Research Paper

PAIN 00 (2026) 1–14

PAIN®

Peripheral kappa opioid receptors modulate cold hypersensitivity in a mouse model of chemotherapy-induced neuropathy

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Abstract

Noxious cold sensation is commonly associated with peripheral neuropathies; however, there has been limited progress in understanding the mechanism. Here, we identify a novel role for peripherally expressed kappa opioid receptors [KORs] in noxious cold hypersensitivity in a mouse model of chemotherapy. In this model, we show that oxaliplatin-induced cold hypersensitivity is attenuated using norbinaltorphimine (NorBNI, KOR antagonist (10 mg/kg, i.p.) in both male and female mice. To further examine the role of KORs in cold hypersensitivity, we show that activation of KOR with U50,488 (KOR agonist, 5 mg/kg i.p.) significantly increases the number of jumps on a cold plate at 3˚C as compared to controls, which is attenuated by KOR antagonist NorBNI. We have determined that this effect is mediated through peripheral KORs using both pharmacology and a peripherally restricted KOR agonist, ff(nle)r-NH2. Using a conditional knockout mouse model of KOR in the dorsal root ganglia (using PirtCre;Oprk$^{fl/fl}$), we see reduced U50488-induced noxious cold hypersensitivity as compared to controls in male and female mice, confirming the role of KORs in the dorsal root ganglia in mediating cold hypersensitivity. To confirm the role of peripheral KORs in oxaliplatin-induced cold allodynia, we use the PirtCre;Oprk$^{fl/fl}$ in our oxaliplatin-induced cold allodynia model. Here, we show that in the absence of KORs in dorsal root ganglia, oxaliplatin-induced cold hypersensitivity is attenuated in male and female mice. Overall, our data suggest that peripheral KORs modulate cold hypersensitivity in a mouse model of chemotherapy.

Keywords: Kappa opioid receptor, Oxaliplatin-induced cold allodynia, Cold hypersensitivity, Cold allodynia, Dorsal root ganglia

1. Introduction

Cold hypersensitivity is defined as an exaggerated or abnormal reaction to cold exposure, causing discomfort or the avoidance of cold.[33] This condition is often associated with neuropathic pain from disorders such as multiple sclerosis, fibromyalgia, complex regional pain syndrome, and chemotherapy-induced peripheral neuropathy.[7,19,32,71,80] Up to 50% of patients with neuropathic pain experience heightened sensory abnormalities, 14% of whom report thermal allodynia.[32] Medications used to treat neuropathic pain are predominantly nonsteroidal anti-inflammatory drugs (NSAIDs) and μ-opioid agonists,[36] all of which have very limited success in relieving cold hypersensitivity. The lack of therapeutic efficacy stems primarily from a limited understanding of the mechanisms involved in the modulation of cold pain.

The underlying mechanisms of cold hypersensitivity associated with chronic pain are unclear, but recent work has shown that silent as well as and cold-sensing neurons become active and contribute to cold allodynia.[42] The unmasking of cold nociceptors has been validated in chronic pain models, such as partial sciatic nerve ligation and ciguatera-poisoning model.[42] This shift in cold-sensing neuronal activity was further elucidated in the acute oxaliplatin-induced cold allodynia model,[30] wherein an acute intraplantar oxaliplatin injection mediates reversible sensory neurotoxicity with cold-triggered paresthesia and dysesthesia of hands and limbs.[14,16,39] To better understand the peripheral mechanisms mediating cold hypersensitivity, researchers have also found that pregabalin reduces the excitability of peptidergic A-fiber nociceptors, which decreases oxaliplatin-induced hypersensitivity on a 10˚C cold plate.[30] Here, we identify a new system involved in cold hypersensitivity and pain: the kappa opioid receptor (KOR) system.

KORs are G_i-protein–coupled receptors expressed centrally in the brain and spinal cord, and peripherally in the dorsal root ganglion (DRG),[49,55] in three main types of sensory neurons, peptidergic afferents, and two populations of low-threshold mechanoreceptors (LTMRs).[69] Selective activation of KORs is antinociceptive for noxious heat stimuli, analgesic in chronic pain

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.painjournalonline.com).

http://dx.doi.org/10.1097/j.pain.0000000000004050

models, and inhibits itch transmission.[10,41,44,46,50,69] We and others have shown that central KOR activation and upregulation modulates the negative affect associated with peripheral nerve injury and inflammatory pain models.[41,44] Conversely, studies suggest that peripherally restricted KOR agonists selectively inhibit chemical pain in capsaicin-induced neurogenic inflammatory pain models and mechanical hypersensitivity induced by surgical incision models.[69] Together, these findings highlight the complex role of the central and peripheral KOR system in different pain and sensation modalities.

Here, we use a model of oxaliplatin-induced cold allodynia[30,42] to investigate the role of KORs in cold hypersensitivity associated with oxaliplatin-induced neuropathy. Our study establishes the role of KORs in mediating cold hypersensitivity associated with the chemotherapy-induced peripheral neuropathy model. We show that activation of peripheral KORs drives cold hypersensitivity in naïve mice and demonstrate that KOR inhibition attenuates cold hypersensitivity associated with oxaliplatin-induced peripheral neuropathy. We then show that conditionally knocking out KORs in the DRG attenuates cold hypersensitivity associated with acute oxaliplatin treatment. Together, these findings identify a novel role for the kappa opioid system in modulation of cold hypersensitivity.

2. Materials and methods

2.1. Animals

Adult C57BL/6J (The Jackson Laboratory #000664) male and female mice (25-30 g) are used for all the behavioral experiments. The PirtCre$^{\pm}$ and Oprk1$^{fl/fl}$ breeder mice were obtained from Dr. Sarah Ross (University of Pittsburgh) developed by Dr. Xinzhong Dong's laboratory[35] and crossed in our laboratory for more than 4 generations to obtain PirtCre; Oprk1$^{fl/fl}$. All animals were 9 to 12 weeks old at the beginning of the experiments. Mice were group-housed in a 12/12-hour dark/light cycle environment (lights are turned on at 6:00 AM) and given access to food pellets and water ad libitum. After weaning, all animals were transferred to a holding facility adjacent to the laboratory and acclimated for at least 7 days before experimentation. Before each experiment, animals were acclimated to the behavior test room for at least 2 hours. All procedures were approved by the Washington University Institutional Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. Drugs

For the behavioral experiments, U50488 (U50, Tocris CAS# 67197-96-0; KOR agonist, 5 mg/kg, i.p.), norbinaltorphimine dihydrochloride (norBNI, Tocris CAS# 113158-34-2; KOR antagonist, 10 mg/kg, i.p.), morphine sulfate (McKesson #996806; μ-opioid receptor [MOR] agonist, 10 mg/kg, s.c.), and CR845 analogues (ff(nle)r-NH2 (D-phenylalanine-D-phenylalanine-D-norleucine-D-arginine-NH2); peripherally restricted KOR agonist, 10 mg/kg, i.p., prepared in the Mclaughlin laboratory)[3–5,53] were dissolved in 0.9% sterile saline. Oxaliplatin (ApexBio, A8648) was dissolved in 5% glucose dH2O solution to achieve a dosage of 100 μg.

2.3. Hot/cold plate assay

The hot/cold plate apparatus was adapted from the operant thermal plantar assay.[61] The floor of the apparatus is made of one 12" × 12" × ¼" aluminum plate (3003, MetalsDepot) fixed to a cold plate Peltier cooler (CP-061, TE Technology), which connects to an independent power supply (PS-12-8, 4A, TE Technology) and temperature controller (TC-48-20, TE Technology). Long cast acrylic tubing (15' height, E-plastics) was used to contain the mice on the plate. Male and female C57BL/6J wild-type (WT) mice were habituated in Plexiglass boxes for 30 minutes before i.p. injection of (1) U50, KOR agonist (5 mg/kg), (2) norBNI, KOR antagonist (10 mg/kg), (3) saline (0.25 mL), or (4) ff(nle)r-NH2, peripherally restricted KOR agonist (10 mg/kg). After treatment, mice were placed on the plate for 5 minutes, and the latency to jump and the number of jumps was recorded as nocifensive responses. The plate temperature varied from 3°C to 42°C and was continuously monitored with a visual temperature strip (liquid crystal temperature indicating sheets, Telatemp) and a surface probe thermometer (Pro-surface Thermapen, Thermoworks).

2.4. Rectal temperature measurements

Core body temperature was measured with a homeothermic monitoring system attached to a flexible rectal probe (TCAT2LV controller, Physitemp Instruments). On the test day, mice were acclimated to the test room for 90 minutes and subsequently injected i.p. with saline (0.25 mL), U50 (5 mg/kg), or ff(nle)r-NH2 (10 mg/kg). Immediately after injection, temperature was recorded by rectal probe; an additional reading was obtained 30 minutes after injection.

2.5. Paw surface temperature measurements

Paw surface temperature readings were collected using a FLIR One Pro LT (Teledyne, FLIR-Android version). On the test day, mice were acclimated to the test room for 90 minutes and subsequently injected i.p. with U50 (5 mg/kg) or saline (0.25 mL). Immediately after injection, the mouse was mildy restrained and an image was taken ensuring exposure of the paws to obtain a clear reading of paw temperatures. An additional image was acquired 30 minutes after injection. Data were processed on the FLIR One app (Windows10, 64 bit version) capturing walking pad, digits, center of the paw, and heels on the ventral side of paws to calculate the paw surface temperatures.

2.6. Tail withdrawal assay

Mice were habituated to the experimenter for a week before the assay by daily handling to minimize any stress that might impact the assay.[23] On the day of experiment, after U50 (5 mg/kg) or saline (0.25 mL) i.p. injection, the mice were scruffed, and one-third of the distal end of the tail was immersed in the hot (54.5°C) water bath. The time taken for the tail to twitch or flick was recorded. Single readings were recorded at 15, 30, 45, 60, 90, and 120 minutes after treatment.

2.7. Von Frey test

Mechanical sensitivity was determined using manual von Frey filaments after U50 (5 mg/kg) or saline (0.25 mL) i.p. treatments. On the test day, individual mice were placed in an acrylic cylinder on a mesh floor and covered with a rectangular acrylic lid to avoid escape. The mice were habituated to the apparatus for 2 hours before testing. During the assay, a monofilament was applied perpendicular to the plantar surface of the hind paw for 2 to 5 seconds. If the animal exhibited any nocifensive behaviors,

including brisk paw withdrawal, licking, or shaking of the paw, either during or immediately after the stimulus, a positive response was recorded.[23] We used the "ascending stimulus" method, in which monofilaments with increasing force are applied until a withdrawal response is elicited. This was quantified as the mechanical withdrawal threshold (g). The stimulus was repeated 3 times alternatively for each hind paw, and the mean of the 6 readings was calculated. A 10-minute break was included between each reading to prevent hypersensitivity.

2.8. Acetone evaporation test

The acetone evaporation test was used to measure aversive behaviors triggered by evaporative cooling.[23] On the test day, mice were placed on the mesh floor for 90 minutes for acclimatization, and subsequently i.p. injected with U50 (5 mg/kg) or saline (0.25 mL). Postinjection, acetone (5-6 μL) was applied to the plantar surface of the hind paw using the open end of a blunt 1-mL syringe, eliciting a rapid paw withdrawal response (ie, elevation, shaking, or licking). The response was recorded for 1 minute after each acetone application. Application was repeated 3 times alternatively for each hind paw, and the mean of the 6 readings was calculated; a 10-min break was included between each reading to prevent hypersensitivity.[21,68] Sensitivity to cold was determined either by quantifying paw directed behavior, scoring the severity of the response (ie, 0, no response; 1, brisk withdrawal or flick of the paw; 2, repeated flicking of the paw; 3, repeated flicking of the hind paw and licking of the paw), or measuring the latency to lick the paw.

2.9. Open field test

Mouse locomotor behaviors were assessed using an open field test (OFT) apparatus consisting of a 2500 cm^2 arena, 50% of which we defined as the center.[2,45] Lighting was stabilized at ~25 lux for anxiety-like behaviors. On the test day, mice were acclimated to the OFT room for 90 minutes and subsequently i.p. injected with U50 (5 mg/kg) or saline (0.25 mL). Mice were then placed in the OFT arena and allowed to roam for 20 minutes, during which their movements were continuously recorded and tracked using EthoVision 13 software (Noldus Information Technologies). The total distance moved in the apparatus and the time spent exploring the center were quantified to determine sedative and anxiety-like behaviors, respectively.

2.10. Intracerebroventricular injections

Mice received intracerebroventricular (ICV) injections as previously described.[12] Briefly, mice are anesthetized in an induction chamber (3% isoflurane) and placed into a stereotaxic frame (Kopf Instruments, Model 942), where they were maintained under continuous anesthesia (2%-2.5% isoflurane). A craniotomy was performed unilaterally and either KOR antagonist norBNI (30 μmol, 2 μL) or artificial cerebrospinal fluid (aCSF) was injected into the lateral ventricle at 200 nL/minute for 10 minutes with a beveled Hamilton syringe (10 μL, 701 N) using the stereotaxic coordinates from bregma: A/P: +0.40 mm, M/L: +1.5 mm, D/V: −3 mm. Sterile nylon sutures (6.0 mm) were used to suture the skin after the injection, and mice were allowed to recover for at least week before behavioral experiments. On the experimental day, postsurgery mice were i.p. injected with either U50 (5 mg/kg) or saline (0.25 mL) and exposed to the cold plate.

2.11. Immunohistochemistry

Histological verification of the ICV injection site was performed as described.[1] Mice were briefly anesthetized with 0.2 mL of a cocktail containing 100 mg/mL ketamine, 20 mg/mL xylazine, and 10 mg/mL acepromazine and perfused intracardially with ice-cold 4% paraformaldehyde in phosphate buffer (PB). Brains were dissected, postfixed for 24 hours at 4°C, and cryoprotected with 30% sucrose solution in 0.1 M PB at 4°C for at least 24 hours, cut into 30-μm sections, and processed for immunostaining. The ICV injection placements were confirmed using a Leica fluorescent microscope (DM6 series scope) with the Paxinos-Watson atlas[54] as a reference. In total, 76 out of 85 WT mice (male and female) had verified injections into the lateral ventricle, and the rest were excluded from the analysis.

2.12. Acute oxaliplatin paw injection

We used the oxaliplatin-induced neuropathy model in mice.[24,42] The rapid onset of cold hypersensitivity after a single therapeutic dose of oxaliplatin (3 mg/kg) mirrors the quick development of cold allodynia seen in patients.[24] In this study, we modified the model to elicit a stronger response and to prevent the lateralization of effects by injecting oxaliplatin into both paws. Oxaliplatin (ApexBio, A8648) was dissolved in 5% glucose dH2O solution to achieve a dose of 100 μg. Mice received intraplantar oxaliplatin injection of 50 μL per paw into both their hind paws. Nocifensive behaviour was assessed on a cold plate at 3°C at 1 and 3 hours after oxaliplatin exposure. Nocifensive behaviors recorded included a number of paw-directed responses such as paw licking, biting, flinching, and shaking.

2.13. In situ hybridization

$Oprk1^{fl/fl}$ and PirtCre;$Oprk1^{fl/fl}$ male and female mice were euthanized under isoflurane by decapitation, and lumbar DRG were removed.[65,67] Dorsal root ganglia were harvested, rapidly frozen on dry ice in mounting medium (Tissue-Tek O.C.T. Compound, Sakura) and stored at −80°C for 1 week. DRG sections (5-7 μM) were cut at −20°C and thaw-mounted onto Super Frost Plus slides (Fisher). Slides were stored at −80°C until the following day. Fluorescent in situ hybridization (ISH) was performed according to the RNAScope 2.0 Fluorescent Multiple Kit User Manual for Fresh Frozen Tissue (Advanced Cell Diagnostics), as described.[78] Briefly, sections were fixed in 4% paraformaldehyde and dehydrated with ethanol at increasing concentrations 50%, 75%, and 100% (vol/vol in deionized water). Sections were pretreated with hydrogen peroxide for 15 minutes at room temperature and washed in PBS twice for 2 minutes each. Post-wash, sections were pretreated with protease IV solution and incubated with mouse *Cre* (312,281) (C1), *oprk1* (316,111) (C2) target probes (Advanced Cell Diagnostics). After probe hybridization, sections underwent probe signal amplification steps followed by incubation with fluorescently labeled probes for *Cre* (fluorescein), *oprk1* (cyanin 3). Slides were counterstained with DAPI, and coverslips were mounted with Vectashield Hard Set mounting medium (Vector Laboratories). All samples were imaged under a Keyence BZ microscope with BZ-X800 analyzer software (Keyence). Quantification of RNAscope fluorescence was performed using RNAscope HiPlex Image Registration Software (ACD) for background reduction, and fluorescence quantification was performed using HALO image analysis software (Indica Labs) at 20x magnification. Parameter settings: Image Zoom: 1; Micron per Pixel: 0.75,488. Number of

Probes: 2; Nuclear Stain: DAPI; Nuclear Contrast Threshold: 0.5; Minimum Nuclear Intensity: 0.049; Nuclear Segmentation Aggressiveness: 0.65; Fill Nuclear Holes: False; Nuclear Size: 11.3571.7; Minimum Nuclear Roundness: 0; Maximum Cytoplasm Radius: 7.5; Cell localization: True. Probe 1 KOR: Contrast Threshold: 0.547; Signal Minimum Intensity: 0.15; Spot Size: 0.5288; Copy Intensity: 0.15; Spot Segmentation Aggressiveness: 0.95. Probe 2 Cre: Contrast Threshold: 0.25; Signal Minimum Intensity: 0.1; Spot Size: 0.5298; Copy Intensity: 0.15; Spot Segmentation Aggressiveness: 0.95. Publication-quality images were acquired on a Nikon AXR confocal microscope using a 20x and 60x oil-immersion objective, controlled by NIS-Elements AR software (Nikon).

2.14. Statistical analysis

Datasets were tested for homogeneity of variance and normality before being assigned to any parametric analysis. All experiments are performed in multiple cohorts, including all treatment groups in each round, to avoid nonspecific day or condition effects. Sex differences were observed in behavior data; after which males and females were analyzed separately. Treatments were randomly assigned to animals before testing. G*Power was used to estimate effect sizes and compute power analyses. Statistical significance was considered *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, determined by parametric or nonparametric analysis as appropriate. We used a repeated measures 2-way ANOVA for tail withdrawal assay analysis and 2-way ANOVA for mechanical sensitivity analysis. For assessment of the cold plate assay data, we used (1) Kruskal–Wallis followed by the Dunn multiple comparison test for ordinal ranking, such as scoring the number of jumps (nonparametric data); (2) nonparametric mixed-effects 2-way ANOVA followed by Sidak *post-hoc* test for the temperature curve; and (iii) Mann–Whitney *U* test for experiments using peripheral novel agonists. For characterization data analysis, we used 2-way ANOVA. Statistical analyses were performed in GraphPad Prism 8.0. All data were expressed as mean ± SEM.

3. Results

3.1. Kappa opioid receptors modulate cold hypersensitivity associated with oxaliplatin-induced neuropathy

We used an acute oxaliplatin-induced peripheral neuropathy model to assess whether KORs modulate cold hypersensitivity linked to oxaliplatin-induced neuropathy[30,42] (**Fig. 1A**, Fig. S1A, supplemental digital content, http://links.lww.com/PAIN/C533). Sixty minutes after the administration of oxaliplatin, we observed an increase in paw-directed responses on a 3°C cold plate compared to saline-injected controls in both male (**Fig. 1B**) and female mice (**Fig. 1C**), as expected. The number of paw-directed responses after oxaliplatin injection was significantly higher in females compared to males at 60 minutes (Fig. S1B, supplemental digital content, http://links.lww.com/PAIN/C533) and 180 minutes (Fig. S1C, supplemental digital content, http://links.lww.com/PAIN/C533), suggesting a potential sex difference in response to oxaliplatin-induced cold hypersensitivity. To determine whether potentiation of noxious hypersensitivity was selectively induced by KOR, we pharmacologically blocked KOR with norBNI (KOR antagonist, 10 mg/kg) and recorded oxaliplatin-induced paw-directed responses on a 3°C cold plate. NorBNI significantly attenuated the number of paw-directed responses compared to saline controls in both male and female mice (**Figs. 1D, E**). The number of paw-directed responses was significantly higher in females than in males after norBNI treatment (Fig. S1C, supplemental digital content, http://links.lww.com/PAIN/C533). Together, these findings suggest that

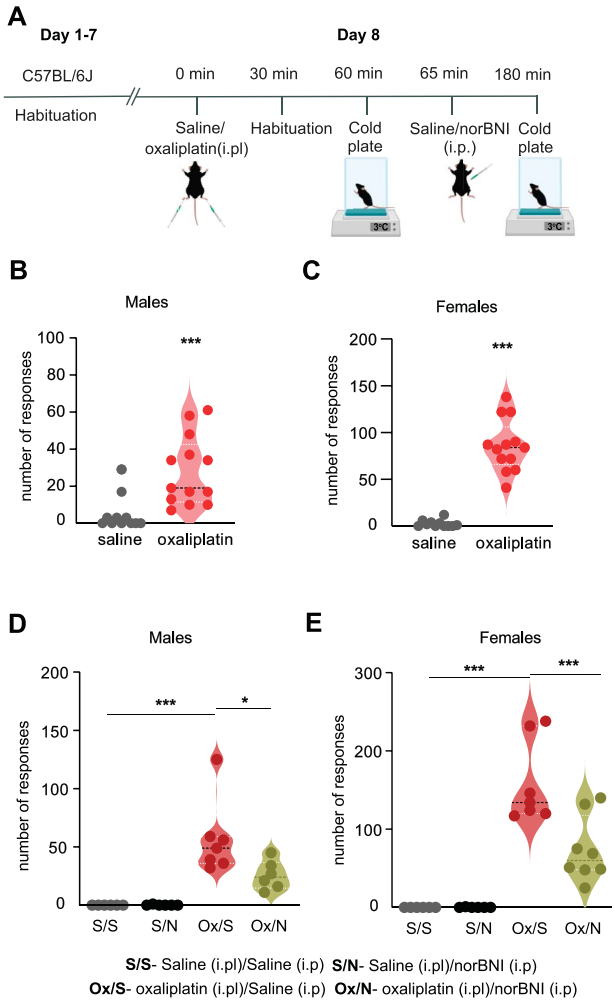

Figure 1. Kappa opioid receptors modulate cold hypersensitivity associated with oxaliplatin-induced neuropathy. (A) Outline of the experimental procedure. C57BL/6J mice were intraplantarly (i.pl.) injected with saline or oxaliplatin (50 μg each paw) after 7 days of handling. Sixty minutes after injection, mice were subjected to cold plate temperatures of 3°C, and the number of responses was recorded. Mice were injected with saline or norBNI (10 mg/kg i.p.) post cold plate behavior, and again subjected to the cold plate. (B) In males, oxaliplatin significantly increased the number of responses at 3°C at 60 minutes. Mann–Whitney *U* test analysis revealed an oxaliplatin effect $z = 12$ (***$P < 0.001$ vs respective saline treatment). Data are presented as mean ± SEM; n = 11 to 13 mice per group. (C) In females, oxaliplatin significantly increased the number of responses at 3°C at 60 minutes. Mann–Whitney *U* test analysis revealed an oxaliplatin effect $z = 0$ (***$P < 0.001$ vs respective saline treatment). Data are presented as mean ± SEM; n = 11 to 13 mice per group. (D) In males, norBNI significantly attenuated oxaliplatin-induced cold responses at 3°C. Two-way ANOVA revealed oxaliplatin paw injection effect $F_{(1, 21)} = 32.08$, systemic norBNI injection effect $F_{(1, 21)} = 4.501$ and an interaction of oxaliplatin and norBNI effect $F_{(1, 21)} = 4.599$ followed by Tukey *post-hoc* test (***$P < 0.001$ oxaliplatin–saline vs saline–saline, *$P < 0.05$ oxaliplatin–norBNI vs oxaliplatin–saline). Data are presented as mean ± SEM; n = 6 to 8 mice per group. (E) In females, norBNI significantly attenuated oxaliplatin-induced cold responses at 3°C. Two-way ANOVA revealed oxaliplatin paw injection effect $F_{(1, 23)} = 71.48$, systemic norBNI injection effect $F_{(1, 23)} = 9.563$, and an interaction of oxaliplatin and norBNI effect $F_{(1, 23)} = 9.639$ followed by Tukey *post-hoc* test (***$P < 0.001$ oxaliplatin–saline vs saline–saline, ***$P < 0.001$ oxaliplatin–norBNI vs oxaliplatin–saline). Data are presented as mean ± SEM; n = 6 to 8 mice per group.

KOR modulates cold hypersensitivity in our model of chemotherapy-induced neuropathy.

3.2. Activation of kappa opioid receptors induces noxious cold hypersensitivity in naïve mice

To determine whether activation of KOR mediates temperature-dependent hypersensitivity, we recorded jumping behavior across a range of temperatures (3°C, 10°C, 15°C, 20°C, 30°C, and 42°C) (**Fig 2A**, Fig. S2A, supplemental digital content, http://links.lww.com/PAIN/C533), a well-established measure of nocifensive behavior.[6,15,23] At 3°C, treatment with the KOR agonist, U50, significantly increased the number of jumps compared to controls in male and female mice (**Figs. 2B, C**). This was accompanied by a significant decrease in latency to jump (**Figs. 2D, E**). We did not observe sex differences in KOR-induced hypersensitivity at 3°C (Fig. S2B&C, supplemental digital content, http://links.lww.com/PAIN/C533). Interestingly, U50 also increased the number of jumps and decreased latency to jump in females but not males at 10°C (Fig. S2D&E, supplemental digital content, http://links.lww.com/PAIN/C533). However, KOR activation had no effect on the number of jumps or the latency to jump at 15°C (Fig. S2F&G, supplemental digital content, http://links.lww.com/PAIN/C533), 20°C (Fig. S2H&I, supplemental digital content, http://links.lww.com/PAIN/C533), or 30°C (Fig. S2J&K, supplemental digital content, http://links.lww.com/PAIN/C533). Notably, at 42°C, the number of jumps increased across all treatments (Fig. S2L, supplemental digital content, http://links.lww.com/PAIN/C533), a well-known response to noxious heat[81] rather than KOR activation specifically. Interestingly, we observed significantly increased sensitivity to noxious heat in females compared to males (Fig. S2L&M, supplemental digital content, http://links.lww.com/PAIN/C533), suggesting that female mice may be more sensitive to KOR activation than male mice at noxious hot temperatures. Together, these results show that KOR activation selectively induces hypersensitivity to noxious cold in naïve mice.

To determine whether activation of KOR affects body temperature, we recorded core body temperature and paw-surface temperature using a rectal thermometer and thermal camera (**Fig. 2F**). We showed that U50 did not alter core body temperature (**Figs. 2G, H**) or paw surface temperature (**Figs. 2I–K**) in males or in females at 0 minutes vs 30 minutes. However, we observed that male mice injected with U50 have a significantly higher core body temperature at 0 minutes and 30 minutes than saline controls. Although the initial temperature is higher, this did not change over time (**Figs. 2G, H**). Also, we observed that male mice injected with U50 have a significantly lower paw surface temperature at 0 minutes and 30 minutes compared with saline controls. Although the initial temperature is lower, this did not change over time (**Figs. 2J, K**). These findings confirm that KOR-mediated cold hypersensitivity effects are unlikely due to impaired central thermoregulation.

3.3. Activation of kappa opioid receptors is antinociceptive at noxious hot temperatures

To determine whether activation of KOR mediates antinociception at hot temperatures, we corroborated our findings with published work showing that activation of KOR was antinociceptive in a warm water tail-withdrawal assay (**Figs. 3A–C**) and the von Frey test of mechanical sensitivity (**Figs. 3D–F**).[46] To further substantiate that KOR-induced noxious cold hypersensitivity is temperature-dependent, we used the acetone evaporation test (**Fig. 3G**). Acetone is known to mimic cool temperatures in the range of 15°C to 21°C but not noxious cold temperatures.[21,40] We showed that U50-mediated KOR activation did not alter paw withdrawal behavior (**Fig. 3H**) or the duration of nocifensive responses (**Fig. 3I**). The dose of U50 we used in this study (5 mg/kg) did not have the sedative effect observed at higher doses,[25] as demonstrated by the absence of differences in locomotor activity between the groups (**Figs. 3J–M**).

3.4. Potentiation of noxious cold hypersensitivity is kappa opioid receptor–selective

To determine whether potentiation of noxious hypersensitivity was selectively induced by KOR, we pharmacologically blocked KOR with norBNI (10 mg/kg i.p.) and recorded U50-potentiated jumping response. Pharmacologically blocking KOR prevented U50-induced noxious cold hypersensitivity at 3°C in both male and female mice (**Figs. 4A–C**). NorBNI alone did not alter any nocifensive responses, irrespective of sex (**Figs. 4B, C**). Furthermore, activation of μ-opioid receptors with morphine (10 mg/kg) did not drive noxious cold hypersensitivity in male or in female mice (**Figs. 4D–F**), suggesting that cold hypersensitivity is a KOR-selective effect.

3.5. Peripheral kappa opioid receptors mediate noxious cold hypersensitivity

To determine whether centrally and/or peripherally expressed KOR mediates the observed increase in noxious cold hypersensitivity, we first blocked central KOR by injecting norBNI (KOR antagonist) intracerebroventricularly (ICV) and administered U50 systemically before placing the mice on the cold plate (**Fig. 4G**). After systemic injection of U50 with central aCSF (aCSF + U50), the number of jumps increased, compared to vehicle treatment (aCSF + Sal) at 3°C in both male and female mice (**Figs. 4H, I**), indicative of cold hypersensitivity. Central delivery of norBNI and systemic administration of U50 (norBNI + U50) resulted in noxious cold hypersensitivity in male and female mice (**Figs. 4H, I**). Centrally administered norBNI alone (norBNI + saline) did not alter cold sensitivity, as compared to the controls (aCSF + saline) (**Figs. 4H, I**). Although we did not include a positive control for central KOR inhibition after ICV administration of norBNI, previous studies indicate that norBNI effectively produces prolonged KOR blockade.[11,28] Together, these data suggest that central KOR does not mediate noxious cold hypersensitivity.

To then determine whether the KOR-induced noxious cold hypersensitivity is modulated by peripherally expressed KOR, we used the peripherally restricted KOR agonist ff(nle)r-NH2 (10 mg/kg) (**Fig. 4J**).[3–5,53] Treatment with ff(nle)r-NH2 significantly increased jumping on the cold plate at 3°C compared to saline controls in both males and females (**Figs. 4K, L**) but did not alter core body temperature (Fig. S3A-C, supplemental digital content, http://links.lww.com/PAIN/C533) or paw surface temperature (Fig. S3D&E, supplemental digital content, http://links.lww.com/PAIN/C533). These findings suggest that peripherally expressed KOR induces noxious cold hypersensitivity.

3.6. Peripheral kappa opioid receptors modulate cold hypersensitivity in the oxaliplatin-induced neuropathy

To selectively target peripheral KOR in the primary sensory neuron in the DRG, we generated a conditional knockout mouse line by crossing PirtCre$^{\pm}$ to Oprk1$^{fl/fl}$ (**Fig. 5A**) in which KOR expression is excised out in the Pirt expressing mice. Pirt,

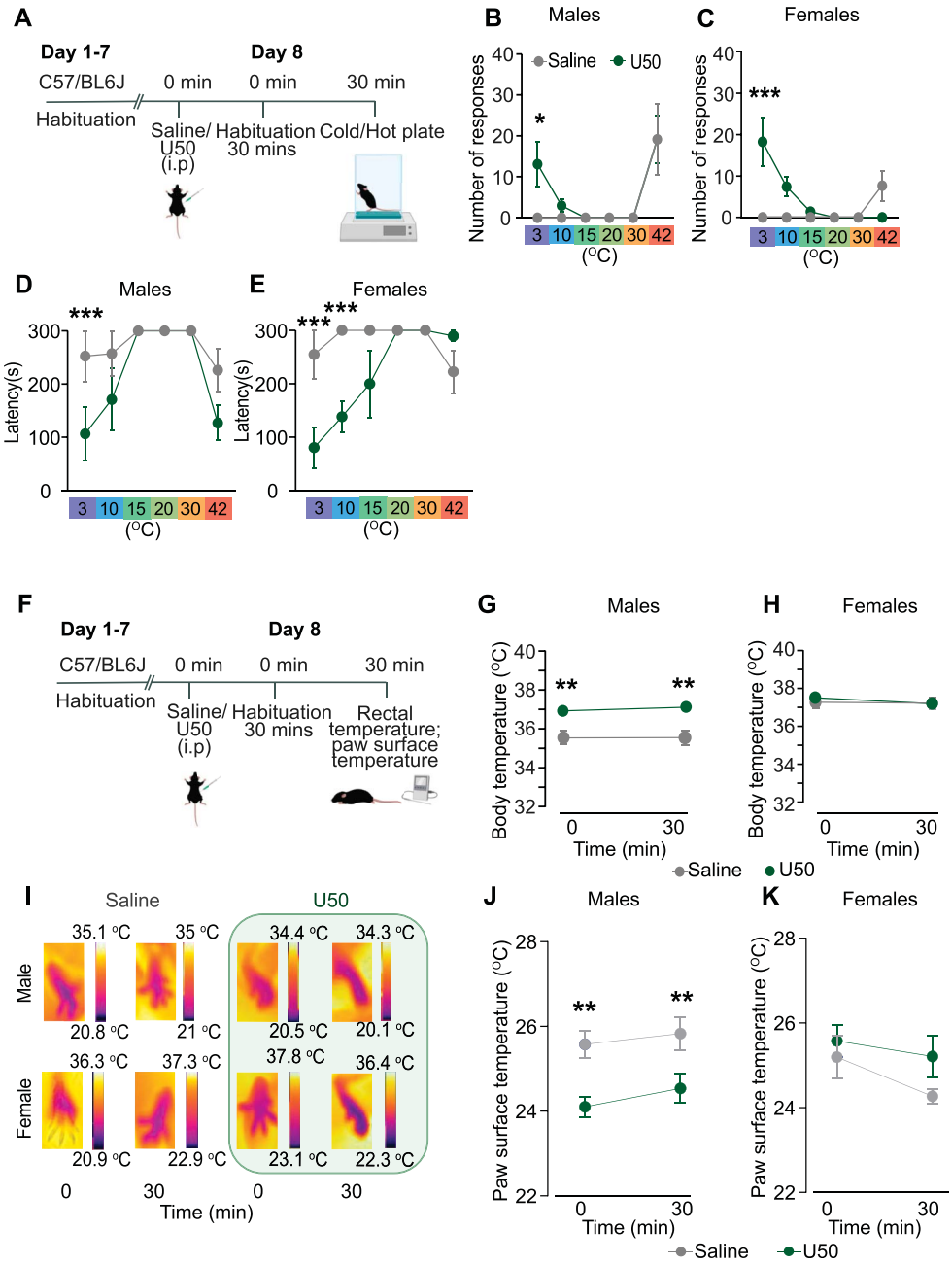

Figure 2. Activation of kappa opioid receptors induces noxious cold hypersensitivity in naïve mice. (A) Outline of the experimental procedure. C57BL/6J mice were intraperitoneally (i.p.) injected with saline or KOR agonist U50 (5 mg/kg) after 7 days of handling. Postinjection, mice were subjected to cold/hot plate temperatures of 3°C, 10°C, 15°C, 20°C, 30°C, and 42°C, and the number of jumps and latency to jump was recorded. (B) In males, U50 significantly increased the number of jumps at 3°C. Nonparametric mixed-effects analysis (2-way ANOVA) revealed temperature effect $F_{(5, 52)} = 8.92$ followed by Sidak *post-hoc* test (*$P < 0.05$ vs respective saline treatment). Data are presented as mean ± SEM; n = 5 to 7 mice per group. (C) In females, U50 significantly increased the number of jumps at 3°C. Nonparametric mixed-effects analysis (2-way ANOVA) revealed temperature effect $F_{(5, 57)} = 3.937$, treatment effect $F_{(1, 57)} = 4.956$, and a treatment × temperature $F_{(5, 57)} = 6.191$ followed by Sidak *post-hoc* test (***$P < 0.001$ vs respective saline treatment). Data are presented as mean ± SEM; n = 5 to 7 mice per group. (D) Latency to jump after KOR activation was decreased in males at 3°C only. Nonparametric mixed-effects analysis (2-way ANOVA) revealed temperature effect $F_{(5, 53)} = 4.792$, treatment effect $F_{(1, 53)} = 8.665$, and a treatment × temperature $F_{(5, 53)} = 2.446$ followed by Sidak *post-hoc* test (***$P < 0.001$ vs respective saline treatment). Data are presented as mean ± SEM; n = 5 to 7 mice per group. (E) Latency to jump after KOR activation was decreased in females at 3°C and 10°C. Nonparametric mixed-effects analysis (2-way ANOVA) revealed temperature effect $F_{(5, 30)} = 5.917$, treatment effect $F_{(1, 29)} = 14.82$, and a treatment x temperature $F_{(5, 29)} = 6.722$ followed by Sidak *post-hoc* test (***$P < 0.001$ vs respective saline treatment). Data are presented as mean ± SEM; n = 5 to 7 mice per group. (F) Outline of the experimental procedure. C57BL/6J mice were habituated, and baseline rectal temperature and thermal imaging of paw surface temperatures were measured, followed by systemic injections of saline or U50 and post-treatment temperature recordings. (G) KOR agonist U50 did not alter core body temperature 30 minutes after drug administration in males. U50 mice had higher baseline core body temperatures compared to the saline counterparts at both 0 minutes and 30 minutes. Two-way repeated measures ANOVA (mixed-effects analysis) revealed a treatment effect $F_{(1, 23)} = 10.70$, followed by Fisher LSD *post-hoc* test (**$P < 0.01$ vs respective saline treatment). Data are expressed as mean ± SEM; n = 6 to 10 mice per group. (H) KOR agonist U50 did not alter core body temperature 30 minutes after drug administration in females. Data are expressed as mean ± SEM; n= 6 to 10 mice per group. (I) Representative FLIR infrared camera images from right hind paws of male and female mouse pre- and postsaline or U50 administration. (J) KOR agonist U50 did not alter paw surface temperatures in males. U50 mice had lower baseline paw surface temperatures compared to the saline counterparts at both 0 minutes and 30 minutes. Two-way repeated measures ANOVA (mixed-effects analysis) revealed a treatment effect $F_{(1, 15)} = 17$, followed by Fishers LSD *post-hoc* test (**$P < 0.01$ vs respective saline treatment). Data are expressed as mean ± SEM; n = 8 to 9 mice per group. (K) KOR agonist U50 did not alter paw surface temperatures in females. Data are expressed as mean ± SEM; n = 8 to 9 mice per group.

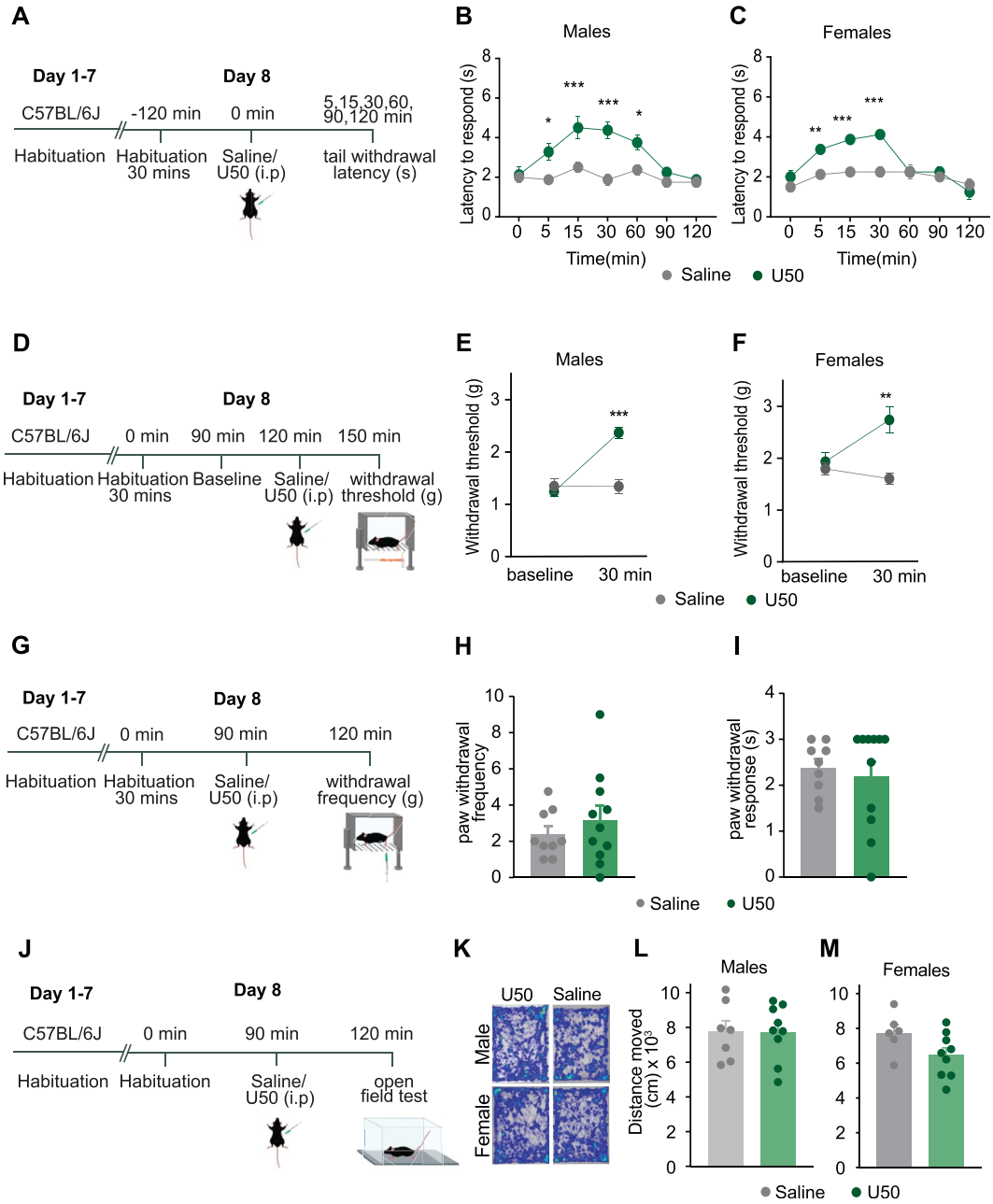

Figure 3. Kappa opioid receptor activation is antinociceptive at noxious hot temperatures in naïve mice. (A) Outline of experimental procedure. C57BL/6J mice were intraperitoneally (i.p.) injected with saline or U50 after 7 days of handling. Postinjection, mice were subjected to tail withdrawal assays at 5 minutes, 15 minutes, 30 minutes, 60 minutes, 90 minutes, and 120 minutes. (B) U50-mediated antinociception in males (increase in tail withdrawal latency). Parametric repeated measures ANOVA (2-way ANOVA) revealed time effect $F_{(6, 97)} = 4.054$, dose effect $F_{(6, 97)} = 8.036$, and time x dose effect $F_{(1, 97)} = 42.85$ followed by Sidak *post-hoc* test ($^{*}P < 0.05$, $^{***}P < 0.001$ vs respective saline treatment). Data are expressed as mean ± SEM; n = 7 to 8 mice per group. (C) U50-mediated antinociception in females (increase in tail withdrawal latency). Parametric repeated measures ANOVA (two-way ANOVA) revealed time effect $F_{(6, 98)} = 5.620$, dose effect $F_{(6, 98)} = 13.33$, and time x dose effect $F_{(1, 98)} = 28.63$ followed by Sidak *post-hoc* test ($^{**}P < 0.01$, $^{***}P < 0.001$ vs respective saline treatment). Data are expressed as mean ± SEM; n = 7 to 8 mice per group. (D) Timeline of experimental procedures for von Frey assays. C57BL/6J mice were habituated, and baseline withdrawal threshold was scored. After systemic injection of saline or U50, a 30-minute post-treatment response to mechanical stimulus was recorded. (E) U50 blocked mechanical sensitivity in males. Parametric repeated measures ANOVA (2-way ANOVA) revealed time effect $F_{(1, 15)} = 36$, dose effect $F_{(1, 15)} = 10.96$, and time × dose effect $F_{(1, 15)} = 36.54$ followed by Sidak *post-hoc* test ($^{***}P < 0.001$ vs respective saline treatment). Data are expressed as mean ± SEM; n = 6 to 8 mice per group. (F) U50 blocked mechanical sensitivity in females. Parametric repeated measures ANOVA (2 way ANOVA) revealed dose effect F (1, 12) = 15.26 and time × dose effect F (1, 12) = 5.127 followed by Sidak post-hoc test ($^{**}P < 0.001$ vs respective saline treatment). Data are expressed as mean ± SEM; n = 6 to 8 mice per group. (G) Outline of the acetone evaporation test experimental procedure. C57BL/6J mice were habituated to the apparatus used in acetone drop test after 7 days of handling. After habituation at 90 minutes, mice were intraperitoneally (i.p.) injected with either saline or U50 (KOR agonist, 5 mg/kg). Postinjection at 120 minutes, mice hind paws were dabbed with a drop of acetone or saline to evoke a withdrawal response. U50 had no effect on acetone-evoked aversive behavior, as compared to saline, in males. The acetone-evoked aversive behavior was measured by quantifying (H) the nocifensive responses (scoring the paw withdrawal frequency) and (I) duration of nocifensive response (scoring the latency of paw withdrawal response). Data are expressed as mean ± SEM; n=9 to 11/group. (J) Outline of the experimental procedure. C57BL/6J mice were habituated for 90 minutes to the room used for open field test (OFT) assay after 7 days of handling. After habituation, mice were intraperitoneally (i.p.) injected with either saline or U50 (KOR agonist, 5 mg/kg). Postinjection at 120 minutes, mice were left in the OFT for 30 minutes to monitor the locomotor behavior. (K) Representative heat maps of OFT behavior in male and female saline and U50 treatment groups. U50 had no sedative effect in the open field test in (L) males (M) females. Data are expressed as mean ± SEM; n = 6 to 9/group.

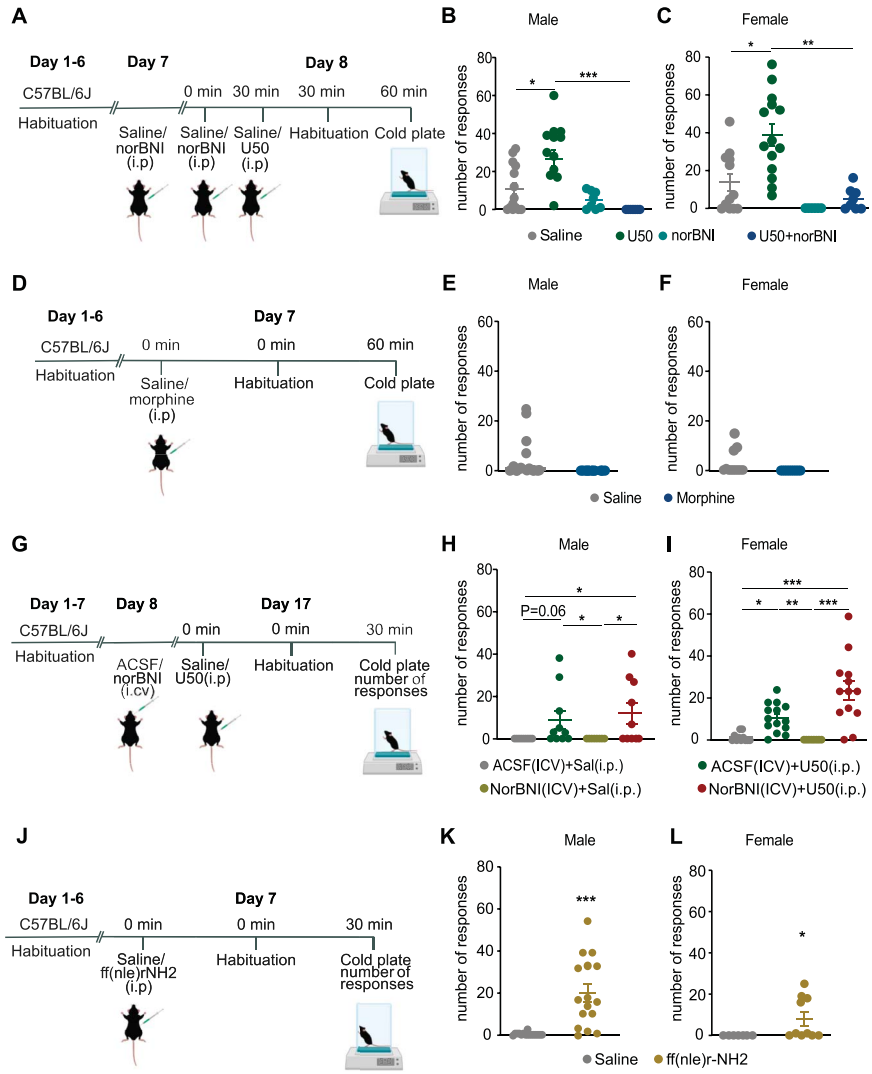

Figure 4. Potentiation of noxious cold hypersensitivity is kappa opioid receptor–selective and peripherally mediated. (A) Outline of the experimental procedure. On day 7, C57BL/6J mice were intraperitoneally (i.p.) injected with saline or KOR antagonist norBNI (10 mg/kg) after 6 days of handling. On day 8, mice were again injected with saline or norBNI (10 mg/kg) to block the KOR completely. Thirty minutes after saline/antagonist injection, the mice were injected with saline or U50 (5 mg/kg) followed by habituation and cold plate assay at 3°C, and the number of jumps was recorded. (B) In males, activation of KOR by U50 significantly increased the number of jumps on a 3°C cold plate. Kruskal–Wallis test revealed treatment effect $H = 23.35$ followed by Dunn *post-hoc* test (*$P < 0.05$ U50 vs saline). KOR antagonism with norBNI blocked cold hypersensitivity on a cold plate at 3°C (***$P < 0.001$ U50 vs U50+norBNI). Data are presented as mean ± SEM; n = 8 to 14 mice per group. (C) In females, activation of KOR by U50 significantly increased the number of jumps on a 3°C cold plate. Kruskal–Wallis test revealed treatment effect $H = 23.28$ followed by Dunn *post-hoc* analysis (*$P < 0.05$ U50 vs saline). KOR antagonism with norBNI blocked cold hypersensitivity on a cold plate at 3°C (**$P < 0.01$ U50 vs U50+norBNI). Data are presented as mean ± SEM; n = 8 to 14 mice per group. (D) Outline of the experimental procedure. C57BL/6J mice were subcutaneously (s.c.) injected with saline or morphine (10 mg/kg) after 7 days of handling. Postinjection, mice were habituated for 30 minutes followed by cold plate test at 3°C, and the number of jumps was recorded. (E) Morphine did not affect cold sensitivity in males. Data are expressed as mean ± SEM; n = 10 to 13 mice per group. (F) Morphine did not affect cold sensitivity in females. Data are expressed as mean ± SEM; n = 12 mice per group. (G) Outline of the experimental procedure. After habituation, mice were intracerebroventricularly (ICV) injected with (norBNI or aCSF) (2 nmol/2 µL), followed by recovery for 7 days. Postrecovery on day 17, mice received systemic U50 or saline injections, followed by 30 minutes habituation and cold plate assay at 3°C. (H) Male mice infused with aCSF and injected with U50 showed cold hypersensitivity, as compared to the control group in 3°C cold plate assays. Kruskal–Wallis test revealed treatment effect $H = 12.45$ followed by Dunn *post-hoc* analysis ($P = 0.06$ aCSF [ICV] + saline [i.p.] vs aCSF [ICV] + U50 [i.p.]). Central KOR antagonism with norBNI had no effect on cold hypersensitivity in males on a cold plate at 3°C (aCSF [ICV] + saline [i.p.] vs norBNI [ICV] + U50 [i.p.]). Central KOR antagonism without peripheral activation did not elicit cold hypersensitivity on a cold plate at 3°C (aCSF [ICV] + saline [i.p.] vs norBNI [ICV] +saline [i.p.]). Central KOR antagonism with peripheral activation elicited cold hypersensitivity (*$P < 0.05$ aCSF [ICV]+saline [i.p.] vs norBNI [ICV] + U50 [i.p.]). Peripheral activation of KOR-mediated cold hypersensitivity was no different to central and peripheral KOR activation–mediated cold sensitivity (norBNI [ICV]+U50 [i.p.] vs aCSF [ICV] + U50 [i.p.]). Data are presented as mean ± SEM; n = 8 to 10 mice per group. (I) Female mice infused with aCSF and U50 showed cold hypersensitivity, as compared to the control group in 3°C cold plate assays. Kruskal–Wallis test revealed treatment effect $H = 28.67$ followed by Dunn *post-hoc* analysis (*$P < 0.05$ aCSF [ICV] + U50 [i.p.] vs aCSF [ICV] + saline [i.p.]). Central KOR antagonism with norBNI had no effect on cold hypersensitivity in females on a cold plate at 3°C (***$P < 0.01$ aCSF [ICV] + saline [i.p.] vs norBNI (ICV) + saline [i.p.]). Central KOR antagonism with peripheral activation elicited cold hypersensitivity on a cold plate at 3°C (**$P < 0.01$ aCSF [ICV] + saline [i.p.] vs norBNI [ICV] + U50 [i.p.]). Peripheral activation of KOR-mediated cold hypersensitivity was no different to central and peripheral KOR activation–mediated cold sensitivity (norBNI [ICV] + U50 [i.p.] vs aCSF [ICV] + U50 [i.p.]). Data are presented as mean ± SEM; n = 8 to 14 mice per group. (J) Outline of the experimental procedure. C57BL/6J mice were intraperitoneally (i.p.) injected with saline or ff(nle)r-NH2 (10 mg/kg) after 7 days of handling. Postinjection, mice were habituated for 30 minutes followed by cold plate test at 3°C, and the number of jumps was recorded. (K) In male mice, peripherally restricted agonist ff(nle)r-NH2 significantly increased jumping on the cold plate at 3°C, compared to controls. Mann–Whitney *U* test revealed a treatment effect (***$P < 0.001$ saline vs ff(nle)r-NH2). Data expressed as mean ± SEM; n = 16 mice per group. (L) In female mice, peripherally restricted agonist ff(nle)r-NH2 significantly increased jumping on the cold plate at 3°C, compared to controls. Mann–Whitney *U* test revealed a treatment effect (*$P < 0.05$ saline vs ff(nle)r-NH2). Data expressed as mean ± SEM; n = 7 to 10 mice per group.

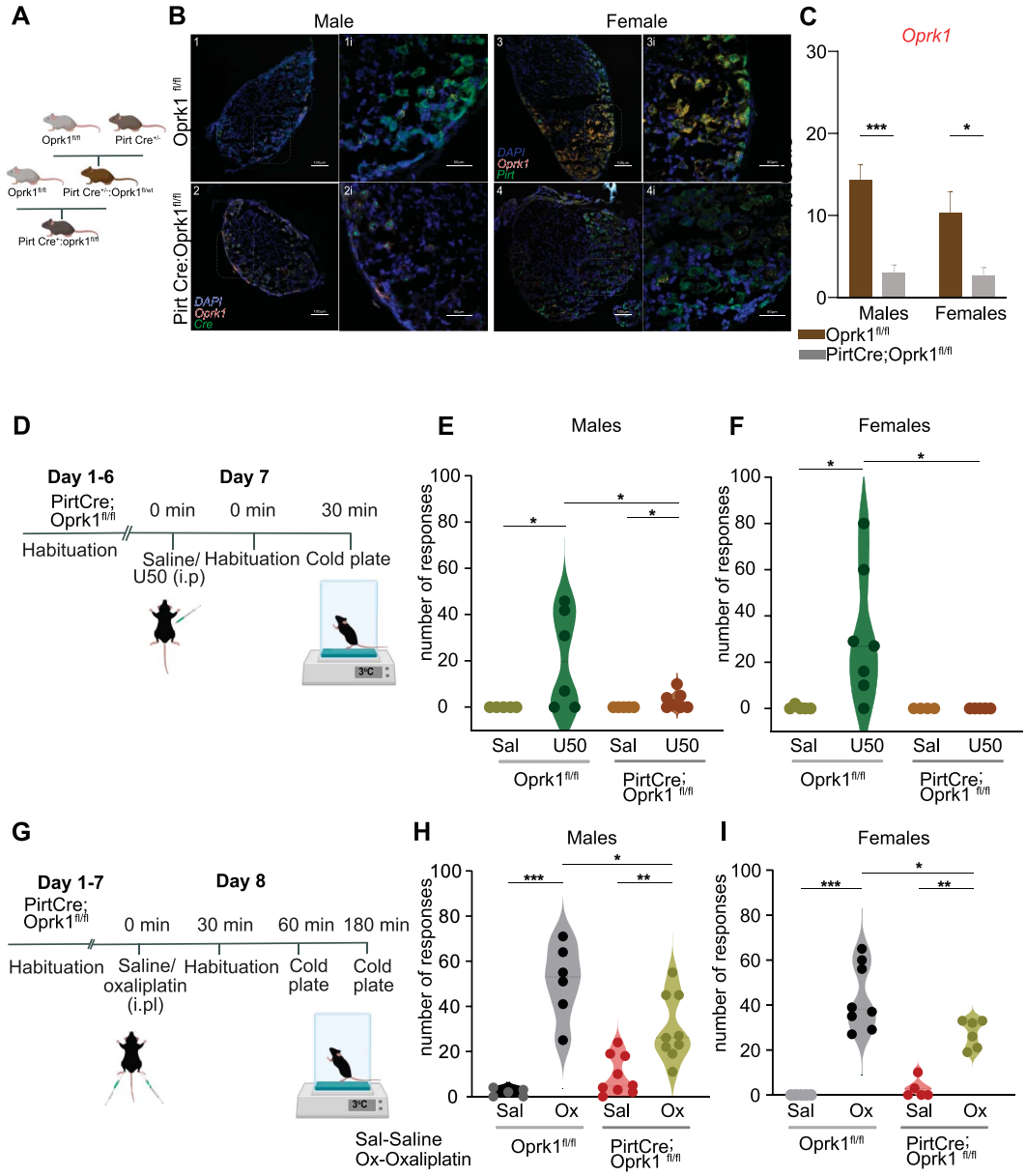

Figure 5. Peripheral kappa opioid receptors modulate cold hypersensitivity in the oxaliplatin-induced neuropathy. (A) Generation of the conditional knock-out mouse line of the PirtCre;Oprk1$^{fl/fl}$ using PirtCre$^{+/-}$ and the oprk1$^{fl/fl}$ mice. (B) Representative images of fluorescent in situ hybridization in DRG section of the transcripts *Oprk1*, *Cre*, and DAPI in (1) Oprk1$^{fl/fl}$ and (2) PirtCre;Oprk1$^{fl/fl}$ male mice. Representative image showing colocalization of *Oprk1* and *Cre* at 60x in (1i) Oprk1$^{fl/fl}$ and (2i) PirtCre;Oprk1$^{fl/fl}$ male mice. Representative image of fluorescent in situ hybridization in a DRG section of the transcripts *Oprk1*, *Cre* and DAPI in (3) Oprk1$^{fl/fl}$ and (4) PirtCre;Oprk1$^{fl/fl}$ female mice. Representative image showing colocalization of *oprk1* and *Cre* at 60x in (3i) Oprk1$^{fl/fl}$ and (4i) PirtCre^{+};Oprk1$^{fl/fl}$ female mice. (C) Expression of Oprk1 transcripts in the PirtCre^{+};Oprk1$^{fl/fl}$ was significantly lower compared to Oprk1$^{fl/fl}$ in the DRG of both male and female. Two-way ANOVA revealed genotype effect $F_{(1, 55)} = 29.29$ followed by *post-hoc* test (***$P < 0.001$ PirtCre;Oprk$^{fl/fl}$ vs Oprk$^{fl/fl}$ males, *$P < 0.05$ PirtCre;Oprk$^{fl/fl}$ vs Oprk$^{fl/fl}$ females). Data are presented as mean ± SEM; n = 3/group. (D) Outline of the experimental procedure. PirtCre;Oprk1$^{fl/fl}$ or Oprk1$^{fl/fl}$ mice were intraperitoneally (i.p.) injected with saline or U50 after 7 days of handling. Postinjection, mice were habituated for 30 minutes followed by cold plate test at 3°C, and the number of jumps was recorded. (E) In males, U50-potentiated cold nocifensive responses were attenuated in PirtCre;Oprk1$^{fl/fl}$ compared to U50-treated Oprk1$^{fl/fl}$ mice at 3°C. Mixed-effects analysis (2-way ANOVA) revealed treatment effect $F_{(1, 18)} = 5.792$ followed by *post-hoc* test (*$P < 0.05$ PirtCre;Oprk$^{fl/fl}$ U50 vs PirtCre;Oprk$^{fl/fl}$ saline treatment, *$P < 0.05$ Oprk$^{fl/fl}$ U50 vs Oprk$^{fl/fl}$ saline treatment, *$P < 0.05$ PirtCre;Oprk$^{fl/fl}$ U50 vs Oprk$^{fl/fl}$ U50 treatment). Data are presented as mean ± SEM; n = 4 to 7/group. (F) In females, U50-potentiated cold nocifensive responses were attenuated in PirtCre;Oprk1$^{fl/fl}$ compared to U50-treated Oprk1$^{fl/fl}$ mice at 3°C. Mixed-effects analysis (2-way ANOVA) revealed genotype effect $F_{(1, 17)} = 4.523$ followed by *post-hoc* test (*$P < 0.05$ Oprk$^{fl/fl}$ U50 vs Oprk$^{fl/fl}$ saline treatment, *$P < 0.05$ PirtCre;Oprk$^{fl/fl}$ U50 vs Oprk$^{fl/fl}$ U50 treatment, *$P < 0.05$ PirtCre;Oprk$^{fl/fl}$ U50 vs PirtCre;Oprk$^{fl/fl}$ saline treatment). Data are presented as mean ± SEM; n = 5 to 7/group. (G) Outline of the experimental procedure. PirtCre;Oprk1$^{fl/fl}$ mice were intraplantarly (i.pl.) injected with saline or oxaliplatin (50 μg each paw) after 7 days of handling. About 180 minutes postinjection, mice were subjected to cold plate temperatures of 3°C, and the number of responses was recorded. (H) In males, cKO of KOR (PirtCre;Oprk$^{fl/fl}$) significantly attenuated the number of responses to oxaliplatin injection compared to Oprk$^{fl/fl}$ at 3°C. Mixed-effects analysis (two-way ANOVA) revealed treatment effect $F_{(1, 25)} = 56.94$, treatment X genotype interaction effect $F_{(1, 25)} = 9.356$, followed by *post-hoc* test (***$P < 0.001$, Oprk$^{fl/fl}$ U50 vs Oprk$^{fl/fl}$ saline treatment, **$P < 0.01$, PirtCre;Oprk$^{fl/fl}$ U50 vs PirtCre;Oprk$^{fl/fl}$ saline treatment, *$P < 0.05$ vs PirtCre;Oprk$^{fl/fl}$ U50 vs Oprk$^{fl/fl}$ U50 treatment). Data are presented as mean ± SEM; n = 5 to 9/group. (I) In females, cKO of KOR (PirtCre;Oprk$^{fl/fl}$) significantly attenuated the number of responses to oxaliplatin injection compared to Oprk$^{fl/fl}$. Mixed-effects analysis (2-way ANOVA) revealed treatment effect $F_{(1, 20)} = 75.61$, treatment × genotype interaction effect $F_{(1, 20)} = 5.719$, followed by *post-hoc* test (***$P < 0.001$ Oprk$^{fl/fl}$ U50 vs Oprk$^{fl/fl}$ saline treatment, **$P < 0.01$ PirtCre;Oprk$^{fl/fl}$ U50 vs PirtCre;Oprk$^{fl/fl}$ saline treatment, *$P < 0.05$ PirtCre;Oprk$^{fl/fl}$ U50 vs Oprk$^{fl/fl}$ U50 treatment). Data are presented as mean ± SEM; n = 5 to 8/.group.

a Phosphoinositide-Binding Protein, is predominantly expressed in the primary sensory neurons.[22,35] To validate this genetic cross, we used *FISH* to characterise the expression of KOR in PirtCre cells (**Fig. 5B**). In PirtCre;Oprk1$^{fl/fl}$ mice, *oprk1* expression was 3% in males and 2.7% in females, compared to 14% in males and 10.37% in females of the Oprk1$^{fl/fl}$ mice (**Fig. 5C**). The *FISH* data indicate a reduction in KOR expression in PirtCre;Oprk1$^{fl/fl}$ mice compared to Oprk1$^{fl/fl}$ mice.

We first wanted to determine whether reduced KOR expression (Oprk$^{fl/fl}$ mice) attenuated cold hypersensitivity on the cold plate assay (**Fig. 5D**). We showed that U50 significantly increased the number of jumps as compared to controls on a cold plate at 3˚C in male and female Oprk$^{fl/fl}$ mice, as expected (**Figs. 5E, F**). In contrast, selective loss of KOR in primary afferents using PirtCre; Oprk1$^{fl/fl}$ mice reduced cold hypersensitivity after U50 in both male and female mice as compared to U50 treated Oprk1$^{fl/fl}$ mice, indicating that conditional knock out of KOR in the DRG attenuates noxious cold hypersensitivity (**Figs. 5E, F**). These findings suggest that (1) KOR in the DRG mediate cold hypersensitivity and (2) KOR deletion in Pirt cells is specific for cold hypersensitivity, as KOR-mediated mechanical (Fig. S4A-C, supplemental digital content, http://links.lww.com/PAIN/C533) and thermal antinociception remained unaffected (Fig. S4D-F, supplemental digital content, http://links.lww.com/PAIN/C533).

To further determine whether KOR in the DRG mediates cold hypersensitivity associated with acute oxaliplatin-induced peripheral neuropathy, we used the same conditional knockout mouse line of KOR in primary afferents using PirtCre;Oprk1$^{fl/fl}$ mice and injected oxaliplatin into the paw (**Fig. 5G**). We wanted to first validate that oxaliplatin induces nocifensive responses to noxious cold in our PirtCre;Oprk1$^{fl/fl}$ mouse, which we observed in male and female mice as compared to saline controls (**Figs. 5H, I**). As predicted, oxaliplatin significantly decreased the number of responses in PirtCre;Oprk1$^{fl/fl}$ mice compared to Oprk1$^{fl/fl}$ (control mice), suggesting that KOR in the DRG mediates the cold hypersensitivity associated with acute oxaliplatin injection (**Figs. 5H, I**). Overall, these data suggest a novel role for peripheral kappa opioid receptors in the modulation of cold hypersensitivity in the oxaliplatin-induced neuropathy.

4. Discussion

Here, we uncover and characterise a novel role for peripheral KORs in the modulation of cold hypersensitivity. We extend these findings in a model of oxaliplatin-induced neuropathy to show that blockade of KOR attenuates oxaliplatin-induced cold hypersensitivity in both male and female mice. In naïve mice, we show that activation of KOR increases jumping on the cold plate compared to controls at noxious temperatures (3˚C), suggesting that activation KOR drives cold hypersensitivity. Moreover, this cold hypersensitivity appears to be KOR selective as it is blocked by NorBNI (KOR antagonist). Interestingly, central NorBNI administration does not block U50-induced cold hypersensitivity, suggesting a peripheral mechanism of action, further corroborated by using the peripherally restricted agonist ff(nle)r-NH2, which increases cold sensitivity on the cold plate. Neither U50 nor ff(nle)r-NH2 affect central thermoregulation or paw surface temperature, as measured by rectal body temperature and thermal camera, respectively. In the conditional knockout of KOR in the DRG (PirtCre; Oprk1$^{fl/fl}$), we show that KOR in the DRG mediates cold hypersensitivity at 3˚C. Importantly, we validate these findings in a mouse model of oxaliplatin-induced neuropathy and associated cold hypersensitivity. PirtCre;Oprk$^{fl/fl}$ (KORs are knocked out in the DRG) show significant attenuation of oxaliplatin-induced cold nocifensive responses compared to Oprk$^{fl/fl}$ (KORs are intact) on a cold plate at 3˚C in male and female mice, suggesting that KORs in the DRG drive the cold hypersensitivity associated with oxaliplatin cold hypersensitivity. Overall, our data suggest that peripheral KORs modulate cold hypersensitivity, which in turn can be attenuated in the oxaliplatin-dependent model of cold hypersensitivity.

4.1. Peripheral kappa opioid receptor mediates cold hypersensitivity

We demonstrate that systemic KOR activation by U50 mediates temperature-dependent hypersensitivity, specifically at noxious cold temperatures. This response is KOR selective, stimulus-dependent, and peripherally mediated. Studies using peripheral injection of oxaliplatin have been shown to activate specific silent cold-sensing neurons, without increasing the proportion of heat- or mechanically sensitive cells in the DRG. This finding further emphasizes the distinction between cold responses and those evoked by mechanical or thermal stimuli.[42,43] In addition, studies in which U50 is used at doses eightfold higher than here suggest that activation of KOR causes hypothermia in mice,[51] which is markedly influenced by tolerance developed upon repeated administration of KOR agonists.[48,59,77] In contrast, another study revealed that a 30 mg/kg dose of U50 did not affect rectal body temperature in mice.[31] Together, these studies corroborate that KOR activation selectively mediates noxious cold hypersensitivity. Importantly, both pharmacological activation (U50, ff(nle)r-NH2) and genetic ablation of KOR in the DRG (PirtCre;Oprk1$^{fl/fl}$) converge to show that peripheral KOR is necessary and sufficient to modulate noxious cold hypersensitivity, further corroborated in our model of oxaliplatin-induced neuropathy. This supports a model in which peripheral, rather than central, KOR signaling drives cold hypersensitivity, highlighting a therapeutically relevant target that avoids centrally mediated aversive effects.

4.2. Sex differences in kappa opioid receptor–mediated cold hypersensitivity

To fully evaluate sex as a biological variable, we investigated KOR modulation of noxious cold hypersensitivity in both males and females using behavioral assays. We show that activation of KOR potentiates both noxious and non-noxious cold hypersensitivity in WT female mice but only noxious cold hypersensitivity in WT male mice. In addition, we show that sex differences in KOR-mediated cold hypersensitivity have been observed in oxaliplatin-induced peripheral neuropathy. After oxaliplatin injection, female mice exhibited significantly increased paw-directed responses compared to their male counterparts, both at 60 minutes and 180 minutes postinjection. This highlights the sexually dimorphic response to oxaliplatin-induced cold hypersensitivity between male and female mice. Similarly in rats, females develop oxaliplatin-induced hyperalgesia more quickly than male rats, suggesting that the effects of oxaliplatin treatment differ between the sexes.[70] Furthermore, systemic oxaliplatin also induces the development of cold hypersensitivity in both male and female rats, with female subjects showing more severe responses.[47] Studies investigating the mechanisms of paclitaxel-induced hyperalgesia, show that paclitaxel-induced neuropathy is exclusively mediated through nociceptor glucocorticoid receptors, in males but not females.[26] Furthermore, studies exploring the role of female sex hormones in paclitaxel-induced neuropathy have observed that increased levels of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, along with their receptors in the

DRG of female rats, are influenced by 17β-estradiol and progesterone. This further highlights sexual dimorphism associated with chemotherapy-induced neuropathy.[79] In addition to neuropathy-driven sex differences, KOR-mediated pain processing in animal models and clinical studies has been shown to be differentially modulated in males and females.[41,72,76] In the lateral sensitization rat model, for example, intraplantar administration of U50 potentiated antihyperalgesic activityin males only, suggesting a sex-dependent effect.[72] Similarly, administration of U50 increased tail withdrawal latency at 49°C in male but not female mice.[72] In peripheral nerve injury, negative affect is potentiated after KOR activation and subsequently blocked with KOR antagonist JDTic in male but not female mice.[41] Similarly, positron emission tomography clinical studies suggest that KOR receptor binding is higher in men than in women, specifically in the anterior cingulate cortex, a region associated with pain affect.[76] There is mounting evidence that KOR modulation of pain is sex-dependent emphasizing the need to consider sex as a potential variable in the study of pain.

4.3. Potential mechanisms mediating peripheral kappa opioid receptor–induced cold hypersensitivity

The complex interplay between central and peripheral mechanisms mediating cold hypersensitivity associated with neuropathic pain remains largely unaddressed. One plausible mechanism by which KOR regulates cold hypersensitivity involves modulation of primary sensory neurons in the DRG. KORs are known to be expressed in low-threshold mechanoreceptors and primary afferent neurons in the DRG,[49,69] which is consistent with our findings showing KOR expression in primary sensory neurons. This is also consistent with DRG single-cell analyses, which localize KOR expression predominantly to nociceptor and nonpeptidergic (NP) neuron populations rather than to canonical TRPM8-expressing cold-sensing neurons.[52,60] Research shows that KOR activation by compounds like U50 triggers the activation of heterotrimeric G proteins, leading to the release of Gβ/γ dimers that bind to VGCCs.[62,63] Snyder et al. have shown that activation of KOR by dynorphin mediates the inhibition of VGCC,[69] in the primary afferent neurons, suggesting a direct relationship between KOR activation and VGCC modulation in the primary afferent neurons of naïve mice. In neuropathic pain models, the role of peripheral nociceptors in the DRG is unmasked and contributes to cold hypersensitivity in chemotherapy-induced peripheral neuropathy (oxaliplatin and ciguatoxin-2) and partial sciatic nerve injury.[42,43] Consistent with the model, research by Issepon et al. has shown that pregabalin alleviates cold hypersensitivity in chemotherapy-induced peripheral neuropathy by silencing large-diameter, silent cold-sensing neurons.[30] This occurs through its action on the α2δ1 subunit, which blocks voltage-gated calcium channels (VGCCs) and modulates calcium responses associated with cold hypersensitivity.[30] In addition, in our study, we highlight KOR expression in the DRG and nociceptors, as evidenced by the absence of KOR in PirtCre;Oprk1$^{fl/fl}$ mice. In addition, research shows that KOR activation reduces cAMP and PKA signaling[82,83]; however, KOR-mediated release of Gβγ subunits, modulation of VGCCs might contribute to the sensitization of TRP channels, further elucidating the link between the KOR and TRP channels. In addition, Pirt modulates TRPV1 channel function[35] in the DRG, and we show that there is a decrease in KOR expression in the DRG of PirtCre;Oprk1$^{fl/fl}$ mice, suggesting potential functional interaction between KOR signaling and TRP channel-mediated pathway. We hypothesize that KOR may mediate cold hypersensitivity through the TRP channels via PKA/cAMP/Pirt activation in the primary sensory neurons. Together, these findings support a model in which activating peripheral KOR signaling may modulate TRP channel activity through VGCCs, leading to unmasking cold nociceptors and thereby driving cold hypersensitivity in chemotherapy-induced neuropathy.

4.4. Limitations

Further investigation into polymorphisms in the prodynorphin gene[18,20] and the heterodimerization of MOR and KOR,[17] which might play a role in the differential KOR-mediated cold hypersensitivity associated with the oxaliplatin model, is warranted. Research has shown that KOR polymorphism has been associated with predisposition to voluntary alcohol-drinking behavior in experimental animals and have also been clinically associated with addiction.[64,75,84] In addition, KOR polymorphism has been linked to the opioid abstinence phenotype because of its antireward mechanism, which contributes to mood regulation both preclinically[13,73,85] and clinically.[27] Most polymorphism studies link variants to behavioral phenotypes rather than direct receptor activation states. Although constitutive KOR activity has been shown in heterologous expression systems,[11,38] there is currently no in vivo evidence that naturally occurring Oprk1 polymorphisms produce clearly demonstrated constitutively active receptor activity. Importantly, the role of endogenous dynorphin in mediating cold hypersensitivity has not been explored. The observation that KOR inhibition attenuates oxaliplatin-induced cold hypersensitivity does not, by itself, distinguish between endogenous dynorphin-driven KOR signaling and constitutive KOR activity. Therefore, this could be resolved by testing the phenotype and effect of antagonists in prodyn-KO mice.

4.5. Peripheral kappa opioid receptor activation in current treatment and future therapeutic potential

Kappa opioid receptors have long been considered promising pain and itch-relieving targets because of their nonaddictive profile.[56,57,66] However, the most significant limitation to targeting the KOR system has been the negative affect, primarily mediated by the central activation of KOR,[29,58] which has prompted investigation into the therapeutic potential of peripherally expressed KOR.[9,69,74] For example, peripherally restricted KOR agonists asimadoline, CR845, and TRK-820 (nalfurafine) have demonstrated efficacy in clinical trials for pruritus.[8,34,37] Similarly, we used ff(nle)r-NH2,[3–5,53] to show that activation of peripheral KOR increases cold sensitivity in male and female mice, identifying a novel role for the peripheral KOR system in noxious cold hypersensitivity.

In conclusion, we have identified a novel role for the KOR system in mediating noxious cold hypersensitivity. The findings presented suggest that this is restricted to peripheral KOR, which is encouraging for the development of potential therapies as the aversive centrally mediated side effects (that have limited translational traction) can be avoided.

Conflict of interest statement

The authors have no conflict of interest to declare.

Acknowledgements

The authors thank all members of the Al-Hasani and McCall laboratories for helpful insight and discussion, and in particular the technical staff for managing their mouse colony. The authors are

thankful for technical assistance from the Pradhan's Laboratory (WashU) Gereau laboratory (WashU), Hongzhen Hu (Mount Sinai), Steve Davidson (University of Cincinnati), and D.P. Mohapatra (WashU). The authors especially thank Michael R. Bruchas and Gina M. Story for early support in pursuit of this line of work. Editing assistance was provided by InPrint: A Scientific Communication Network at Washington University in St. Louis.

Funding: National Institute on Drug Abuse R00DA038725 (RA); National Institute on Neurological Disorders and Stroke R01NS123070 (RA); National Institute on Neurological Disorders and Stroke R01NS117899 (JGM); National Institute on Neurological Diorders and Stroke R01NS135401 (JGM and RA); The Rita Allen Foundation with help from the Open Philanthropy Projection (JGM).

Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request. All data in this manuscript have been made available at Dryad https://datadryad.org/submission/364837.

Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/C533.

Article history:
Received 4 September 2025
Received in revised form 22 April 2026
Accepted 7 May 2026
Available online 7 July 2026

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