THE ROLE OF KAPPA OPIOID RECEPTORS IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS ON THE AFFECTIVE AND SENSORY COMPONENTS OF CHRONIC NEUROPATHIC PAIN IN MICE

By

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ABSTRACT:

Chronic pain is a widely recognized debilitating condition worldwide that has been associated with disrupted sleep and increased usage of opioids to relieve pain. Our laboratory has interest in exploring the role of kappa opioid agonists and receptors (KOR) signaling in chronic pain. One area that has been brought to our attention is the KOR signaling in the paraventricular nucleus (PVN) which may play a key role in promoting pain and management of it. Our primary aim is to investigate the role of KOR neurons in the PVN on the affective and sensory components of chronic pain induced by partial sciatic nerve ligation (PSNL) surgery in male mice. Secondary aims propose to (a) evaluate the systemic blocking of KOR signaling using the KOR antagonist, nor-Binaltorphimine (nor-BNI) on the affective and sensory components of pain as well as (b) to evaluate the consequences of specific blockade of KOR in the PVN using KOR CRISPR/Cas9 gene editing technology on the affective and sensory components of pain and (c) potential consequences of KOR manipulations on learning process. Our data demonstrate that systemic and local (PVN) blockade of KOR neurons blocked conditioned place preference (CPP) to an analgesic drug without affecting learning behavior, suggesting that PVN-KOR may play a role not only in sleep, but also in the affective component associated with chronic pain. Thus, KOR blockade in the PVN might diminish the aversive component of chronic pain and additionally contribute to the restoration of sleep in the PSNL mice.

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INTRODUCTION:

The definition of pain from the International Association for the Study of Pain is defined as "unpleasant sensory and emotional experience associated with or resembling actual or potential tissue damage"[1]. Pain can be separated into multiple components including sensory, affective and cognitive dimensions. The sensory component of pain, often referred as the "intensity" includes the spatial, temporal characteristics, and quality of pain[32]. The affective component captures the "unpleasantness" often associated with pain and can best be described as how "bad" the pain is, which leads to the motivational aspect of this component, which demands that we take action to protect ourselves from the pain[32]. Pain can be acute or chronic, but unlike acute pain which has an essential role in survival, chronic pain does not present the same survival value and it has a great personal and economic burden to those that are affected[5]. It is not completely clear when acute pain becomes chronic, but the common consensus is pain that persists for more than 3 months [6].

Chronic pain is the leading cause of disability and disease worldwide [4]. Because of the widespread prevalence, this has caused a rapid increase in usage of opioid medications to relieve or manage pain in the United States prescribed by physicians. However, this potential long-term exposure often leads to misuse, abuse, and ultimately the possibility of addiction in these patients which contributes to the ongoing Opioid Crisis in the United States[7]. In 2016, over 66% of the total overdoses were opioid-related[7] and the total number of deaths in the United States in 2021 from illicit drugs or prescription opioids was 106,000 deaths,[8] a number that is increasing every year. Even though chronic pain has a huge prevalence worldwide, the mechanisms that promote and sustain pain are not well known. It is speculated that the central nervous system plays a role in transmission and modulation of pain after injuries. Researching

these mechanisms and the pathways that are involved is crucial to improve understanding on this topic and ultimately improving the therapeutic options.

Opioids can be divided into two groups, endogenous and exogenous. Endogenous opioids are naturally produced in the body and can include examples such as enkephalins, endorphins, endomorphins, dynorphins, and nociception/orphanin[9]. These endogenous opioids bind to opioid receptors such as the mu (MOR), delta (DOR) and kappa opioid receptor (KOR) which are G protein-coupled receptors (GPCRs) and are found extensively throughout the periphery, the dorsal root ganglion, the spinal cord, and in supraspinal regions[10]. KOR consists of seven transmembrane regions that couple to intracellular G proteins and play a role in mediating the body's response to hormones, drugs, and neurotransmitters[9]. Dynorphin, which is an endogenous ligand to the KOR, has been shown in previous studies to play a key role in stress responses[11,12]. Stress leads to release of stress-related neuropeptide corticotropin-releasing factor (CRF). Release of CRF has a downstream effect, where CRF receptors induce dynorphin to be released. The activations of the dynorphin/KOR system play a pivotal and key role in the stress-induced behavioral responses such as anxiety and drug-seeking behaviors in research animal models[11]. KOR antagonists could play a potential role in relieving the negative affective states attributed to anxiety, depression, and drug-seeking behavior. Recent studies done in preclinical and clinical settings have also shown that activation of dynorphin/KOR signaling might produce aversive effects in several conditions, including pain. Thus, KOR antagonists could potentially play a role in modulating the affective component of chronic pain[13-16].

Previous studies from our group demonstrate that partial sciatic nerve ligation (PSNL) surgery induces the development of pain-like behavior in mice as well as disruption of sleep. In addition, we have revealed that systemic blockade of KOR, using selective antagonists, nor-binaltorphimine (nor-BNI) and CYM-53093, restored sleep in the male mice that underwent

PSNL surgery, suggesting that KOR might play a role in sleep disruption in chronic pain states. Using the gene editing technique, our group developed a CRISPR/Cas9 plasmid that targeted KOR and following stereotaxic injection of KOR CRISPR/Cas9 plasmid into the PVN, we observed the reduction of PVN KOR neurons restored sleep in PSNL mice, observing the role of hypothalamic KOR in sleep disruption in a chronic pain model[2]. Further findings of the study are that sleep restoration decreases pain[2]. Data from our group suggests that KOR antagonists might be useful to restore sleep and relieve pain in chronic conditions.

This has led to the question: does KOR neurons in the PVN region of the brain promote a direct positive effect on sleep and/or on pain components? Since we know that blockade of KOR into the PVN restores sleep affected after chronic pain in mice, our group decided to investigate the direct effect of KOR into the PVN in pain signaling. For this thesis, the role of KOR neurons in the paraventricular nucleus (PVN) of the hypothalamus will be investigated in chronic pain conditions. Partial sciatic nerve ligation (PSNL) surgery on male mice is a well-known pain model to induce chronic (neuropathic) pain in mice[17].

From the gathered data, we hypothesized the blocking KOR in the PVN will remove the affective component of pain that might be contributing to the restoration of sleep in the PSNL mice. We therefore hypothesize that the sensory component of pain might involve PVN-KOR neurons. The primary aim of this thesis is to investigate the role PVN KOR in the components of chronic pain induced by PSNL surgery on naive male mice. We plan to: (a) evaluate the effect of systemic blockade of KOR on the affective and sensory components of pain; (b) the effect of specific blocking of KOR in the PVN on the affective and sensory components of pain using KOR CRISPR/Cas9 plasmid, (c) verify if this specific genetic approach disrupts the learning process.

PROCEDURE AND METHODS:

Animals

The study was conducted in accordance with the NIH guidelines for use of laboratory animals and approval from the Institutional Animal Care and Use Committee at the University of Arizona. Male C57BL/6J mice (8 weeks) or KOR^{Cre} mice (8-10 weeks) were used for all experiments. KOR^{Cre} mice were generated as previously described and[22] were backcrossed to C57BL/6 background for at least 8 generations. Every effort was made to minimize numbers and suffering of animals used in the experiments. Mice were randomly assigned to the treatment groups and the experiments were conducted in a blind fashion.

Drug administrations

Norbinaltorphimine (nor-BNI) was purchased from Tocris, Bristol, United Kingdom and dissolved in 0.9% saline to 10 mg/kg just before injection. Gabapentin (Spectrum Chemical MFG; Gardena, CA) was dissolved in water and administered at 30 mg/kg. Lithium was purchased from Sigma Aldrich, St. Louis, Missouri, and dissolved in 0.9% saline to 150 mg/kg before administration. Nor-BNI, gabapentin and lithium were administered intraperitoneally (i.p.). Control groups received their respective vehicles at 10 mL/Kg.

Partial sciatic nerve ligation surgery

We produced a partial sciatic nerve ligation (PSNL) model as described previously. Briefly, mice[20] were anesthetized with 2-5% isoflurane. The right sciatic nerve was exposed and ligated by a tight ligature with 8–0 silk suture around approximately one-half of the diameter. In sham-operated mice, the nerve was only exposed without ligation. The muscle was sutured with a 5-0 braided suture and the skin was closed with an Auto-clip.

Design and in vivo transfection of CRISPR/Cas9 plasmid targeting KOR

To edit the KOR neurons we targeted the second exon of the *oprk1* common to all KOR splice variants (ENSMUSG00000025905, gRNA: gTCCCCCATTCAGATCTTCCG, on-score 65.7, off-score 78.8). The indicated gRNA sequence was inserted into the Esp3I restriction site of the pLCRISPR.EFS.tRFP lenti plasmid (Cat# 57819, Addgene, Cambridge, MA) as described before[21]. Plasmids were verified by Sanger sequencing (Eurofins, Louisville, KY). Five hundred nL of KOR CRISPR plasmid was injected into the paraventricular nucleus (PVN) of the hypothalamus (bregma: 0.8 mm posterior, 1.2 mm lateral, 4.9 mm ventral, at an angle of 10°).

Stereotaxic plasmid injections

Mice were anesthetized with isoflurane (induction, 5%; maintenance 2%) and placed into a 25 stereotaxic apparatus (David Kopf Instruments). After exposing the skull, a small hole was drilled for injection. A pulled-glass pipette with a 20–40 μm tip diameter (FIVEphoton Biochemicals; World Precision instruments) was inserted into the PVN (coordinates, bregma: AP: −1.8 mm, DV: −4.9 mm, ML: +1.2 mm, Angle: 10°) and KOR or control plasmids (500 nL) was injected using a Nanoliter2010 (World Precision instruments) at a flow rate of 50 nL/min. The glass pipette was kept in place for at least 5 min to ensure proper diffusion. The skin incision was closed with suture and surgical glue, the mouse was then allowed to recover on a heating pad. PSNL was performed 10 days after plasmid injection to allow proper gene editing.

Sensory (evoked) pain measurements

Mechanical or tactile allodynia was assessed at different time points before and after PSNL/sham surgery using von Frey filaments. Mice were individually placed into clear

Plexiglass chambers on top of a wire mesh floor. They were left for an hour to acclimate to the surrounding environment before measurements were taken. Von Frey filament was applied between the wire mesh to the plantar surface of the animal's right hind paw to reach the threshold which is defined as the mouse flicking its hind paw away from the filament. To reach threshold, increasing gauges of Von Frey filaments were used until it elicited a hind paw withdrawal response from each mouse (up and down methodology), and measurements were recorded. Withdrawal thresholds were determined by Dixon nonparametric test and expressed as the mean tactile threshold. Mechanical threshold was evaluated prior and on days 7, 10, 11 and 15 after PNSL or sham surgery.

Affective (spontaneous) pain measurements

Conditioned place preference (CPP) was used in this study to assess the affective component of pain. CPP chambers have one neutral chamber and two conditioning chambers with different visual, tactile and special cues (different wall patterns and different floor textures). The CPP test consists of 5 days: acclimation, baseline, two conditioning days and a test. For the acclimation, BL and test days, animals are individually placed in the CPP chambers with free access to the neutral and conditioning chambers and allowed to explore for 15 minutes. Time spent in each conditioning chamber was quantified in the BL and test days. A cut off time of more than 720 seconds or less than 180 seconds spent in one of the conditioning chambers during BL was established as an exclusion criterion. In the two conditioning days, mice were treated with saline in the morning and placed in one of the conditioning chambers for 30 minutes. Four hours later, all animals received gabapentin (30 mg/kg, i.p.) administration and immediately placed in the opposite conditioning chamber of the morning for 30 minutes. Conditioning chambers were defined by the counterbalance after the BL. Test day defined the time spent in each of the conditioning chambers. CPP is demonstrated by an increase of the time spent in the

gabapentin-paired chamber on the test day in comparison to BL. CPP test was performed on days 11 to 15 after the PSNL surgery.

Learning behavior measurement

Conditioned place aversion (CPA) was used in this study to assess the learning behavior in mice. CPA chambers have 1 neutral chamber and two conditioning chambers with different visual, tactile and special cues (different wall patterns and different floor textures). CPA test consists of 5 days : acclimation, baseline, two conditioning days and test. For the acclimation, BL and test days, animals are individually placed in the CPA chambers with free access to the neutral and conditioning chambers and allowed to explore for 15 minutes. Time spent in each conditioning chamber was quantified in the BL and test days. A cut off time of more than 720 seconds or less than 180 seconds spent in one of the conditioning chambers during BL was used as an exclusion criterion. In the two conditioning days, mice were treated with saline in the morning and placed in one of the conditioning chambers for 30 minutes. Four hours later, all animals received lithium (150 mg/kg, i.p.) administration and immediately placed in the opposite conditioning chamber of the morning for 30 minutes. Conditioning chambers were defined by the counterbalance after the BL. Test day defined the time spent in each of the conditioning chambers. Aversion is demonstrated by a reduction of the time spent in the Lithium-paired chamber on the test day in comparison to BL.

Statistical Analysis

Data are expressed as the mean ± SEM. The statistical significance of differences between the groups was evaluated using one-way (CPP and CPA data) or two-way (mechanical allodynia) ANOVA followed by the Sidak or Tukey multiple comparisons tests when two or more

groups were compared, respectively. $P < 0.05$ was considered significant. All statistical analyses were performed with Prism 9 (GraphPad Software, California, USA).

Study Design

The systemic and local role of KOR signaling in chronic pain was studied using: 1) systemic administration of KOR antagonists; and 2) cell specific manipulation of KOR expressing neurons in the paraventricular nucleus (PVN) using CRISPR/Cas9 gene editing technique. To evaluate the effect of nor-BNI pretreatment on the affective and sensory components of pain in mice with chronic pain, the KOR antagonist was administered on day 10 after PSNL and the CPP test was performed on days 11 to 15 after PSNL. Mechanical allodynia was evaluated prior and on days 7, 10, 11 and 15 after PSNL/sham surgery. The effect of nor-BNI pretreatment on the affective and sensory component of pain in mice with chronic pain, the KOR antagonist was administered on day 10 after PSNL and the CPP test was performed on days 11 to 15 after PSNL. Mechanical allodynia was evaluated prior and on days 7, 10, 11 and 15 after PSNL/sham surgery. The possible effect of PVN administration of KOR CRISPR/Cas9-gene editing on affective and sensory components of pain in mice with chronic pain, microinjection was performed to deliver the KOR or control CRISPR/Cas9 plasmid. Ten days later, PSNL surgery was performed. CPP test was performed on days 11 to 15 after PSNL. Mechanical allodynia was evaluated prior and on days 7, 11 and 15 after PSNL surgery. To assess if PVN-KOR editing could affect the learning behavior of mice, KOR or control CRISPR/Cas9 plasmid were microinjected in the PVN followed by a lithium-CPA test 10 days after the microinjection. Lithium was chosen because it is widely known to induce discomfort or aversive state without affecting pain components[19].

RESULTS:

The first result of noteworthy conclusion from the experimentation is that gabapentin produced CPP in PSNL male mice. Intraperitoneal administration of vehicle control did not alter the CPP to gabapentin. On the other hand, systemic pretreatment with nor-BNI was able to prevent increase of the time spent in the gabapentin-paired chamber on the CPP test after PSNL in male mice (Fig. 1A). The difference score confirmed that pharmacological blockade of KOR prevented the CPP to gabapentin after PSNL (Fig. 1B), suggesting that the systemic KOR blockade was sufficient to prevent CPP to gabapentin after PSNL. Time spent in the CPP chambers were not altered in sham control animals pretreated with systemic vehicle or nor-BNI when compared to their baseline (Fig. 1C). The difference between test and baseline scores confirmed that the time spent in gabapentin-paired chamber was not affected in sham male mice (Fig. 1D). These data demonstrated that systemic KOR blockade was able to prevent spontaneous pain-behavior in PSNL mice.

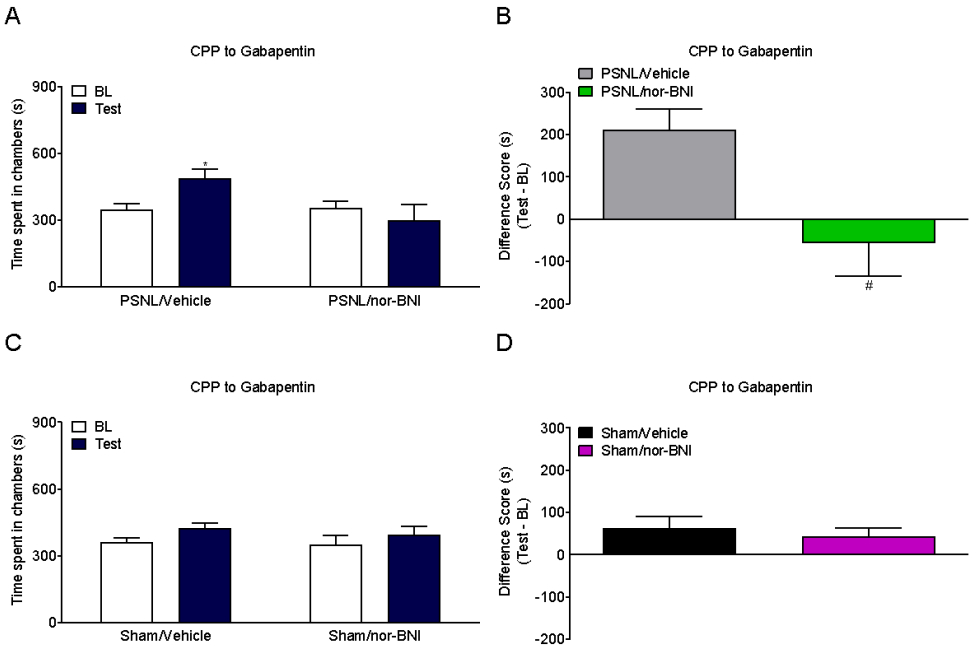

Figure 1. Systemic single pretreatment with the KOR antagonist, nor-BNI, abolished the CPP in PSNL male mice. Nor-BNI (10 mg/kg) or saline (vehicle) were administered via intraperitoneal administration (i.p.) on day 10 after PSNL or sham surgery. CPP test was

performed from day 11 to day 15 after PSNL or sham surgery. The CPP test consisted of acclimation followed by 2 conditioning and test days. On the acclimation, baseline (BL) and test days, mice were placed into the middle/neutral chamber with free access to all chambers and allowed to explore the chambers for 15 minutes. On the conditioning days, mice were treated with saline in the morning and immediately placed into one of the conditioning chambers for 30 minutes with no access to other chambers. Four hours later, the animals received gabapentin and were immediately placed into the opposite chamber from the morning conditioning for 30 minutes. (**A** and **C**) Time spent in chambers during the CPP test. (**B** and **D**) Difference between test and baseline. Data represent mean ± SEM of 7 - 8 animals per group. Two-way ANOVA followed by Tukey post-hoc test for panels A and C; Student t-test for Panels B and D.

Hind paw allodynia (pain-like behavior) can be observed by reduction of sensory (tactile) threshold. In the male mice that underwent PSNL surgery, the tactile threshold for the evaluation of evoked pain-like behavior in their hind paw region was measured prior and on days 7, 10, 11 and 15 after PSNL or sham surgery. It can be observed that PSNL surgery induced hind paw allodynia in comparison to sham animals, shown by decreased in the tactile threshold by almost 3 grams after surgery (Fig. 2). Evoked pain-like behavior was not affected by either vehicle control or nor-BNI treatment on day 10 after surgery, demonstrated by the lack of analgesia (increased tactile thresholds) after the treatment (Fig. 2). Control mice that underwent sham surgery showed no statistical difference in evoked pain threshold prior and after pretreatment with nor-BNI or vehicle. Overall, this result demonstrated that KOR blockade did not relieve or reduce the sensory component of pain (evoked pain) unlike the effect on the affective component of pain. Thus, systemic nor-BNI treatment reduced the affecting component of chronic pain without affecting the sensory component after PSNL.

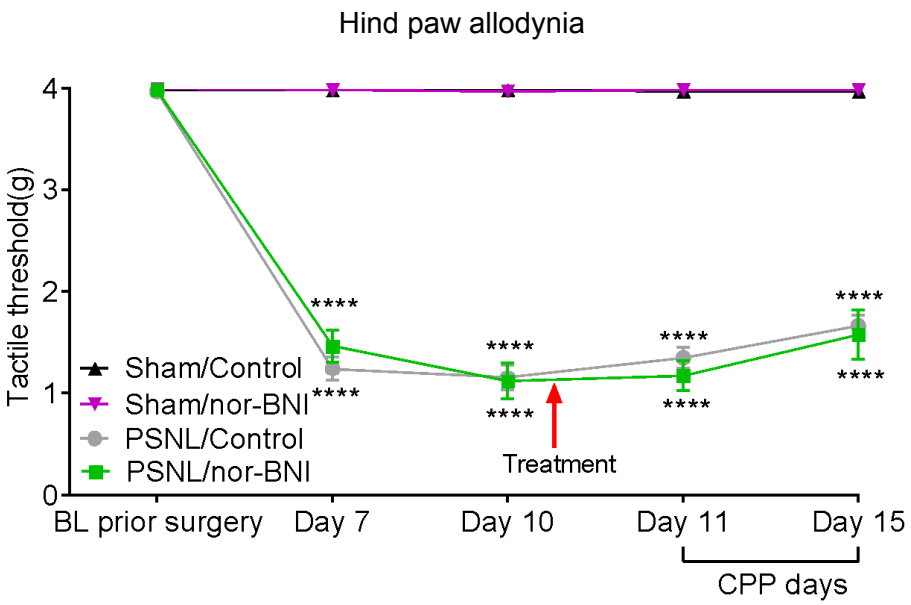

Figure 2. Hind paw tactile thresholds before and after systemic administration of nor-BNI in male mice submitted to PSNL surgery and CPP test. Tactile thresholds were evaluated prior to PSNL or sham surgery and on day 7, 10, 11 and 15 after. Nor-BNI (10 mg/kg) or saline (vehicle) were administered via i.p. on day 10. CPP test was performed on days 11 to 15 after surgery, with tactile thresholds assessed after acclimation on day 11 (day 1 of CPP) and after the CPP test on day 15 (day 5 of CPP) after surgery. Data represent mean ± SEM of 7 - 8 animals per group. Two-way ANOVA followed by Tukey post-hoc test. **** represents $p < 0.0001$ in comparison to sham/vehicle or sham/nor-BNI groups.

To investigate further the role of KOR in the PVN, we performed bilateral administration of either KOR CRISPR/Cas9 plasmid or control plasmid into the PVN via stereotaxic surgery 10 days prior to PSNL. KOR CRISPR/Cas9 was able to edit the expression of KOR into the PVN in comparison to animals injected with plasmid control. For this experiment, all mice were submitted to PSNL and the only variable was the reduction or not of the expression of KOR cells into the PVN. Animals submitted to the administration of control plasmid in the PVN and subsequently PNSL surgery demonstrated a significant CPP to gabapentin, demonstrated by increased time spent in the gabapentin-paired chamber in the test when compared to baseline (BL) (Fig. 3A). Surprisingly, KOR CRISPR/Cas9 administration was able to prevent the CPP to the gabapentin after PSNL, observed by no difference between the time spent in the gabapentin-paired chamber in the BL and test (Fig. 3A). The difference between test and

baseline scores confirmed that the time spent in gabapentin-paired chamber was not affected in KOR CRISPR/Cas9 male mice (Fig. 3B). This result implies that PSNL KOR CRISPR group mice had alleviation or reduction in the spontaneous pain, suggesting that PVN KOR might be playing a role in the affective component of chronic pain.

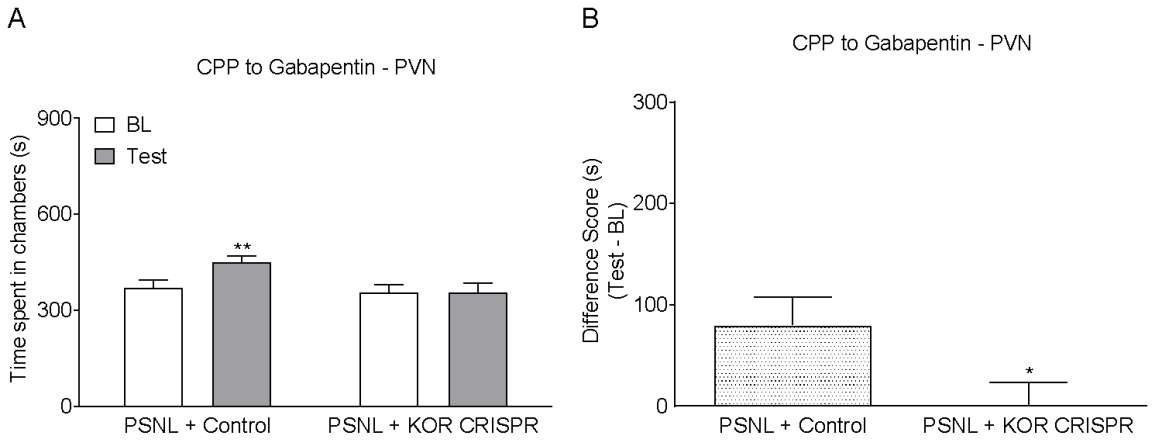

Figure 3. KOR CRISPR into the PVN abolished the CPP in PSNL male mice. Stereotaxic surgery for bilateral administration of KOR CRISPR or control (500 nl, 50 nL/min/site) into the PVN was performed 10 days prior to PSNL surgery in male mice. CPP test was performed from day 11 to day 15 after PSNL or sham surgery. The CPP test consisted of acclimation followed by 2 conditioning and test days. On the acclimation, baseline (BL) and test days, mice were placed into the middle/neutral chamber with free access to all chambers and allowed to explore the chambers for 15 minutes. On the conditioning days, mice were treated with saline in the morning and immediately placed into one of the conditioning chambers for 30 minutes with no access to other chambers. Four hours later, the animals received gabapentin and were immediately placed into the opposite chamber from the morning conditioning for 30 minutes. Data represent mean ± SEM of 16 animals per group. Two-way ANOVA followed by Sidak post-hoc test for panel A and t-test for panel B. * and ** represent $p < 0.05$ and $p < 0.01$ in comparison to baseline (Panel A) or in comparison to PSNL + control group (Panel B), respectively.

The same cohort of animals underwent hind paw tactile threshold evaluation prior PVN microinjection of CRISPR (Baseline 1; BL1), prior (Baseline 2; BL2) and on days 7, 11 and 15 after PSNL surgery. There were no statistical differences between groups on the tactile threshold on BL1 and BL2. PSNL surgery was able to induce pain-like behavior, demonstrated by a reduction of the tactile threshold, suggesting the development of hind paw allodynia (Fig.

4). Neither KOR CRISPR/Cas9 plasmid nor control plasmid altered the development of hind paw allodynia after PSNL (Fig. 4). Overall, this result demonstrated that the sensory component of pain was not affected by genetic reduction of the expression of KOR into the PVN. Thus, PVN KOR seems to reduce the affecting component of chronic pain without affecting the sensory component after PSNL.

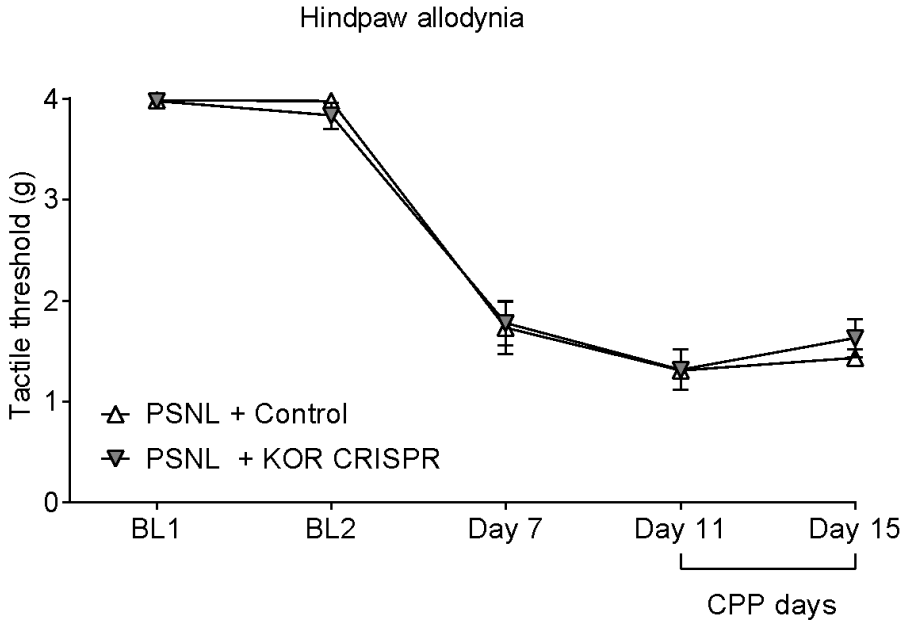

Figure 4. Hind paw tactile thresholds prior and after KOR or control CRISPR administration into the PVN, PSNL surgery and CPP test. Tactile thresholds were evaluated prior stereotaxic administration of KOR or control CRISPR into the PVN (BL1), 10 days prior PSNL surgery), right before PSNL surgery (BL2) and on days 7, 11 and 15 after PSNL. CPP test was performed on days 11 to 15 after PSNL surgery, with tactile thresholds assessed after acclimation on day 11 (day 1 of CPP) and after the CPP test on day 15 (day 5 of CPP) after surgery. Data represent mean ± SEM of 16 animals per group. Two-way ANOVA followed by Sidak post-hoc test.

One of our questions regarding the genetic manipulation was if the administration of KOR CRISPR/Cas9 plasmid was blocking the learning behavior than actually affecting the affective component of pain. If this were the case, animals that had reduction of the expression of KOR into the PVN would not show CPP to gabapentin because they were not able to learn to associate the specific CPP chamber to the rewarding sensation produced by pain-relief. To evaluate this hypothesis, we needed to evaluate the learning behavior without affecting any pain

component. In this regard, lithium is known to induce an unpleasant or discomfort feeling in users[30]. In addition, systemic administration of lithium has been demonstrated to produce conditioned place aversion (CPA) in naive male mice without altering any pain component[31]. We were able to replicate these previous findings by demonstrating intraperitoneal administration of lithium was able to produce CPA to the lithium-paired chamber, revealed by a reduction of the time spent in the lithium-paired chamber in the test versus BL (Fig. 5A). Vehicle control administration did not induce CPA (Fig. 5A). Difference scored between time spent in the lithium-paired chamber in the test versus BL confirmed the CPA only in lithium treated mice (Fig. 5B). This data demonstrated that mice learned to avoid the lithium-paired chamber due to an aversive state produced by the drug.

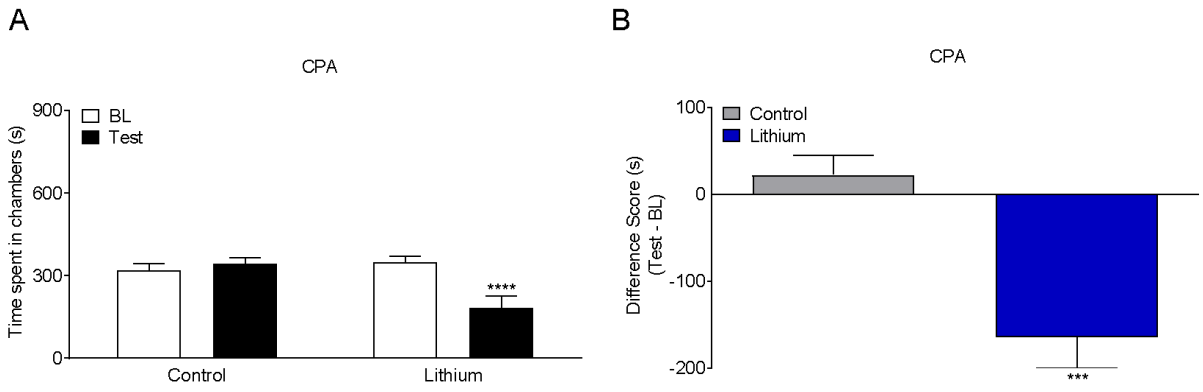

Figure 5. Lithium induced CPA in male mice. The CPA test consisted of acclimation, test, 2 conditioning and test days. On acclimation, baseline (BL) and test days, mice were placed into the middle chamber with free access to all chambers and allowed to explore the chambers for 15 minutes. On conditioning day, animals were paired into one of the CPP chambers defined by counterbalance for 30 minutes after the i.p. injection of vehicle (DiH2O) in the morning and 4 hours later, animals were paired into the opposite chamber from the morning conditioning for 30 minutes, right after i.p. administration of lithium at 150 mg/kg. (**A**) Time spent in chambers on the CPA test. (**B**) Difference between test and baseline. Data represents the mean ± SEM of 12 animals per group. Two-way ANOVA followed by Sidak post-hoc test for panel A and t-test for panel B. *** and **** represent $p < 0.001$ and $p < 0.0001$ in comparison to Control group (Panel B) and to baseline (Panel A), respectively.

We then decided to investigate if reduction of expression of KOR in the PVN would prevent the learning process on CPA using lithium. We performed bilateral administration of either KOR

CRISPR/Cas9 plasmid or control plasmid into the PVN via stereotaxic surgery 10 days prior CPA. Either KOR CRISPR/Cas9 or control plasmids developed CPA to lithium injection. Both groups of mice demonstrated an aversion to the lithium-paired chamber on the test day (Fig. 6A). The difference between test and baseline scores confirmed that the time spent in the lithium-paired chamber was not affected in KOR CRISPR/Cas9 male mice (Fig. 6B). This result confirmed that PSNL KOR CRISPR did not disrupt the learning behavior and the lack of CPP to gabapentin-paired chamber after PSNL previously observed was really due to the significant role in the affective component of chronic pain. Although KOR in the PVN seems to be important to restore sleep deprivation in chronic pain states, it also seems to directly modulate the affective component of chronic pain signaling.

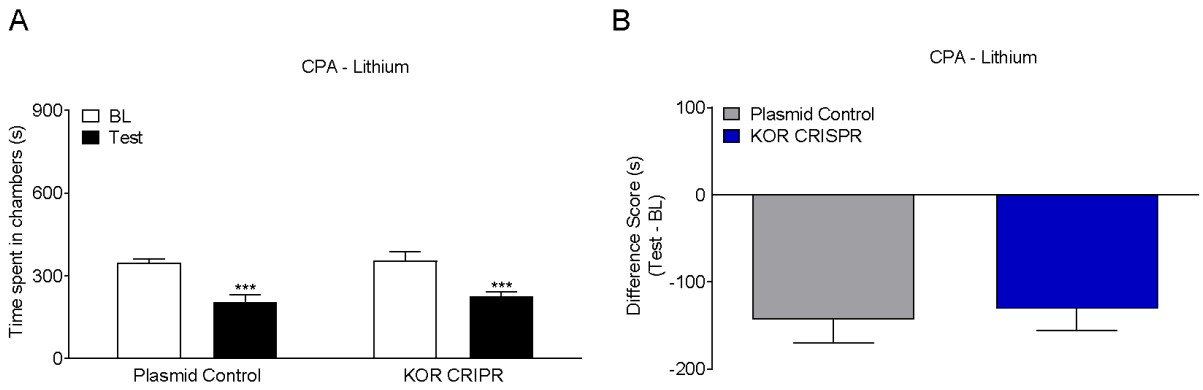

Figure 6. KOR CRISPR into the PVN did not modify the CPA induced by lithium in male mice. Stereotaxic surgery for bilateral administration of KOR CRISPR or control (500 nl, 50 nL/min/site) into the PVN was performed 10 days prior to the CPA test. On acclimation, baseline (BL) and test days, mice were placed into the middle chamber with free access to all chambers and allowed to explore the chambers for 15 minutes. On conditioning day, animals were paired into one of the CPP chambers defined by counterbalance for 30 minutes after the i.p. injection of vehicle (DiH2O) in the morning and 4 hours later, animals were paired into the opposite chamber from the morning conditioning for 30 minutes, right after i.p. administration of lithium at 150 mg/kg. (**A**) Time spent in chambers during the CPA test. (**B**) Difference between test and baseline. Data represent mean ± SEM of 8 animals per group. Two-way ANOVA followed by Sidak post-hoc test for panel A and t-test for panel B. *** represents $p < 0.01$ in comparison to baseline (Panel A).

DISCUSSION:

The aim of our preclinical study was to evaluate the role of KOR in the PVN on chronic pain induced by PSNL in male mice. We first observed that systemic blockade of KOR abolished CPP in PSNL mice, which evaluates the affective component of pain without affecting the development of tactile allodynia induced by the neuropathic pain model, which assesses the sensory component of pain. Moreover, we demonstrated that PVN-KOR plays an important role selectively in the affective component of pain signaling. The genetic reduction of the expression of these receptors in the PVN was able to block the CPP to gabapentin in PSNL animals without affecting the sensory components of pain as well as the learning behavior. The current data contribute to a previous report from our group that revealed that PSNL surgery induced sleep disruption in animals and either systemic or PVN blockade of KOR signaling, restored the sleep pattern after chronic pain induction. Thus, KOR in the PVN plays a direct role in sleep and also in the affective component of pain in chronic pain conditions.

PSNL surgery is a well-known model to cause chronic pain inducing changes in the affective and sensory components of pain[17]. To assess the sensory component of pain, we evaluated the tactile allodynia in the hind paw using von Frey filaments in sham and PSNL mice. Systemic and local (PVN) blockade of KOR signaling did not affect the sensory threshold of PSNL mice. Our group has recently demonstrated that repeated systemic administration of KOR agonists engages the stress signaling pathway producing the development of a transient tactile allodynia in naive mice as well as primed these animals to subthreshold doses of nociceptive stimuli[37]. Obara and his colleagues demonstrated that intrathecal administration of KOR antagonists, nor-BNI and 5'-guanidinonaltrindole (GNTI) enhanced tactile allodynia in rats and mice after sciatic nerve ligation and suggests that in chronic pain conditions allodynia seems to be mediated through nonopioid effect of the endogenous dynorphin, possibly by direct activation of

NMDA receptors[24]. Conversely, we have not observed an enhancement of tactile allodynia after nor-BNI treatment, possibly due to the different route of administration. Since KOR blockade does not seem to modify sensory thresholds in chronic pain states, it is safe to mention that evoked pain signaling in chronic pain conditions differs from non-injury states, including KOR signaling [34,36]. Thus, KOR blockade with nor-BNI, and other KOR antagonists do not seem to play a role in relief or reduction of the sensory component of pain. Several studies support our data revealing that KOR antagonists do not block evoked pain behavior in neuropathic pain conditions[34,35].

Previous studies from our laboratory have demonstrated that KOR in the anterior cingulate cortex (ACC) seems to be a major hub of pain modulation in chronic pain conditions, especially the affective component[25]. Herein, we introduce another important region of the brain in which KOR signaling plays a pivotal role in modulation of the affective component of pain induced by nerve injury. PSNL and sham mice underwent CPP test to evaluate the effect of systemic KOR blockade on the affective component of pain. Previous studies performing CPP tests have demonstrated that animals seek analgesic treatments such as clonidine infusion or electrical stimulation of the motor cortex to relieve the neuropathic pain induced from PSNL surgery[23]. Our group has used gabapentin to produce pain relief in CPP tests after chronic pain induction. In our experimental design, we saw that PSNL mice treated with vehicles showed significant preference to the gabapentin-paired chamber on the test day. Systemic nor-BNI pretreatment in PSNL animals was able to prevent the increased time spent in the gabapentin-paired chamber during CPP test, which demonstrated that systemic KOR blockade was able to relieve the spontaneous pain behavior in chronic conditions. These data revealed that systemic KOR blockade relieves the affective component of pain.

The PVN has also been reported to be a hub for pain modulation[38,39]. Microinjections of

glutamate into the PVN helped reduce the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats[27]. PVN is also being implicated as a key core brain in the hypothalamic-pituitary-adrenal axis (HPA axis), which is well known to activate during emotional or psychological stress and promote anxiety-related behavior, some of the comorbidities associated with chronic pain[29]. In these areas, the PVN^{CRH} neurons (CRF positive neurons that release corticotropin-releasing hormones found in the PVN) are activated during inducing stress[33]. Stimulation of these neurons via optogenetic means can lead to sleep disruption from stress-induced insomnia in naive young adult male mice[28]. Previous research has shown that activation of corticotropin-releasing factor (CRF) positive neurons in PVN can heighten sensitivity and contribute towards visceral pain in irritable bowel syndrome[26]. This suggests that the neural circuit in the PVN that ultimately might involve KOR activation potentially could have an important function in pain modulation, which warrants our interests and research in its role. Our data suggests that local genetic reduction of the expression of KOR neurons in the PVN relieves the affective component of pain which could be directly contributing to the restoration of sleep mice that have underwent PSNL surgery.

Lastly, to verify that the learning process was not affected by the blockade of KOR signaling in the PVN induced by microinjection of KOR CRISPR we have used lithium on the CPA test. Previous research on rodents has shown that lithium can be used to induce aversion without affecting pain state [31]. High doses of lithium can induce negative physiological sensations in rodents promoting an aversive state, which can be used in condition taste or placed aversion tests[31]. This control experiment demonstrated that gene editing of KOR PVN did not affect the learning behavior of mice and the effect observed in the previous CPP experiment was really related to the affective component of pain and not lack of learning the cues of the CPP test. Thus our group demonstrated that PVN-KOR may play a role not only in sleep restoration, but also in the affective component associated with chronic pain.

Conclusion

The current study demonstrated the pathological role of PVN KOR in chronic pain. Our previous studies have shown that systemic and local (PVN) KOR blockade is relevant to restore sleep in preclinical chronic pain conditions, and our new results added that this network also plays a significant role in the affective component of pain. The data suggests that specific (PVN) blocking of KOR neurons will relieve the affective component without interfering with the sensory component of pain in chronic conditions. We suggested that the relief of the affective component might be contributing to the restoration of sleep in the PSNL mice. However, the opposite could be true, blockade of KOR signaling in the PVN could also be restoring sleep disturbance in chronic pain states and ultimately inducing the relief of the affective component of pain. Thus, the relationship between sleep restoration and relief of the affective component of pain remains to be elucidated.

With an increasing amount of the population suffering from chronic pain and its comorbidities such as depression, sleep disruption, and anxiety as well as with the opioid crisis in the US, the need for a new drug and treatment to replace opioids continues to grow. KOR antagonists present a promising prospect that could treat chronic pain without the addictive properties. These antagonists might also contribute to the treatment of chronic pain comorbidities. Two KOR antagonists have completed phase II of clinical trials and one is in phase III trials for the treatment of major depressive disorder, generalized anxiety and anhedonia. If these drugs succeed and advance to the market, they can be studied in clinical trials for chronic pain in the near future.

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