0.001). Data analyzed using two-way ANOVA followed by Bonferroni post-tests where time was treated as "within subjects" factor, while treatment was treated as "between" subjects factor.

Thermal Latency

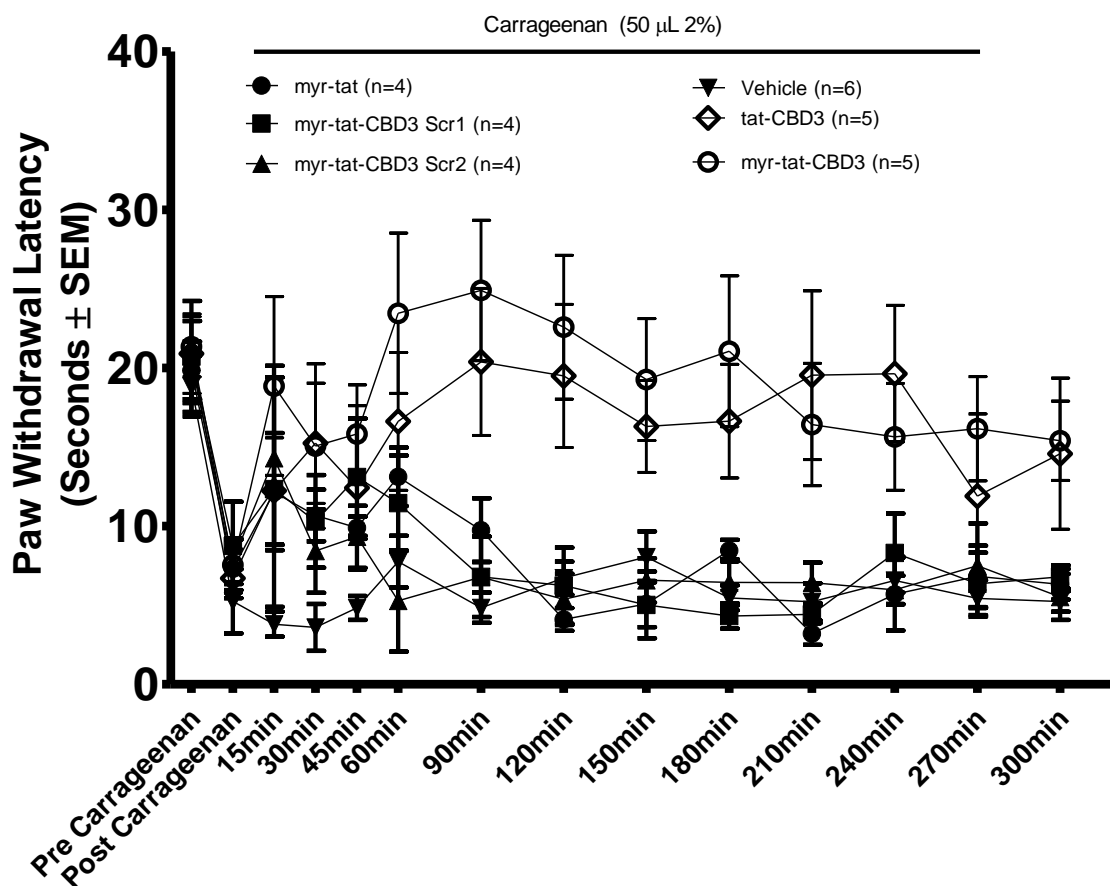


Figure 1C. Spinal administration of control peptides: myr-tat (20 μ g/5 μ L i.th.), myr-tat-CBD3 scr1 (20 μ g/5 μ L i.th.) or myr-tat-CBD3 scr2 (20 μ g/5 μ L i.th.) did not significantly attenuate thermal hypersensitivity in carrageenan-induced inflammatory pain.

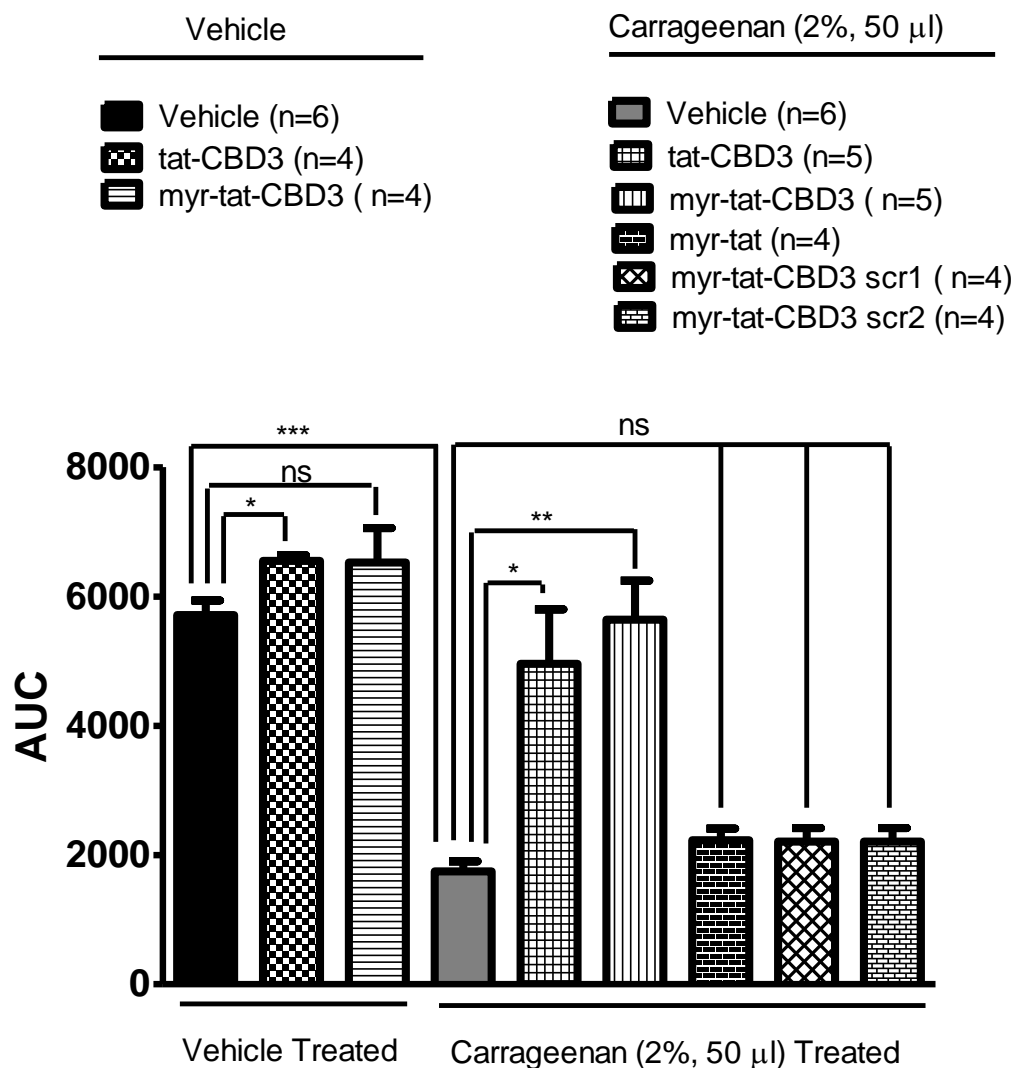


Figure 1D. Summary of the data in figure 1A through figure 1C was plotted as area under the curve (AUC) using the trapezoid method, for PWLs. Thermal hypersensitivity induced via intraplantar administration of carrageenan (50 μ L 2% i.pl.) was significantly attenuated by the spinal administration of tat-CBD3 (20 μ g/5 μ L i.th.) and myr-tat-CBD3 (20 μ g/5 μ L i.th.) but not with the spinal administration of control peptides: myr-tat (20 μ g/5 μ L i.th.), myr-tat-CBD3 scr1 (20 μ g/5 μ L i.th.) or myr-tat-CBD3 scr2 (20 μ g/5 μ L i.th.).

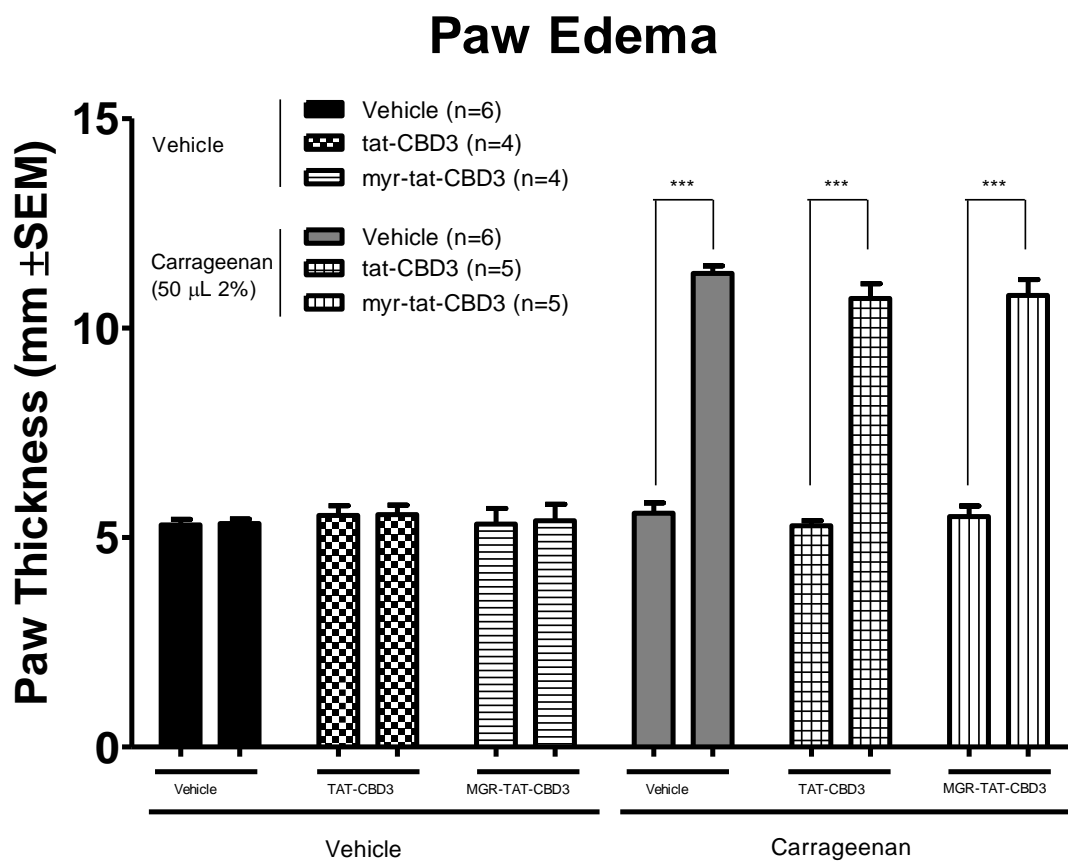


Figure 1E. Intraplantar administration of carrageenan (50 μL 2%) produced significant paw edema in rats 3 hours post-treatment. Data are expressed as mean ± SEM. Pre-carrageenan values were compared using Student t test. *** p < 0.001 when compared with pre-carrageenan values.

3.2 tat-CBD3 and myr-tat-CBD3 reverses paw incision induced post-operative pain

From recent literature, gabapentin, through its presumptive interaction with $\alpha 2\delta$ subunit of voltage gated calcium channels, has been shown to be antinociceptive in pre-clinical model of post-surgical pain [27]. Furthermore, in a clinical study, Ziconotide, a direct CaV2.2 blocker, decreased patient controlled analgesia morphine equivalent consumption and visual analog pain intensity scores [28].

Therefore, the anti-nociceptive potential of tat-CBD3 and myr-tat-CBD3 on thermal and mechanical hypersensitivity induced by an incision of the plantaris muscle of the rat hind paw, a model of post-operative surgical pain, was investigated. Incision of the rat plantaris muscle led to an induction of thermal and mechanical hypersensitivity (Fig. 2A-2H). Both nociceptive responses peaked within 24 h after surgery and were maintained during the 5 to 6 h experimental period in vehicle-treated animals. Spinal administration of tat-CBD3 and myr-tat-CBD3 peptides significantly reversed the thermal and mechanical hypersensitivity in paw incised animals for at least 4 hours post peptide administration (Fig. 2A-2C, 2E-2G). The antihyperalgesic effect of tat-CBD3 waned after 4 hours (Fig. 2E) while that of myr-tat-CBD3 was sustained throughout the 6 hour time course evaluated (Fig. 2F). The reversal of tactile hypersensitivity following injection of myr-tat-CBD3 was far better than tat-cbd3 with observed withdrawal thresholds: $\sim 11 \pm \text{SEM}$ g vs $5 \pm \text{SEM}$ g at 5 h, respectively. Neither peptide altered mechanical thresholds in sham-injured animals. The vehicle (saline) did not significantly increase injury induced paw withdrawal thresholds. Importantly, none of the control peptides (myr-tat, myr-tat-CBD3 scr1, or myrtat-CBD3 scr2) altered thermal or mechanical hypersensitivities (Fig. 2C and 2G). Thermal and mechanical hypersensitivity data are plotted as area under the curve (AUC) using the trapezoid method, for paw withdrawal latencies (PWLs) for studies in figure 2A-2C and for paw withdrawal thresholds (PWTs) for studies in figure 2E-2G, respectively.

Thermal Latency

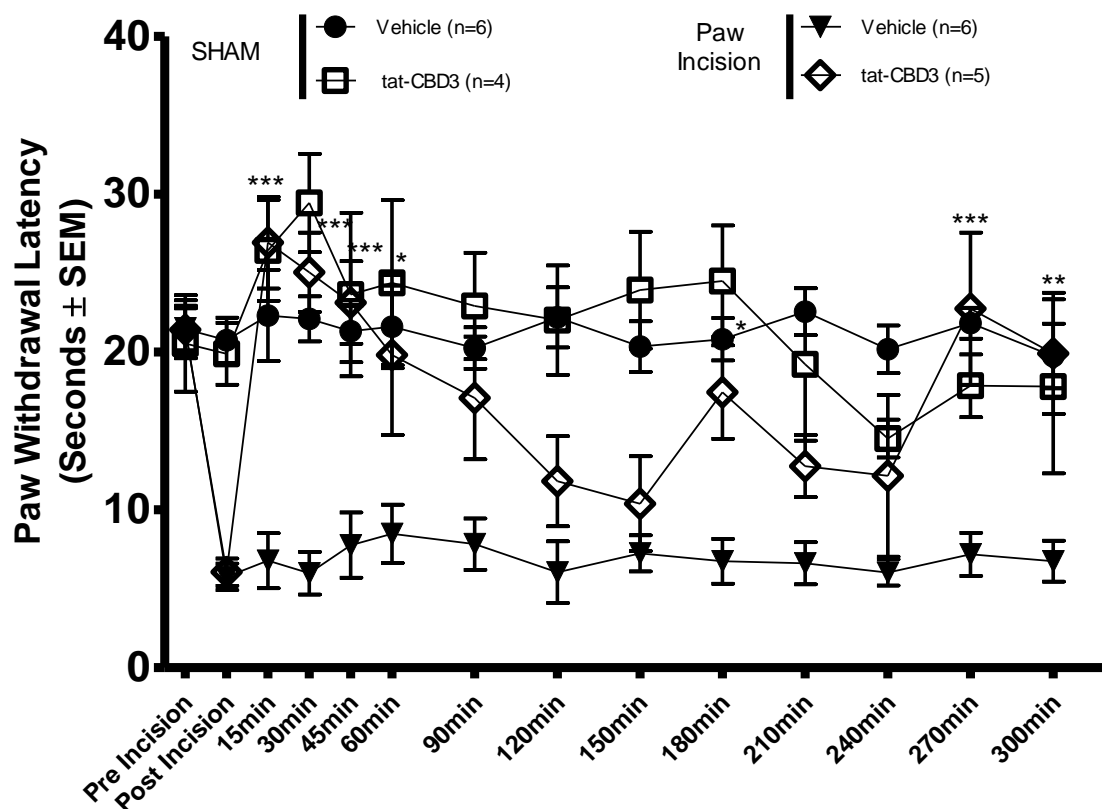


Figure 2A. The spinal administration of tat-CBD3 (20 $\mu\text{g}/5 \mu\text{L}$) significantly reversed thermal hypersensitivity in rat model of post-operative pain. Paw withdrawal latencies resulted in a significant decrease 24 hours post-incision that was significantly reversed at time 15, 30, 45, 60, 180, 270 and 300 minutes following tat-CBD3 administration compared to vehicle treated group (n=5, *p < 0.05, **p < 0.01, ***p < 0.001). Data analyzed using two way ANOVA followed by Bonferroni post-tests where time was treated as "within subjects" factor, while treatment was treated as "between" subjects factor.

Thermal Latency

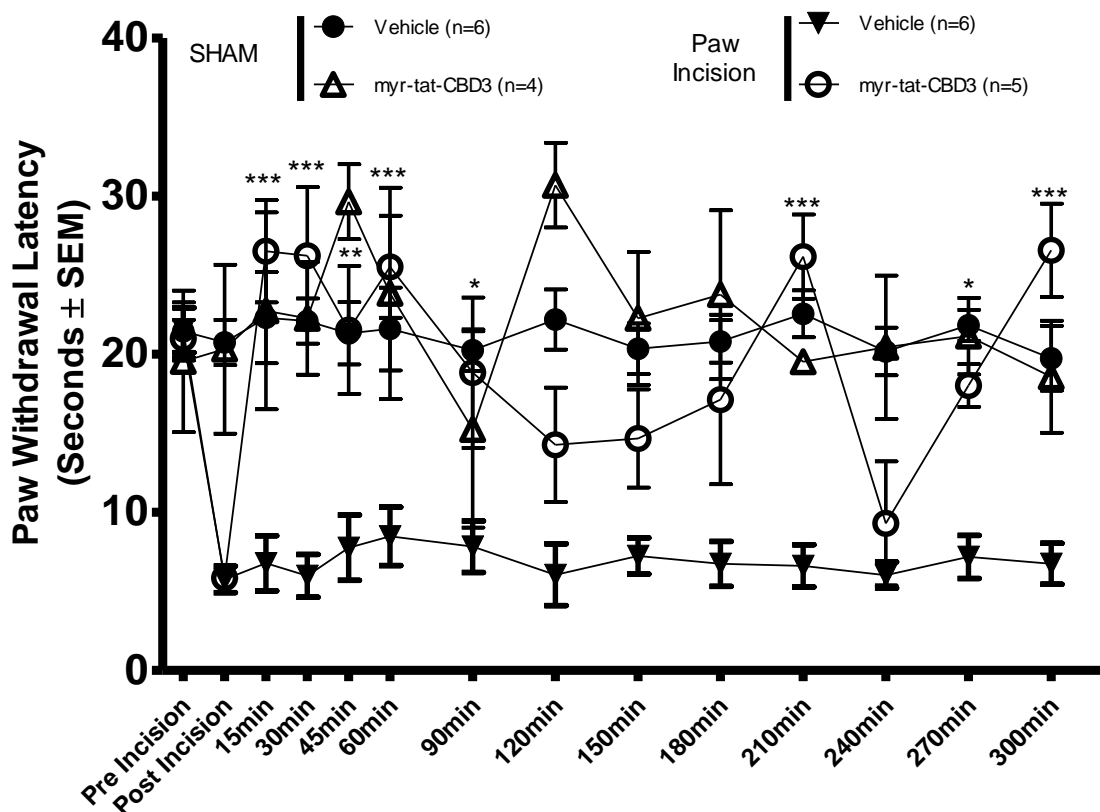


Figure 2B. The spinal administration of myr-tat-CBD3 (20 $\mu\text{g}/5 \mu\text{L}$) significantly reversed thermal hypersensitivity in rat model of post-operative pain. Paw withdrawal latencies resulted in a significant decrease 24 hours post-incision that was significantly reversed at time 15, 30, 45, 60, 210, 270 and 300 minutes following myr-tat-CBD3 administration compared to vehicle treated group (n=5, *p < 0.05, **p < 0.01, ***p < 0.001). Data analyzed using two-way ANOVA followed by Bonferroni post-tests where time was treated as "within subjects" factor, while treatment was treated as "between" subjects factor.

Thermal Latency

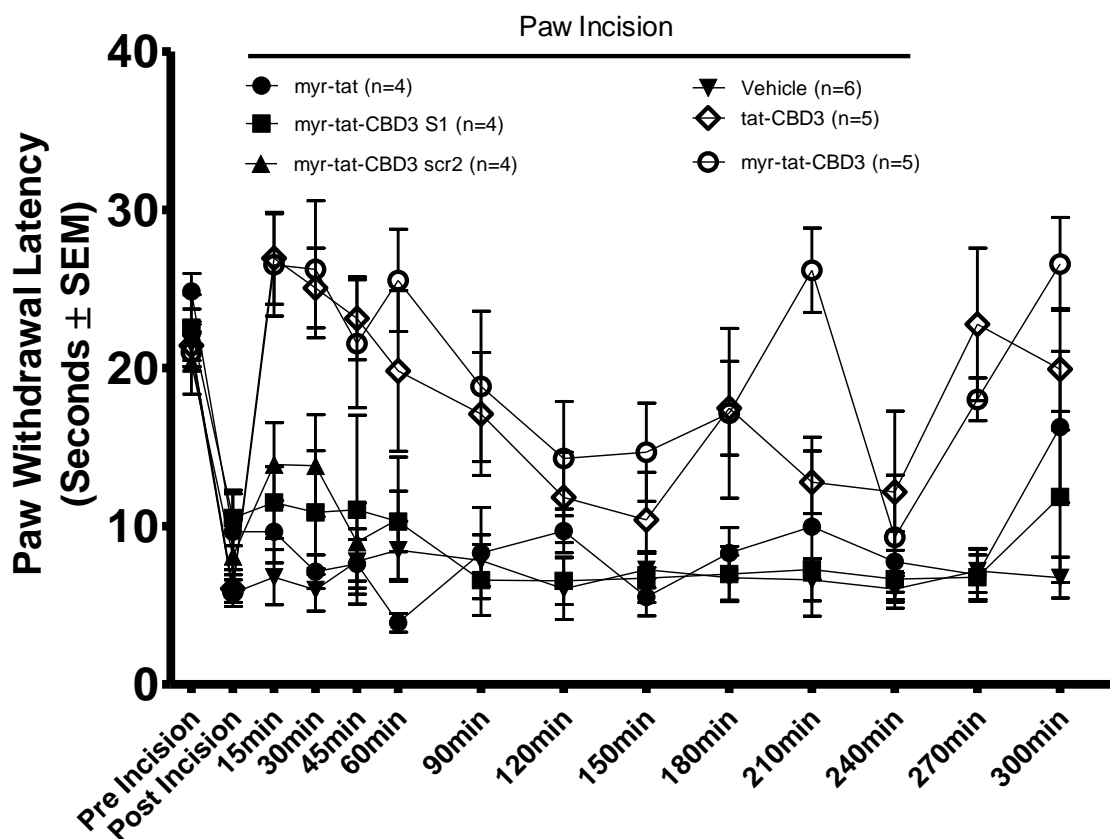


Figure 2C. Spinal administration of control peptides: myr-tat (20 μ g/5 μ L i.th.), myr-tat-CBD3 scr1 (20 μ g/5 μ L i.th.) or my-tat-CBD3 scr2 (20 μ g/5 μ L i.th.) did not produce any significant attenuation of thermal hypersensitivity in paw incision model of post surgical pain.

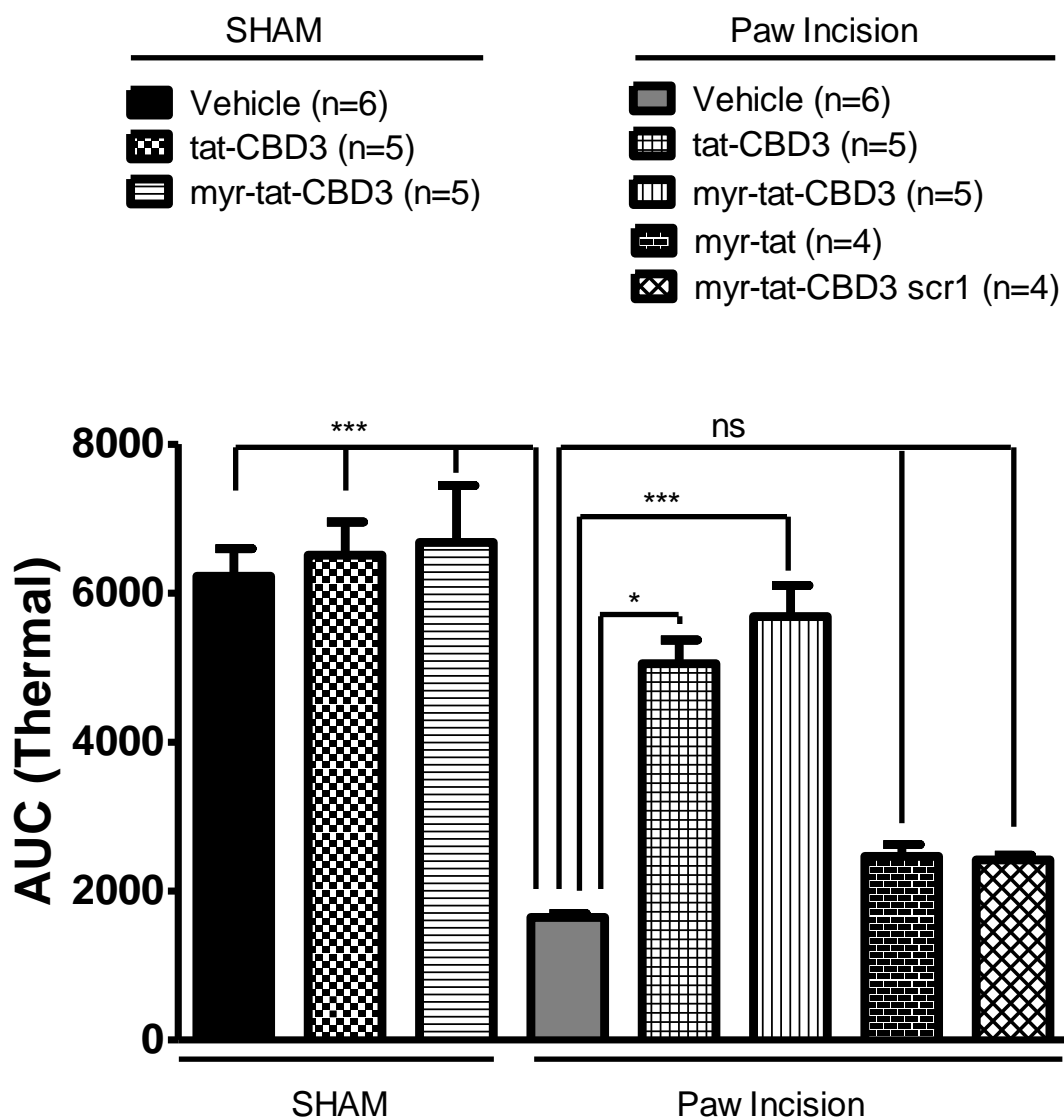


Figure 2D. Summary of the data in figure 2A through figure 2C was plotted as area under the curve (AUC) using the trapezoid method, for PWLs. Thermal hypersensitivity induced via paw incision was significantly attenuated by the spinal administration of tat-CBD3 (20 μ g/5 μ L i.th.) and myr-tat-CBD3 (20 μ g/5 μ L i.th.) but not with the spinal administration of control peptides: myr-tat (20 μ g/5 μ L i.th.), myr-tat-CBD3 scr1 (20 μ g/5 μ L i.th.) or my-tat-CBD3 scr2(20 μ g/5 μ L i.th.).

Mechanical Threshold

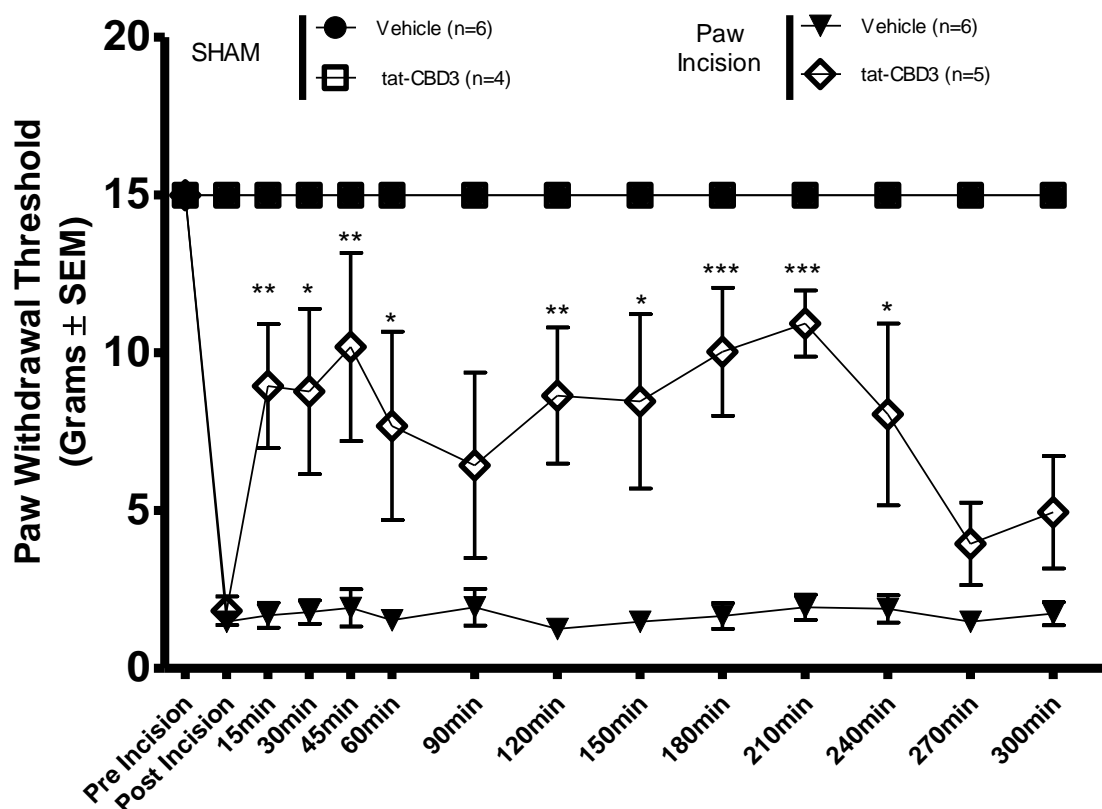


Figure 2E. The spinal administration of tat-CBD3 (20 $\mu\text{g}/5 \mu\text{L}$) significantly reversed mechanical hypersensitivity in rat model of post-operative pain. Paw withdrawal threshold resulted in a significant decrease 24 hours post-incision that was significantly reversed at time 15, 30, 45, 60, 120, 150, 180, 210, and 240 minutes following tat-CBD3 administration compared to vehicle treated group (n=5, *p < 0.05, **p < 0.01, ***p < 0.001). Data analyzed using two way ANOVA followed by Bonferroni post-tests where time was treated as "within subjects" factor, while treatment was treated as "between" subjects factor.

Mechanical Threshold

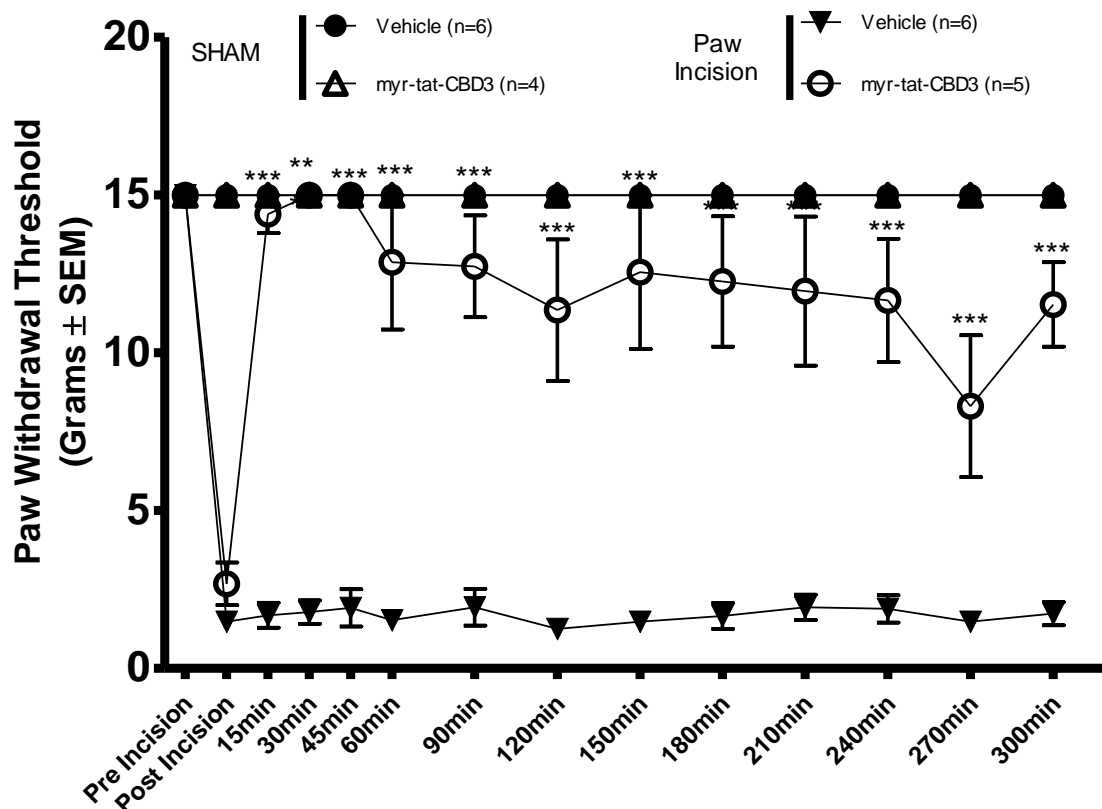


Figure 2F. The spinal administration of myr-tat-CBD3 (20 $\mu\text{g}/5 \mu\text{L}$) significantly reversed mechanical hypersensitivity in rat model of post-operative pain. Paw withdrawal threshold resulted in a significant decrease 24 hours post-incision that was significantly reversed for 5 hours following myr-tat-CBD3 administration compared to vehicle treated group ($n=5$, $***p < 0.001$). Data analyzed using two way ANOVA followed by Bonferroni post-tests where time was treated as "within subjects" factor, while treatment was treated as "between" subjects factor.

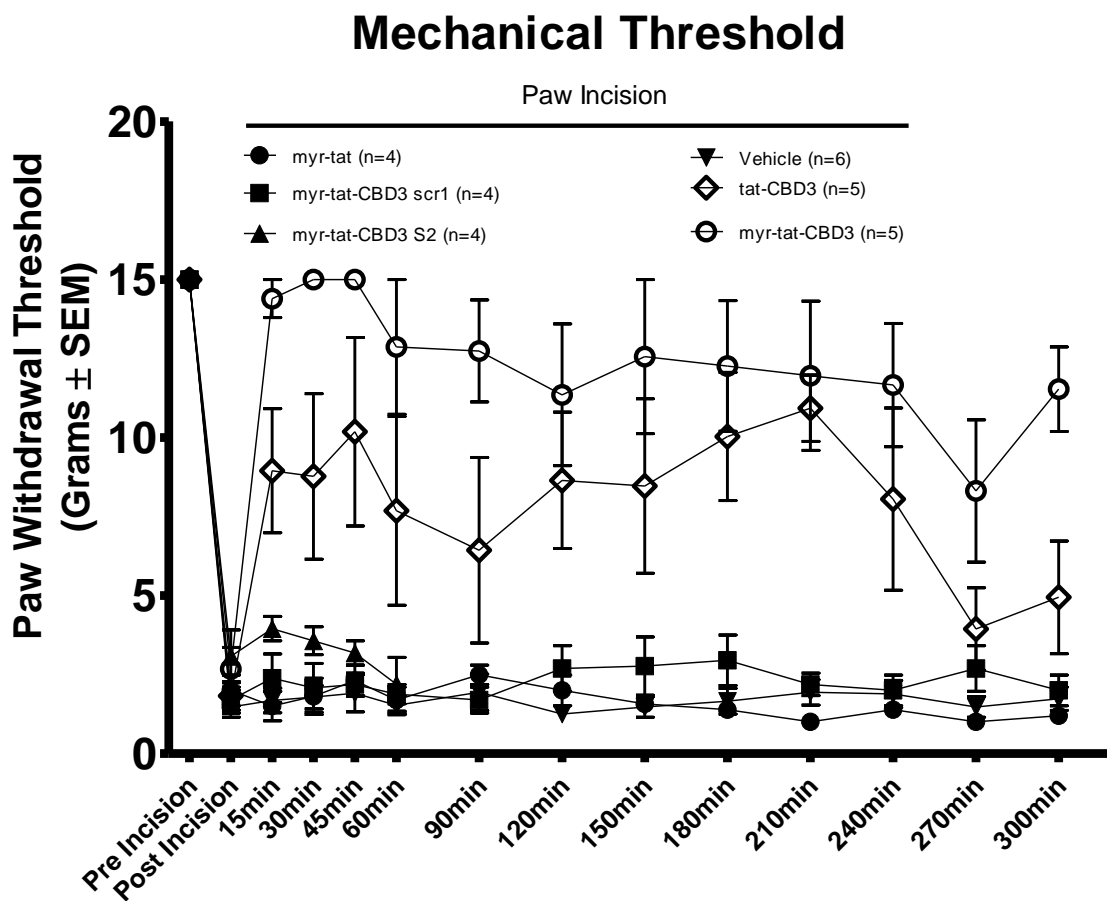


Figure 2G. Spinal administration of control peptides: myr-tat (20 $\mu\text{g}/5 \mu\text{L}$ i.th.), myr-tat-CBD3 scr1 (20 $\mu\text{g}/5 \mu\text{L}$ i.th.) or my-tat-CBD3 scr2 (20 $\mu\text{g}/5 \mu\text{L}$ i.th.) did not produce any significant attenuation of mechanical hypersensitivity in paw incision model of post-surgical pain.

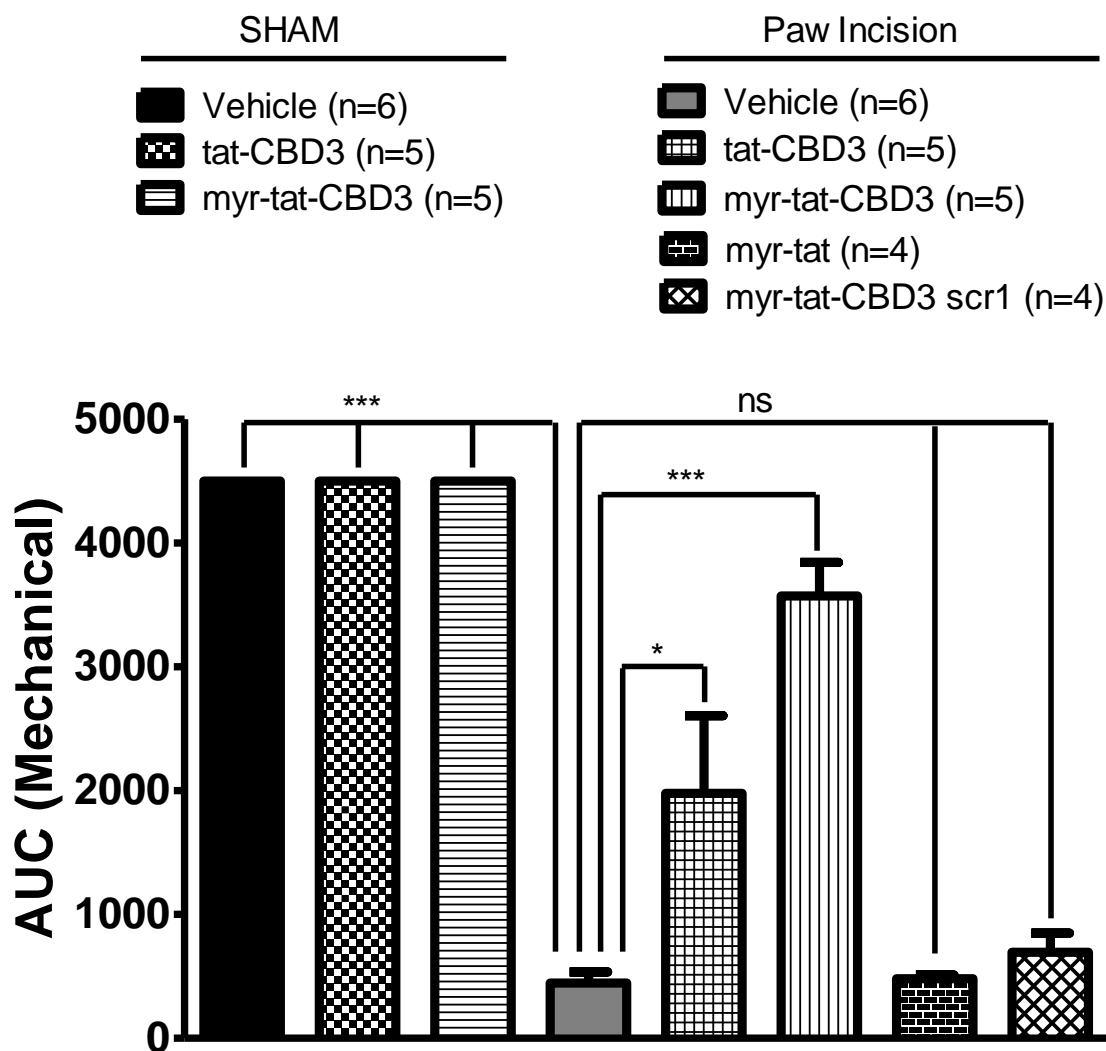


Figure 2H. Summary of the data in figure 2E through figure 2G was plotted as area under the curve (AUC) using the trapezoid method, for PWTs. Mechanical hypersensitivity induced via paw incision was significantly attenuated by the spinal administration of tat-CBD3 (20 μ g/5 μ L i.th.) and myr-tat-CBD3 (20 μ g/5 μ L i.th.) but not with the spinal administration of control peptides: myr-tat (20 μ g/5 μ L i.th.), myr-tat-CBD3 scr1 (20 μ g/5 μ L i.th.) or my-tat-CBD3 scr2(20 μ g/5 μ L i.th.).

3.3 tat-CBD3 and myr-tat-CBD3 does not produce reward like effects as measured using conditioned place preference

The ability of a drug to be reinforcing (potentially rewarding) can be measured using conditioned place preferences [26]. Male SD rats were conditioned using tactile and visual cues to associate one end-chamber of a 3 chamber CPP/CPA apparatus with a given treatment (San Diego Instruments, SDI). Drug administration to rats was paired with a chamber for 5 exposures; total time spent in that paired chamber was measured 24 hours after the final exposure, with animals in a drug-free state (Fig. 3A). At baseline, all rats spent 43-45% of the total time in the putative conditioning chamber over a 20-minute period (526.6 ± 52.8 sec; Figure 3B). Unlike previous studies demonstrating that morphine (i.c.v. dosing) results in a positive conditioned place preference indicative of the potential of rewarding behavior [29], tat-CBD3 and myr-tat-CBD3 ($10 \mu\text{g}$ in $5\mu\text{L}$, i.c.v.) did not produce a significant increase in total time spent per chamber ($45.7 \pm 7.1\%$ and $46.5 \pm 5.9\%$, respectively; $p=0.76$) nor a decrease suggesting lack of conditioned place aversion (Figure 3B). The CPP experiments performed here used an intracerebroventricular route of administration that allowed for direct delivery to the brain centers for rewarding behavior hence giving us the best ability to detect whether these peptides would be abused or avoided. These data suggest that both peptides act neutrally at supraspinal sites and are not likely to produce rewarding or aversive behaviors.

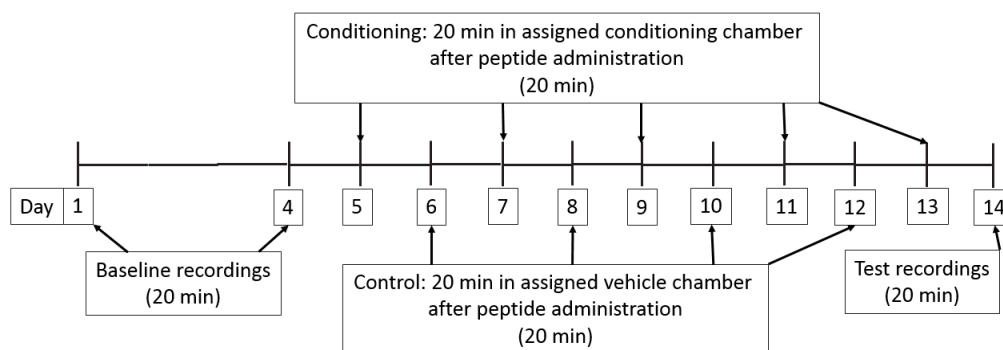


Figure 3A. Timeline schematic of conditioned place preference conditioning schedule. Baseline (BL) values were determined from an average between 2 exposures to an open field of the SDI system prior to compound administration for each animal. On compound administration days, animals were confined to one end chamber (the same throughout the experiment) for 20 min. On alternative days, animals were treated with vehicle and confined to the chamber opposite of the putative conditioning chamber. On day 14, 24 hours after final drug exposure, experimental values were obtained.

Conditioned Place Preference

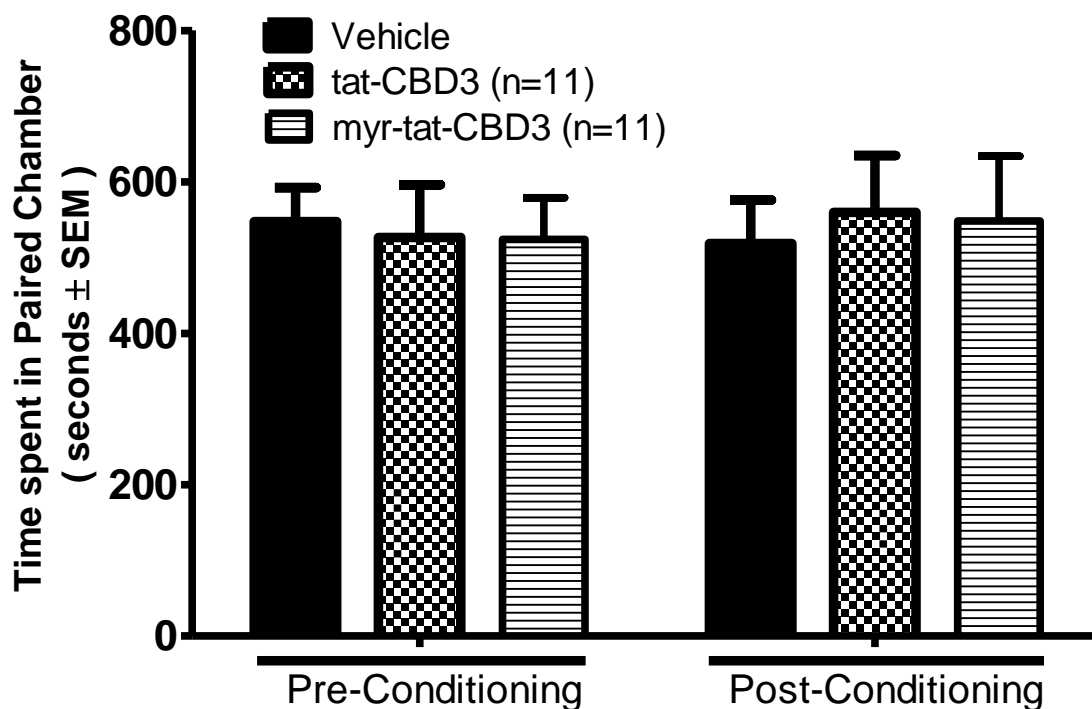


Figure 3B. Intracerebroventricular administration of tat-CBD3 (10 $\mu\text{g}/5 \mu\text{L}$ i.c.v.) and myr-tat-CBD3 (10 $\mu\text{g}/5 \mu\text{L}$ i.c.v.) does not induce conditioned place preference in rats. Baseline prior to drug administration resulted in animals spending approximately equal time on both sides of the CPP chambers (547.5, 550.7 and 526.7 seconds in vehicle, tat-CBD3 and myr-tat-CBD3 chambers respectively). Intracerebroventricular administration of tat-CBD3 (10 $\mu\text{g}/5 \mu\text{L}$ i.c.v.) or myr-tat-CBD3 (10 $\mu\text{g}/5 \mu\text{L}$ i.c.v.) did not result in significant increase in total time spent in putative conditioned chamber 24 hours post five drug exposure (518.7, 548.3 and 559.9 seconds in vehicle, tat-CBD3 and myr-tat-CBD3 chambers respectively. $p=0.73$ (2-tailed paired student's t-test; $n = 11$). Data represent the mean seconds \pm S.E.M.

3.4 tat-CBD3 and myr-tat-CBD3 does not produce any motor impairment or paralysis as measured using rotarod performance test

tat-CBD3 (20 µg/5 µL i.th.) and myr-tat-CBD3 (20 µg/5 µL i.th.) peptides were evaluated for typical motor deficits reported with opioids including sedation. Rats were trained to walk on a rotating rod with a maximal cutoff time of 180 seconds prior to administration of drug or vehicle. The mean baseline latency for all animals was 180 ± 0 seconds. Vehicle treated animals remained on the rotarod for an average of 180 ± 0 seconds at each time point over the course of 300 minutes. Animals treated with the control peptides myr-tat-CBD3 scr1 or myr-tat-CBD3scr2 (20 µg/5 µL i.th.) remained on the rotarod for an average of 172.9 ± 11.0 sec or 175.7 ± 12.2 sec, respectively, over the course of 300 minutes (Fig. 4). Animals treated with tat-CBD3 (20 µg/5 µL i.th.) remained on the rotarod for an average of 180 ± 0 seconds over the course of 300 minutes (Fig. 4). Animals treated with myr-tat-CBD3 (20 µg/5 µL i.th.) remained on the rotarod with minimum value of 147.4 ± 20.0 seconds, not significantly different from vehicle or control peptide-treated animals and baseline values. $p=0.18$ (2-tailed paired student's t-test; $n = 5$). (Fig. 4).

Rotarod

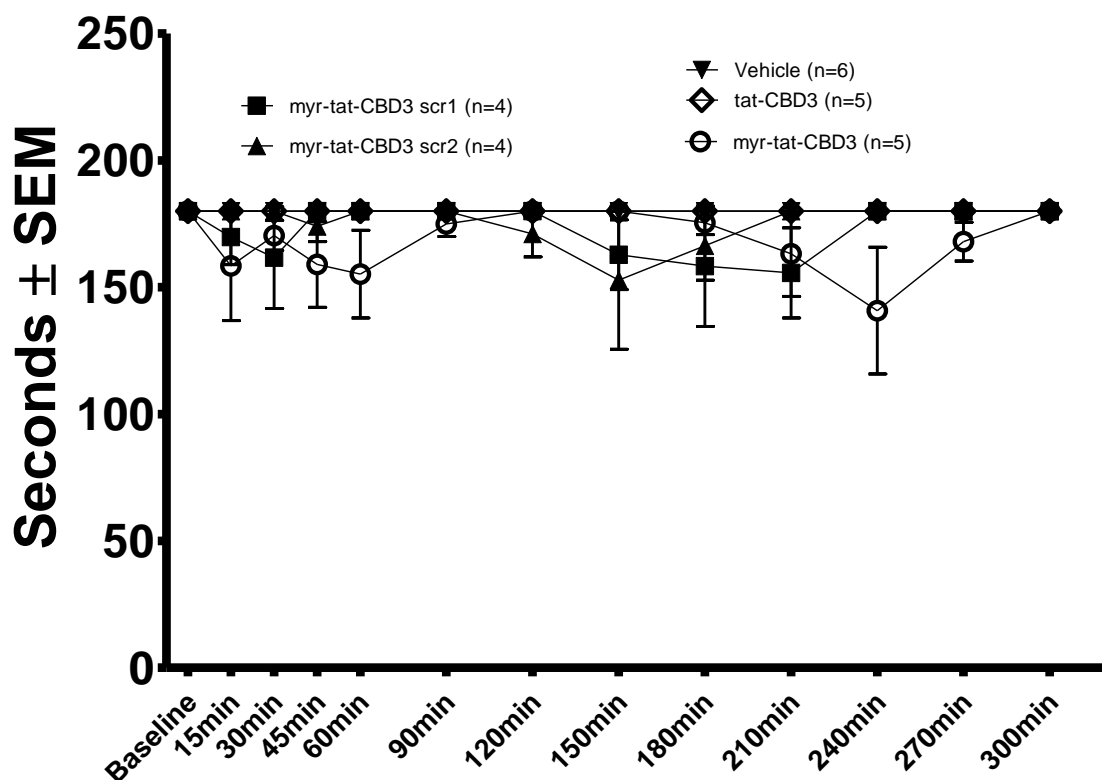


Figure 4. The spinal administration of tat-CBD3 and myr-tat-CBD3 did not produce significant deficits in motor coordination as tested using the rotarod performance test. tat-CBD3 (20 μ g/5 μ l i.th.) and myr-tat-CBD3 (20 μ g/5 μ l i.th.) was evaluated for motor deficits using the rotarod performance test. Vehicle treated animals and tat-CBD3 treated animals remained on the rotarod for an average of 180 ± 0 sec at each time point over the course of 300 min. Animals treated with myr-tat-CBD3 remained on the rotarod for at least an average of 147.4 ± 20.0 sec, a value that was not significantly different from vehicle treated or control peptides-treated animals and baseline values. n =4-6 rats per treatment group.

3.5 Proposed model for N-myristoylated CRMP2 peptide's actions on CaV2.2 trafficking and efficacy in pain models

Application of a N-myristoylated tat-conjugated CRMP2 peptide (myr-tat-CBD3) results in membrane-delimited 'rimming' of the peptide whereas the non-myristoylated version (tat-CBD3) appears to be spatially diffusely distributed in the cell cytoplasm. Analysis of penetration of peptides into giant plasma membrane vesicles (GPMVs), which are 'blebs' of membrane devoid of organelles and actin cytoskeleton, data from the Khanna laboratory revealed an unrestricted distribution of the membrane sensitive dye (di-4-ANNEPDHQ) with tat-CBD3, whereas the myristoylated peptide induces a lateral heterogeneity of the fluorescent signal resulting in dye aggregation into micro domains within these model membranes (1). While CRMP2 has been demonstrated to exist as a tetramer (1) [31], the oligomeric state of membrane proximal CRMP2 is as yet unknown; however, neither peptide appears to affect CRMP2 oligomerization (2) [30]. Whereas the cells take up both forms of the peptide with similar efficiency, the myristoylated peptide demonstrates a lesser degree of efflux (3) [30]. The apparent increase in retention of myr-tat-CBD3 translates into a superior potency and efficacy in inhibition of evoked calcium influx in sensory neurons presumably via greater uncoupling of CRMP2-CaV2.2 interactions at or juxta-membrane (4). The increased inhibition of CaV2.2 surface trafficking induced by myr-tat-CBD3 compared with tat-CBD3 may account (5) for the more pronounced restriction of calcium influx imposed by the myristoylated peptide. Cdk5-phosphorylated CRMP2 has been demonstrated to have an enhanced interaction with CaV2.2 (2) [32]; however *in vitro* data from the Khanna laboratory demonstrated that myr-tat-CBD3 does not affect the levels of Cdk5-phosphorylated CRMP2 (6), thereby ruling a role of phosphorylated CRMP2 in regulating calcium influx. CRMP2 binding to tubulin is strengthened by the peptides (7) [30]; the consequences of this are currently unknown. Importantly, where tat-CBD3 is completely ineffective in reversing mechanical hypersensitivity in a rat neuropathic pain model (tibial nerve injury), the myristoylated peptide reverses this hypersensitivity when administered *in vivo* either systemically or orally (8) [30]. Neither peptide elicits any reward-like addictive behaviors (9)

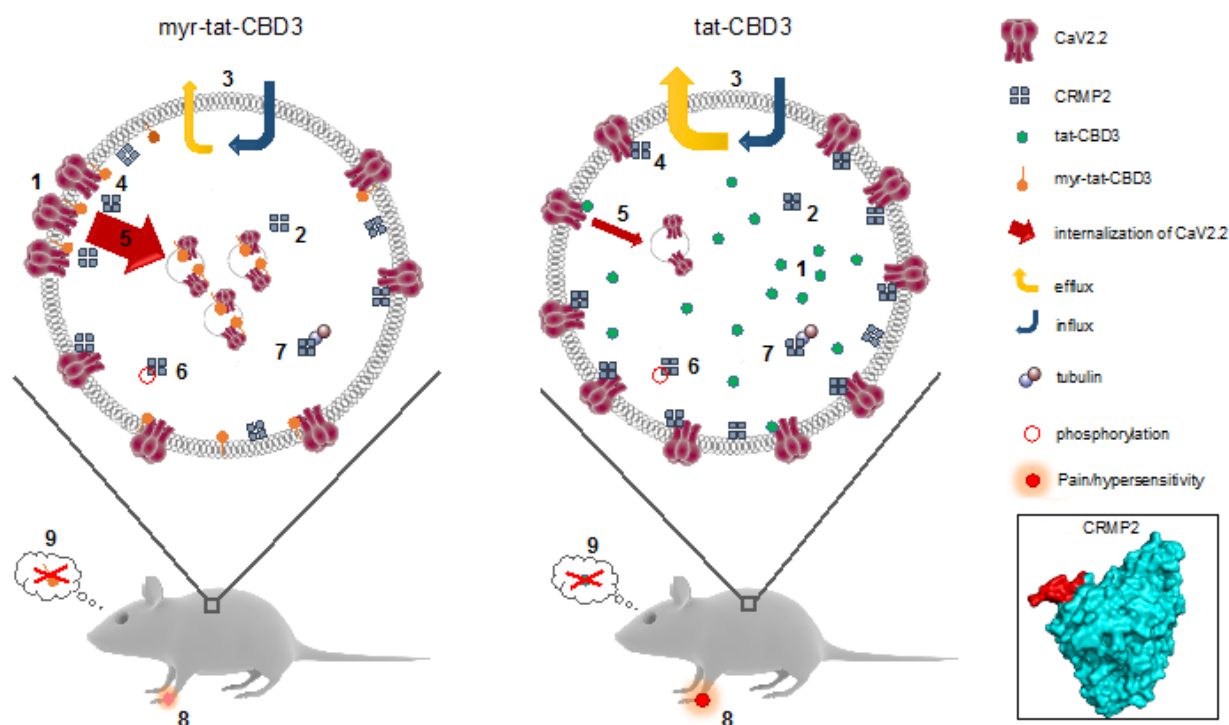


Figure 5. Proposed model for N-myristoylated CRMP2 peptide's actions on CaV2.2 trafficking and efficacy in pain models. Surface representation of the crystal structure of CRMP2 (PDB ID: 2GSE) with CaV2.2 Binding Domain 3 (CBD3) in CRMP2 shown in red. While both peptides, tat-CBD3 and myr-tat-CBD3, diffused across the cellular membrane with similar efficacy, tat-CBD3 exhibited greater efflux, better retention of myr-tat-CBD3 resulted in a greater reduction of CaV2.2 influx from sensory neurons. The more pronounced restriction of calcium influx imposed by myr-tat-CBD3 may account for the more pronounced inhibition of both thermal and mechanical hypersensitivity induced in the pain models. Furthermore, application of myr-tat-CBD3 resulted in membrane-delimited 'rimming' of the peptide whereas the tat-CBD3 appears to be spatially distributed in the cell cytoplasm.

CHAPTER 4: DISCUSSION

There is a clear need for the development of a novel therapeutic in the treatment of pain. While pain is the number one reason patients seek medical care, approximately two-thirds of patients report inadequate pain relief [1, 33]. The Institutes of Medicine and the American Pain Society estimate that in 2010 pain affected more than 100 million adults in the United States costing society \$635 billion each year in medical treatments and lost in productivity [34, 35]. Opioids remains the gold standard in the treatment of pain but opioid use is restricted by undesirable side effects such as nausea, constipation and dependence [36]. Despite a myriad of agents that have long been known to be analgesic: non-narcotics (acetaminophen and aspirin), narcotics (opioids and opiates) and non-steroidal anti-inflammatory drugs (NSAIDs), current pain management therapies alleviate only thirty percent of the pain experienced by patients [37].

Recently, N type voltage gated calcium channel (CaV2.2) has become an attractive target in the development of novel therapeutics in the treatment of pain. Ziconotide has been approved by the FDA in the United States for the management of severe chronic pain in patients for whom are intolerant or refractory to other treatment; gabapentin and pregabalin have been approved by the FDA in the United States for multiple types of neuropathic pain: diabetic neuropathy, postherpetic neuralgia, and fibromyalgia[38-40]; while these drugs are appropriate for the treatment of different modalities of pain, all three drugs target different proteins associated with CaV2.2. Ziconotide exert its analgesic properties as an antagonist for CaV2.2, while gabapentin and pregabalin interact with $\alpha 2\delta$ subunit of CaV2.2 to achieve pain relief.

Despite clinical success of Ziconotide, gabapentin and pregabalin in the management of pain experienced by patients; direct pharmacological interaction with CaV2.2 by these agents result in many adverse side effects such as motor coordination deficits, nausea/sedation and memory impairment [41-43]. In order to circumvent these undesired side effects associated with current therapies, novel strategies indirectly targeting the modulation of CaV2.2 expression may lead to improved therapeutic pharmacology for the treatment of pain.

Recent proteomic studies have identified a new modulator of CaV2.2: collapsin response mediator protein 2 (CRMP2) [17]. CRMP2 canonically directs growth cone collapse, axon number/length and neuronal polarity [44-46]. Following the discovery of the interaction between CRMP2 and CaV2.2, subsequent experiments revealed that the over expression of CRMP2 led to an increase in surface expression of CaV2.2, increased calcium currents and increase in nociceptive neurotransmitter release: calcitonin gene-related peptide (CGRP) from the dorsal root ganglia (DRG) [15, 16] . Conversely, disrupting the interaction between CRMP2 and CaV2.2 using the Cav2.2 Binding Domain 3 (CBD3) peptide conjugated to HIV transduction domain TAT, tat-CBD3 demonstrated efficacy in attenuating dideoxycytidine-induced neuropathic pain behaviors and formalin induced nocifensive behaviors [18].

The findings here extend the examination of tat-CBD3 in postoperative incisional pain model and carrageenan induced acute inflammatory model and revealed that the disruption of the interaction between CRMP2 and CaV2.2 is sufficient to attenuate pain like behaviors in these models. Rats with paw-incision exhibited thermal and mechanical hypersensitivity similar to the human post-operative pain syndrome [47]. Recent literature suggests that CaV2.2 localization is disrupted in chronic pain states [48]. Mechanistically, Cdk5 was shown to have increased activity in DRGs during post-surgical pain [49]. Cdk5 mediated phosphorylation of CRMP2 increases the association between CRMP2 and CaV2.2, which augments calcium influx [32]. The disruption of CRMP2 and CaV2.2 interaction with tat-CBD3, returning CaV2.2 expression closer to pre-surgical level, could underlie the mechanism of anti-nociception in the paw incision model of postoperative pain. Not only has tat-CBD3 demonstrated efficacy in attenuating thermal and mechanical hypersensitivity in the paw incision model, tat-CBD3 was able to alleviate thermal hypersensitivity in the carrageenan-induced inflammatory pain model. CGRP is known to be released during inflammatory pain states [50, 51]. While siRNA targeting to knockdown CRMP2 has shown to significantly reduce CaV2.2 expression and subsequently, CGRP release in DRGs [16]. Thus, the down regulation of CaV2.2 surface expression and CGRP release after administration of tat-CBD3 may underlie the mechanism of anti-nociception in this model of inflammatory pain (Fig. 5) [18].

In addition to expanding the efficacy of tat-CBD3 in attenuating pain in paw incision model of post-operative pain and carrageenan model of inflammatory pain, current findings also make a compelling case for the use of myristoylated tat-CBD3 peptide for the treatment of postoperative and inflammatory pain with better efficacy and specificity in peptide localization. Myristoylation is an irreversible protein lipidation modification where a myristoyl group is attached covalently to the N terminal of proteins so that it functions to anchor the modified proteins to cellular membranes. It is hypothesized that myristoylation, in addition to its anchoring mechanism, allows cargo taken into cell via flip-flop diffusion mechanisms [52]. It can be postulated that addition of myristoyl to tat-CBD3 would anchor it to the membrane, enhance its local concentration near CaV2.2, resulting in greater efficacy. Indeed, myristoylated tat-CBD3 (myr-tat-CBD3) blocked the maintenance of thermal and mechanical hypersensitivity in paw incision model of post-operative pain for the entire duration of the time course (5 hours) while the antihyperalgesic effect of tat-CBD3 disappeared after four hours (Fig 2E, 2F). Additionally, the reversal of mechanical hypersensitivity following administration of myr-tat-CBD3 was far superior to tat-CBD3 with observed withdrawal thresholds: ~11 grams vs 5 grams at 5 hours respectively. Moreover, the absence of effects on hypersensitivity with a peptide lacking CBD3 (myr-tat) or two scrambled versions of the CBD3 peptides (myr-tat-CBD3 scr1 or myr-tat-CBD3 scr2) demonstrate specificity of the anti-nociceptive effect to the CBD3 sequence. However, contrary to current therapies in pain management, CBD3 peptides did not exhibit reward like behavior seen with opioids treatments nor motor coordination deficit seen with gabapentin treatments.

Conditioned place preference (CPP) protocol was carried out as a behavioral measurement of the abuse liability of the CBD3 peptides (tat-CBD3 and myr-tat-CBD3). Tat-CBD3 and myr-tat-CBD3 was administered by the intracerebroventricular route to bathe most components of the mesolimbic reward circuitry. Unlike morphine sulfate, which produces significant increase in the amount of time spent in the putative conditioning chamber [29], i.c.v. tat-CBD3 and myr-tat-CBD3 treated animals did not produce a significant increase in total time spent per putative conditioning chamber (Fig 3). The findings here suggest that both peptides act neutrally at supraspinal sites and are not likely to produce rewarding behaviors.

Rotarod performance test was used to investigate possible motor coordination deficit that could arise after administration of tat-CBD3 or myr-tat-CBD3. While gabapentin is known to cause motor coordination deficits [53], the spinal administration of tat-CBD3 and myr-tat-CBD3 did not produce significant deficits in motor coordination as tested using the rotarod performance test (Fig. 4).

Despite an ample array of analgesics on the market, opioids remain the gold standard in pain management; however the risks associated with narcotic dosing for pain have resulted in 36% of patients and 68% of primary care physicians reporting reservations about taking or prescribing opioids [34, 54]. In the United States, prescribed opioids have become among the most highly abused drugs causing overdose as measured by treatment center admission [55, 56]. Findings here demonstrated novel efficacious compounds to attenuate pain without inducing reward liability or motor coordination deficits. Taken together, these data suggest that pharmacological strategies aimed at CaV2.2 trafficking may represent powerful therapeutic tools in the treatment of post-operative pain while limiting the undesired side effects such as abuse potential and motor deficits.

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