Peripheral kappa opioid receptor activation drives cold hypersensitivity in mice

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Abstract:

Noxious cold sensation is commonly associated with peripheral neuropathies, however, there has been limited progress in understanding the mechanism of cold pain. Transient receptor potential (TRP) A1 channels facilitate the perception of noxious cold at the level of dorsal root ganglia (DRG), where kappa opioid receptors (KOR) are also expressed but have not previously been implicated in cold sensation. Here we identify a new role for KOR in enhancing cold hypersensitivity. First, we show that systemic KOR agonism (U50,488, KOR agonist), significantly potentiates the latency to jump and the number of jumps on the cold plate compared controls at 3°C. Importantly, NorBNI (KOR antagonist) attenuates U50,488-induced cold hypersensitivity. However, the central administration of NorBNI does not block U50,488-induced cold hypersensitivity suggesting that peripheral KOR likely modulate this effect. Furthermore, the peripherally-restricted KOR agonist, ff(nle)r-NH2 also induces cold hypersensitivity. Using fluorescent *in situ* hybridization, we show that KOR mRNA colocalizes with the transcripts for the cold-activated TRPA1 and TRPM8 channels in DRG. Finally, using calcium imaging in DRG, we show that intracellular calcium release is potentiated during the simultaneous application of a TRPA1 agonist, mustard oil (MO), and a KOR agonist (U50,488), when compared to MO alone. This potentiated calcium response is absent in TRPA1 KO mice. Together our data suggest that KOR-induces cold hypersensitivity through modulation of peripheral TRPA1 channels. These findings indicate that whether activation of peripheral KOR is protective or not may be dependent on the pain modality.

Keywords: kappa opioid receptor (KOR), Transient receptor potential ankyrin 1 (TRPA1), cold sensitivity, Transient receptor potential melastatin 8 (TRPM8)

Introduction

Cold sensitivity is an elusive condition that has previously been defined as an exaggerated or abnormal reaction to cold exposure, causing discomfort or the avoidance of cold (Kay, 1985). Kappa opioid receptors (KORs) are inhibitory G protein-coupled receptors that are activated by the endogenous ligand, dynorphin, as well as exogenous KOR agonists such as U50488 (U50), U69,593 and spiradoline (Al-Hasani et al., 2017; Chavkin, James, & Goldstein, 1982; Lahti, Mickelson, McCall, & Von Voigtlander, 1985; Von Voigtlander & Lewis, 1982; Vonvoigtlander, Lahti, & Ludens, 1983). KOR activation is antinociceptive for noxious heat stimuli, analgesic in chronic pain models, and drives pain-induced negative affect (Horan & Porreca, 1993; S. S. Liu et al., 2019; Massaly et al., 2019). However, despite these important findings, little is known as to whether or how KOR activation modulates cold sensation.

KOR are expressed throughout the central nervous system and peripherally in dorsal root ganglia (DRG) sensory neurons (Peckys & Landwehrmeyer, 1999; Snyder et al., 2018). Recent evidence suggests that centrally- and peripherally-expressed KOR modulate different behaviors. For example, our group and others have shown that central KOR activation and upregulation modulates negative affect associated with peripheral nerve injury and inflammatory pain models (S. S. Liu et al., 2019; Massaly et al., 2019). Conversly, recent studies suggest peripherally-restricted KOR agonists selectively inhibit chemical pain and mechanical hypersensitivity associated with capsaicin-induced neurogenic inflammatory pain model and a surgical incision model, respectively (Snyder et al., 2018). Together these findings highlight the complex role of the KOR system in different pain and sensation modalities. Nevertheless, no studies have examined the whether KOR can alter cold hypersensitivity associated with neuropathic pain.

Cold hypersensitivity is often associated with neuropathic pain from disorders such as multiple sclerosis, fibromyalgia, complex regional pain syndrome, and chemotherapy-induced peripheral neuropathy (Attal et al., 2009; Christogianni et al., 2018; Jensen & Finnerup, 2014; Tajerian & Clark, 2016; Wilbarger & Cook, 2011). 15% to 50% of neuropathic pain patients often experience heightened sensory abnormalities (Jensen & Finnerup, 2014). Medications used to treat neuropathic pain are predominantly non-steroidal anti-inflammatory drugs (NSAIDs), mu opioid agonists, and anti-epileptics (Kudel et al., 2019), all which have very limited success in relieving cold hypersensitivity. While little mechanistic insight is available for cold pain, significantly more is known about cold sensation – particularly how it is regulated by transient receptor potential (TRP) channels in DRG (Patapoutian, Tate, & Woolf, 2009).

TRP channels are a group of ion channels in the voltage-gated superfamily which are activated by distinct temperature shifts as well as chemical ligands. TRPA1 (TRP Ankyrin 1) channels are located on small-diameter sensory neurons and are known to be activated by noxious temperatures below 10°C, menthol, mustard oil, and icillin (Story et al., 2003). Another cold transducer, TRPM8 (TRP Melastatin 8) is located alongside TRPA1 in the periphery (Dhaka, Earley, Watson, & Patapoutian, 2008) with menthol and temperatures below 20°C known to activate these channels (McKemy, Neuhausser, & Julius, 2002). TRPA1 knockout mice exhibited reduced cold sensitivity on a cold plate at 0°C (Bautista et al., 2006), inferring the receptor's role at noxious temperatures.

Furthermore, cold sensitivity at noxious cold temperatures (<15 ^{o}C) is preserved in TRPM8 KO mice by TRPA1 in a two-plate preference test (Pogorzala, Mishra, & Hoon, 2013), suggesting the vital role of TRPA1 channels detecting noxious temperatures in the periphery. Both TRPA1 and TRPM8 clearly drive the mechanisms underlying much of cold hypersensitivity.

Alongside TRP channels, G protein-coupled receptors (GPCRs) are known for sensing noxious, irritating, and inflammatory stimulants (Veldhuis, Poole, Grace, McIntyre, & Bunnett, 2015). Studies have shown that downstream intracellular GPCR signaling can regulate calcium homeostasis via the TRP channels (Clapham, 2003). To date, there has been limited traction in the exploration of opioids and TRP channel interaction (Shapovalov et al., 2013; Williams et al., 2013). While there is a clear role for interactions between mu-opioid receptors and TRP channels, but not KOR and TRP channels, even though KOR is known to be involved in neuropathic pain (S. Liu et al., 2016). We have very little understanding of how the KOR system is engaged, maintained, and how it contributes to heightened cold sensation and pain, which is the primary focus of this research.

Here we pharmacologically identify a role for KORs in increasing cold sensitivity in mice. In particular, we use a peripherally-restricted KOR agonist and central KOR antagonism to determine that peripheral KORs regulate this induced cold hypersensitivity. Furthermore, we use *in situ* hybridization to show that KORs colocalize with TRPA1 and TRPM8 in the DRG. Finally, we use calcium imaging to demonstrate that KOR activation potentiates TRPA1-dependent calcium signaling in DRG neurons. In sum, we show that peripheral KOR activation enhances cold sensitivity and this behavior is likely mediated through potentiated TRPA1 activity.

Methods

Animals

Adult C57BL/6J male and female mice (25-30 g) were used for all the behavioral and *in situ* hybridization experiments. For calcium imaging experiments, we used TRPA1$^{-/-}$ male and C57BL/6J male mice. All animals were 9 to 12 weeks at the beginning of the experiments. Mice were group-housed together with a 12/12 h dark/light cycle, given access to food pellets and water ad libitum (lights were turned on at 6:00 AM). Following weaning, all animals were transferred to a holding facility adjacent to the lab and acclimated to this animal facility for at least seven days before the experiments to minimize stress. Furthermore, all animals were acclimated to the behaviour test room for at least two hours prior to each experiment. All procedures were approved by the Washington University in St. Louis Institutional Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Drugs

U50,488 (U50, KOR agonist, 5 mg/kg i.p.) (CAS# 67197-96-0) and nor-Binaltorphimine dihydrochloride (norBNI, KOR antagonist, 10 mg/kg, i.p.) (CAS# 113158-34-2) were obtained from Tocris and dissolved in 0.9% sterile saline. Morphine sulfate (10 mg/kg s.c.) procured from (Mckesson #996806) was dissolved in 0.9% sterile saline. CR845 analogues (ff(nle)rNH2) (peripherally-restricted KOR agonist, 10 mg/kg, i.p.) were generated in the Mclaughlin lab (Alleyne et al, in revision) and dissolved 0.9% sterile saline.

Hot/Cold Plate Assay

The hot/cold plate apparatus was adapted from the operant thermal plantar assay (Reker et al., 2020). The floor of the apparatus is made of one 12" × 12" × ¼" aluminum plate (3003, MetalsDepot), fixed to a cold plate Peltier (Cold plate cooler, CP-061, TE Technology). The Peltier device is independently controlled by a 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 wildtype (WT) mice were habituated in plexiglass boxes for 30 mins prior to treatment with: 1) U50 ,KOR agonist, 5 mg/kg, i.p.) 2) norBNI, KOR antagonist (10 mg/kg, i.p.) 3) Saline (0.25ml) 4) CR845 peripherally-restricted KOR agonist (10 mg/kg i.p.). Following treatment, the 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 temperature of the plate was varied from 3ºC-42ºC depending on the experiment. The plate's temperature was continuously monitored with a visual temperature strip (Liquid crystal temperature indicating sheets-Telatemp) and a surface probe thermometer (Pro-surface thermapen-Thermoworks).

Acetone Evaporation Test

The acetone evaporation test is used to measure aversive behaviors triggered by evaporative cooling (Deuis, Dvorakova, & Vetter, 2017). On the test day, mice are left on the mesh floor for 90 minutes for acclimatization. Post-habituation, mice were injected with U50 (5 mg/kg i.p.) or saline (0.25ml) treatments. Post-injections, 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 ((i.e., elevation, shaking, or licking). The acetone application is repeated for both the paws (three readings from each paw) and their mean calculated. The response was recorded for one minute following each acetone application. A 10-minute break is included between each reading to prevent hypersensitivity (Colburn et al., 2007; Slivicki et al., 2018). Sensitivity to cold is recorded either by quantifying the number paw directed behavior, or scoring the severity of the response (for example: 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 latency to lick the paw.

Tail withdrawal assay

Mice were habituated to the experimenter for a week prior to the assay and scruffed daily to minimize the stress that might impact the assay (Deuis et al., 2017). On experiment day, following U50 (5 mg/kg i.p.) or saline (0.25ml) injection, the mice were scruffed, and one-third of the distal end of the tail was immersed in the hot water bath set at 54.5ºC.

The time taken for the tail to twitch or flick was recorded. A single reading was recorded at 15, 30, 45, 60, 90, and 120 minutes following treatment.

Von Frey test

Mechanical sensitivity was determined using manual von Frey filaments following U50 (5 mg/kg i.p.) or Saline (0.25ml) treatments. On the test day, mice were individually placed in an acrylic cylinder on a mesh floor and covered with a rectangular acrylic lid to prevent them from escaping. The mice were habituated to apparatus for 2 hours prior to testing. During the assay, a monofilament was applied perpendicularly to the plantar surface of the hind paw for 2–5 seconds. If the animal exhibited any nocifensive behaviors, including brisk paw withdrawal, licking, or shaking of the paw, either during or immediately following the stimulus, a response is recorded. In that case, such a response is considered positive (Deuis et al., 2017). We used the "ascending stimulus" method, applying monofilaments with increasing force until it elicits withdrawal response. The von Frey filament force that elicits this positive response represents the mechanical withdrawal threshold. The stimulus is repeated for both the paws (three readings from each paw) and their mean calculated. A 10-minute break is included between each reading to prevent hypersensitivity.

Open field test (OFT)

The OFT apparatus is a 2,500 cm^2 arena, in which the mice are free to explore for 20 minutes. 50% of the total OFT area was defined as the center (Al-Hasani et al., 2015; McCall et al., 2015). Lighting was stabilized at ~25 lux for anxiety-like behaviors. To determine sedative and anxiety-like behavior, distance moved in the apparatus and the time spent exploring the center were quantified, respectively. On the test day, mice are left in the OFT room for 90 mins for acclimatization to the environment. Post-habituation mice are injected with saline or U50, placed in the OFT arena, and behavior was recorded for 20 minutes. Movements were video recorded and analyzed using Ethovision 13 (Noldus Information Technologies).

Rectal temperature measurements

Core body temperature readings were obtained using a homeothermic monitoring system attached to a flexible rectal probe (Physitemp instrument Inc. TCAT2LV controller). On the test day, mice are left to acclimate in the test room for 90 minutes. Post-habituation mice were injected with saline (0.25ml) or U50 (5 mg/kg i.p.) or ff(nle)r-NH2 (10 mg/kg i.p.). Immediately post-injection, the mouse was hand-restrained and the tail lifted, and a lubricated probe gently inserted into the rectum to a fixed depth (typically, up to 1 cm) to obtain a rectal temperature. 30 minutes post-injection, the rectal temperatures are recorded. We made sure the depth of probe insertion is constant for each measurement.

Intracerebroventricular injections

Mice received intracerebroventricular (ICV) injections as previously described (Bruchas, Land, Lemos, & Chavkin, 2009). Briefly, mice were anesthetized in an induction chamber (3% Isoflurane) and placed into a stereotaxic frame (Kopf Instruments, Model 942), where

they were maintained at 2–2.5% isoflurane. A craniotomy was performed unilaterally into the lateral ventricle with either NorBNI (30μmol, 2μl) or artificial cerebrospinal fluid (aCSF) at 200 nl/min for 10 mins using a beveled Hamilton syringe (10 μL-701 N with beveled tip) (stereotaxic coordinates from Bregma: A/P: +0.40mm, M/L: +1.5mm, D/V: –3mm), to block central KORs (Bruchas et al., 2009; Shirayama et al., 2004). The skin was sutured after the injection using sterile nylon sutures (6.0 mm), and mice were allowed to recover for a week before any behavioral experiments. On the experimental day, post-surgery mice were systemically injected with either U50 (5 mg/kg i.p.) or saline and were exposed to the cold plate. The ICV injection placements were confirmed using immunohistochemistry.

Immunohistochemistry

Histological verification of the injection site was performed as described (Al-Hasani, McCall, Foshage, & Bruchas, 2013). Mice were briefly anesthetized with 0.2ml cocktail (ketamine (100 mg/ml), xylazine (20 mg/ml) and acepromazine (10 mg/ml)) and intracardially perfused with ice-cold 4% paraformaldehyde in phosphate buffer (PB). Brains were dissected, post-fixed 24 h at 4 °C and cryoprotected with 30% sucrose solution in 0.1 M PB at 4 °C for at least 24 h, cut into 30-μm sections, and processed for immunostaining. The ICV injection placements were confirmed using the Paxinos-Watson atlas (Paxinos, G. and Watson, 1998) as a reference under a Leica fluorescent microscope (DM6 series scope). In total, n=76 out of 85 WT mice (male and female) had the injections landing in the lateral ventricle and the rest were excluded from the analysis.

In situ Hybridization

Following rapid decapitation of C57BL6/J mice, DRG were rapidly frozen on dry ice in the mounting media, and then the tissue harvested was stored at –80ºC. DRG sections were cut at 5-7 μM at –20ºC and thaw-mounted onto Super Frost Plus slides (Fisher, Waltham, MA). Slides were stored at –80ºC 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, Inc.), as described. Briefly, sections were fixed in 4% PFA, dehydrated with alcohol (50%, 75%, 100%) concentrations in the respective order in accordance with the protocol. Sections were pretreated with hydrogen peroxide for 15 mins at room temperature. Following this, the sections were washed in the 1X PBS solution twice for 2 min each. Post-wash, the sections were pretreated with protease IV solution. Sections were then incubated for target probes for mouse *trpa1*(400211) (C1), *trpm8 (*420451) (C2), *oprk1* (316111) (C3). Probes were obtained from Advanced Cell Diagnostics. Following probe hybridization, sections underwent a series of probe signal amplification steps followed by incubation with fluorescently labeled probes designed to target the specified channel associated with *trpa1* (fluorescein), *trpm8* (cyanin3), *oprk1* (cyanin 5). Slides were counterstained with DAPI, and coverslips were mounted with Vectashield Hard Set mounting medium (Vector Laboratories). Images were obtained on a Leica fluorescent microscope, and the expression was quantified manually using the Leica DM6 series scope by a blinded experimenter. DRGs were imaged on a Leica fluorescent microscope (DM6 series scope) at 5X, 20X, and 40X magnification. 2-3 images were acquired of each mouse DRG section, and 4-5 DRGs were imaged per mouse (n=4 for male, n=2 for females). Total

cell counts for the section were assessed by counting all of the DAPI in the DRG section. In the ISH assay, each punctate dot represents a single target mRNA molecule. To avoid false positives, we set a threshold of a minimum of two puncta expressing cells only. To The target genes expression was quantified manually by counting the DAPI cells expressing the puncta. The quantified expression is averaged within a sample and across the mice and expressed as a pie chart. Each *oprk1* positive cell was assessed for colocalization with *trpm8* and *trpa1* using 40X magnification, represented in the pie chart (**Fig 3P, 3Q**).

Mouse DRG cultures

10-week-old male and female mice were euthanized under isoflurane by decapitation and lumbar DRG were removed (Sheahan et al., 2018; Sleigh, Weir, & Schiavo, 2016). DRG culture media was prepared fresh using Neurobasal A medium (Invitrogen) with 100 U/mL penicillin/streptomycin (Corning), 2 mm GlutaMAX (Life Technologies), 2% B27 (Gibco), and 5% fetal bovine serum (Gibco). DRG were incubated in papain (40 U, Worthington) for 20 min at 37°C, supplemented with 5% CO_2. DRG were then rinsed and incubated in collagenase (Sigma-Aldrich) for 20 min, following which they were manually triturated with Pasteur pipettes to dissociate neurons, passed through a sieve of 40-µm filter, and plated onto collagen (Sigma-Aldrich)-coated 12-mm glass coverslips (Thermo Fisher Scientific). Neurons were maintained in culture media for two days prior to calcium imaging experiments.

Calcium Imaging

To determine calcium dynamics, cultured DRG neurons from C57BL/6J male and female mice were loaded with Fura-2 (3 µg/mL, Life Technologies) and pluronic acid (1:1) for 45-60 min to enable visualization of changes in calcium concentrations (Munanairi et al., 2018; Snyder et al., 2018). Neurons were then incubated in Tyrode's solution for 15-20 min to allow for de-esterification of Fura-2 AM. Tyrode's solution consisted of (in mm): 130 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 30 glucose, and 10 Hepes and was made fresh on the experimental day. On the day of recording, coverslips were placed into a temperature-controlled chamber (37°C) with Tyrode's solution. The cultures were treated with either KOR agonist (U50488, 10 µM) alone, TRPA1 agonist (Mustard oil (MO) 100µM), or a combination of both. The change in the intensity of the Ca^{2+}indicator is quantified to estimate the change in the concentration of free Ca^{2+} using calcium imaging software suite (Leica Systems) to record fluorescence emission at alternating excitation wavelengths of 357 and 380 nm. For detailed timelines, refer to calcium imaging figure sets.

Statistical Analysis

All data samples were tested for homogeneity of variance and normality before being assigned to any parametric analysis. All experiments were performed in multiple cohorts, including all treatment groups in each round, to avoid any unspecific day/condition effect. Treatments were randomly assigned to animals before testing. G*Power was used to estimate effect sizes and to compute power analyses. Statistical significance was considered $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$, as determined by parametric or nonparametric analysis. Parametric data was analyzed using two-way

ANOVA repeated measures for tail withdrawal assay analysis, two-way ANOVA for mechanical sensitivity analysis. For ordinal ranking such as scoring the number of jumps (nonparametric data), Kruskal-Wallis followed by the Dunn's multiple comparison test for analyzing the cold plate assay data. For the temperature curve data, nonparametric (two-way ANOVA) mixed-effects analysis was followed by Sidak's *post-hoc* test for the cold plate assay analysis. Mann Whitney U test for cold plate data using peripheral novel agonist. Statistical analyses were performed in GraphPad Prism 8.0. All data are expressed as mean ± SEM.

Results

Activation of KOR induces cold hypersensitivity

To determine whether activation of KOR mediates temperature-dependent hypersensitivity, we recorded jumping behavior across a range of temperatures (3ºC, 10ºC, 15ºC, 20ºC, 30ºC, 42ºC) (**Fig 1A**). Jumping action in this assay has been well-established as a measure of nocifensive behavior (Allchorne, Broom, & Woolf, 2005; Castellanos et al., 2020; Deuis et al., 2017). We show that the KOR agonist U50 (5 mg/kg i.p.) significantly increases the number of jumps when compared to controls at 3ºC in females (**Fig 1B**). This jumping behavior is supported by a significant decrease in latency to jump (**Fig 1C**). Together, these results suggests that KOR activation selectively induces hypersensitivity to noxious cold. An increase in the number of jumps is also observed at 42ºC, but this effect also occurs in the saline treated groups, and the U50 male mice - suggesting that this is not due to activation of KOR, but rather the noxious hot temperature itself (Yalcin, Charlet, Freund-Mercier, Barrot, & Poisbeau, 2009) **(Fig 1C**).

To further substantiate that KOR-induced noxious cold hypersensitivity is temperature-dependent, we used the acetone evaporation test. Acetone is known to mimic cool, but not noxious cold temperatures in the range of 15–21°C (Colburn et al., 2007; Leith, Koutsikou, Lumb, & Apps, 2010). In this test, we show that U50-mediated KOR activation does not alter paw withdrawal behavior nor number, severity, or duration of nocifensive responses (**Fig S1**). As a positive control to be certain that KOR activation is occurring as expected, we demonstrate the known effect that U50 (5 mg/kg i.p.) is antinociceptive in both a warm water tail-withdrawal procedure (**Fig S2**) and the von Frey test of mechanical sensitivity (**Fig S3A,B**). Importantly, we show that this dose of U50 (5 mg/kg i.p) does not have sedative effects (common at higher doses), as demonstrated by no differences in locomotor activity between the groups (**Fig S4**). Furthermore, we also demonstrate that U50 does not alter core body temperature, as compared to saline controls (**Fig S5**), suggesting these effects are not due to detection relative temperature differences. Finally, to determine the kappa opioid selectivity of KOR-mediated cold hypersensitivity, we show that a prototypical mu opioid agonist, morphine (10 mg/kg s.c.), did not drive cold hypersensitivity in male and female mice (**Fig S6**).

To determine the necessity of KORs role in cold hypersensitivity at 3ºC, we pharmacologically blocked KORs using norBNI (KOR antagonist, 10 mg/kg, i.p.), which blocked U50-induced cold hypersensitivity on the cold plate (**Fig 1F-H**). This effect was

consistent in both male and female mice. Systemic norBNI (i.p.) administration alone did not alter any nocifensive response irrespective of the sex (**Fig 1G&H**). These data suggest that KOR exclusively potentiates cold hypersensitivity at noxious cold temperatures (3ºC).

Peripheral KORs mediate cold hypersensitivity

To determine whether KORs expressed centrally and/or peripherally mediate the observed increase in cold hypersensitivity, we used two distinct approaches. Firstly, we blocked central KOR by injecting norBNI intracerebroventricularly (ICV) and administering U50 systemically (i.p) before placing them on the cold plate (**Fig 2A**). Central administration of norBNI (norBNI (ICV)+U50(i.p.)) did not attenuate or block the previously observed U50-induced cold hypersensitivity, as compared to the aCSF (ICV) and U50 (i.p.) injected mice (**Fig 2B&C**). Central norBNI administration alone did not alter cold sensitivity, as compared to the controls (aCSF (ICV)+saline(i.p.)). Mice infused with aCSF(ICV) and U50 (i.p.) showed increased cold sensitivity, as compared to the control group (aCSF (ICV)+saline(i.p.)) (**Fig 2B&C**).The effects were similar in male and female mice (**Fig 2B&C**).

Secondly, we show that the peripherally restricted KOR agonist ff(nle)r-NH2 (10 mg/kg i.p.) significantly increased jumping on the cold plate at 3ºC, compared to controls, in males and females (**Fig 2E&F**). Together this data KOR increases cold hypersensitivity through peripherally expressed KOR.

KOR transcripts colocalize with TRPA1 and TRPM8 transcripts in DRG

TRP channels are necessary for temperature sensation. Specifically, the TRPA1 and TRPM8 channels have been most widely associated with cold sensitivity (MacDonald, Wood, & Emery, 2020). To determine whether KORs are expressed on the same cells as TRPA1- and/or TRPM8-expressing cells in DRG, we performed *in situ* hybridizations against the mRNA for KOR (*oprk1*), TRPA1 (*trpa1*), and TRPM8 (*trpm8*) in male (**Fig 3A-F**) and female (**Fig 3G-L**). We found that in male DRGs, 4.2% of all DRG cells *oprk1* (**Fig 3M**), 17% of the cells expressed *trpa1* (**Fig 3N**), and 5.2% of the cells expressed *trpm8*. 2% of the cells expressing *oprk1* colocalized with *trpa1* positive cells (**Fig 3O**). 0.6 % of *oprk1* expressed alongside with *trpm8* and 0. % with both the *trpa1* and *trpm8* channels (**Fig 3P**). In female DRGs, 17% of the cells expressed *oprk1* (**Fig 3M**), 42% of the cells expressed *Trpa1*(**Fig 3N**), and 15.7% of the cells expressed *trpm8*. 7.4% of the cells expressing *oprk1* colocalized with *trpa1* positive cells (**Fig 3O**). 5.75% of *oprk1* expressed alongside *trpm8* and 1% with both the *trpa1* and *trpm8* channels (**Fig 3Q**). The co-expression of the KORs and TRPA1 receptors is higher in females than the males in the DRG (**Fig 3M&N**), suggesting possible crosstalk between the receptors.

KOR potentiates Ca^{2+} mobilization via TRPA1

To understand how peripheral KOR might induce cold hypersensitivity, we measured calcium responses in cultured DRG following simultaneous application of MO (TRPA1 agonist) and U50 (**Fig 4A-C**) in male WT mice. A KCl response at the end of an experiment served as a positive control for cell health and activity. U50, together with MO, significantly increased Ca^{+2} mobilization when compared to MO alone in DRG neurons in WT male mice (**Fig 4C**). In TRPA1 knockout mice ($TRPA1^{-/-}$), MO application alone and together with U50 caused no response in Ca^{+2} signaling (**Fig 4D&E**). Likewise, the potentiated calcium response was absent in the $TRPA1^{-/-}$ mice (**Fig 4F**), together our data suggest that KOR induces cold hypersensitivity through enhancing activity at TRPA1 channels.

Discussion

Here we report that peripheral KOR activation increases cold sensitivity in mice. We show that transcripts for KORs are present in the same cells as TRPA1 and TRPM8 in DRG. Furthermore, activation of KOR and TRPA1 together in cultured DRG potentiates calcium mobilization compared to activation of TRPA1 alone. This suggests that KORs are likely able to modulate cold hypersensitivity through modification of TRPA1 in DRG; however, the intracellular mechanism between these two receptors has yet to be established.

KORs have long been considered promising targets for pain and itch relief due to their non-addictive profile (Porreca & Burks, 1983; Porreca, Mosberg, Hurst, Hruby, & Burks, 1984; Shippenberg, Stein, Huber, Millan, & Herz, 1988). However, the most significant limitation to targeting the KOR system has been the induction of negative affect, primarily mediated by the central activation of KOR (Horan & Porreca, 1993; Porreca, Mosberg, Omnaas, Burks, & Cowan, 1987), particularly in the nucleus accumbens (Al-Hasani et al., 2015; Massaly et al., 2019; Shippenberg et al., 1988). As a result, this has prompted the investigation into the therapeutic potential of peripherally-expressed KOR (Beck, Reichel, Helke, Bhadsavle, & Dix, 2019; Snyder et al., 2018; Togashi et al., 2002). Progress has been constrained by the lack of peripherally restricted KOR compounds, as well as short-acting antagonists. However, more recently, there has been a focused effort to develop both short-acting reversible antagonists (Page et al., 2019) and peripherally restricted agonists (Barber et al., 1994; Olesen et al., 2013; Paton, Atigari, Kaska, Prisinzano, & Kivell, 2020; Shaw, Carroll, Alcock, & Main, 1989; Suzuki et al., 2017; Alleyne et al, in revision) to understand the role of KOR in pain- and itch-related behaviors.

Thus far, in the itch field, Asimadoline (peripherally-restricted KOR agonist) has demonstrated efficacy in animal models of pruritus (Barber et al., 1994). The drug candidate is now in Phase 2 Proof-of-Concept clinical study to treat pruritus associated with atopic dermatitis by Tioga Pharmaceuticals (ClinicalTrials.gov, NCT01513161). TRK-820 (nalfurafine) manufactured by Toray Industries, Inc. is approved and used in Japan to treat uremic pruritus (Kumagai et al., 2010). In addition, recent studies have shown that peripherally-restricted KOR agonists reduce mechanical hyperalgesia at the early inflammatory phase in a plantar incision model (Snyder et al., 2018). JT09, peripherally-restricted KOR agonist, is as effective as morphine in alleviating pain without any sedative effects (Beck et al., 2019).

CR845, a D-amino-acid tetrapeptide, is a highly selective peripherally-restricted KOR agonist, shown to alleviate pain and inflammation (Keppel Hesselink, 2017). The peptide is well-tolerated and is proven to be as effective as oxycodone in a human model of acute visceral pain (Arendt-Nielsen et al., 2009). CR845, in humans, has no reports of dysphoria or hallucinations (Keppel Hesselink, 2017) and is currently in phase II/III development to treat acute post-operative uremic pruritus [ClinicalTrials.gov] (Keppel Hesselink, 2017). In our current studies, we use an analogue of this compound, ff(nle)r-NH2 (Alleyne et al, in revision), to show that activation of peripheral KORs increases cold sensitivity in male and female mice. Our findings identify an important role for the KOR system in cold hypersensitivity.

Though little is known about the KOR system in cold sensation, it has been widely shown that the role of KOR in pain is dependent on the type of pain and sex. For example, in chronic pain states, KOR has been shown to induce negative affect (S. S. Liu et al., 2019; Massaly et al., 2019) that is absent following acute pain (Bagdas et al., 2016; Leitl, Onvani, et al., 2014; Leitl, Potter, et al., 2014). In both preclinical animal models and human imaging studies, KOR modulation of pain is sex-dependent. In preclinical studies, intraplantar administration of U50 mitigated pain in the lateral sensitization rat model, without any systemic side effects. The effect at the highest dose of 100 µg/20µl had better anti-hyperalgesic activity in males than females, implying a sex-dependent effect (Auh & Ro, 2012). Clinically, positron emission tomography studies show that KOR receptor binding is higher in men than in women, especially in the anterior cingulate cortex, a region associated with pain affect (Vijay et al., 2018). To fully evaluate sex as a biological variable, we investigated the KORs role KOR-modulation of cold hypersensitivity in both males and females. In this context, we report no significant differences. We also considered KORs somewhat controversial role in thermoregulation (Rawls & Benamar, 2011). KOR agonist U50 at higher doses than we use here (20 mg/kg; 40 mg/kg) has been shown to cause hypothermia in mice (Nemmani, Gullapalli, & Ramarao, 2001), and this response to KOR activation is markedly influenced by tolerance developed by receptors upon repeated administration of KOR agonists (Milanés, Gonzalvez, Fuente, & Vargas, 1991; Rawls, Robinson, Patel, & Baron, 2008; Von Voigtlander & Lewis, 1982). In contradiction, another study revealed that the 30 mg/kg dose of U50 did not affect rectal body temperature in mice (Itoh, Ukai, & Kameyama, 1993). Here we show that neither KOR agonists (U50, ff(nle)r-NH2) alter body temperature in male and female mice.

To understand how activation of KOR mediates cold hypersensitivity, we investigated the TRP channels, TRPA1 and TRPM8 channels, both well known for their role in cold sensitivity (MacDonald et al., 2020). Interestingly, there has been targeted research exploring mu-opioid, but not kappa-opioid, receptors, and TRP channel interaction (Shapovalov et al., 2013; Williams et al., 2013). In mice, mu opioid receptor activation leads to internalization of TRPM8 channels, and in TRPM8$^{-/-}$ mice, opioid-induced cold analgesia was absent (Shapovalov et al., 2013). Similarly, spinal TRPA1 is shown to facilitate the morphine antinociceptive effect on a hot plate assay (Wei et al., 2016), suggesting the interaction of mu-opioid receptors and TRP channels in modulating thermal sensitivities. Furthermore, administration of opioids has been shown to distort thermal sensation; for example, some patients experience waves of warmth upon opioid

administration (Chu, Clark, & Angst, 2006), and drug withdrawal is often characterized by cold chills in combination with hyperalgesia (Pud, Cohen, Lawental, & Eisenberg, 2006).

Co-localization and electrophysiological studies have confirmed the presence of KORs on C- and A-fibers on DRG neurons expressing the TRP channel, TRPV1, and calcitonin gene-related peptide (Snyder et al., 2018). KORs are most commonly Gi-coupled GPCRs. Studies have shown that TRPM3 (Mucopilin 3), another TRP channel known to detect temperature and pain, interacts with the βγ subunits of the G-protein in the DRG to regulate calcium currents (Badheka, Borbiro, & Rohacs, 2015; Quallo, Alkhatib, Gentry, Andersson, & Bevan, 2017). Calcium imaging studies in human DRG neurons show a decrease in Ca^{2+} influx in the presence of the endogenous KOR ligand, dynorphin (Snyder et al., 2018). However, the calcium activity of KOR has not been evaluated in tandem with any TRP channels. These findings substantiate our work exploring KOR's ability to modulate calcium activity via TRPA1, which appears to likely be the mechanism that driving cold hypersensitivity. In summary, we show that activation of KOR and TRPA1 together in DRG potentiates calcium signals when compared to activation of TRPA1 alone. This suggests that cold hypersensitivity may be driven by peripheral activation of KOR that subsequently enhances the function of TRPA1, perhaps through receptor operation. Bautista et al. have shown similar mechanisms where Ca^{2+} released in response to inflammatory mediators acts as a co-factor in activating TRPA1. Inflammatory mediators such as bradykinin activate phospholipase-C β signaling systems resulting in inositol triphosphate-mediated Ca^{2+} release (Bautista et al., 2006). Further exploration of the downstream signaling pathway mediating the calcium homeostasis via the KOR is warranted.

Cold hypersensitivity is a chronic, debilitating, and poorly treated condition prevalent in neuropathic pain conditions such as multiple sclerosis, fibromyalgia, complex regional pain syndrome, and neuropathy following chemotherapy treatment. Our results identify a potential role for the KOR system in the mediation of cold hypersensitivity. Here we show KOR's role to be restricted to the activation of peripheral KOR, which is encouraging and allows the study of this system without any centrally-mediated side effects.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

Author contributions:

Conceptualization, M.K.M, T.S.S., A.M.F., and R.A.; Methodology, M.K.M., L.V.T, J.G.M., and R.A.; Investigation, M.K.M., L.V.T, P.C., S.P., J.S.A., R.A.H., and R.A. Manuscript preparation, M.K.M., J.G.M., and R.A.; Funding acquisition, R.A.; Supervision, J.P.M., J.G.M., and R.A.; Project administration, R.A.

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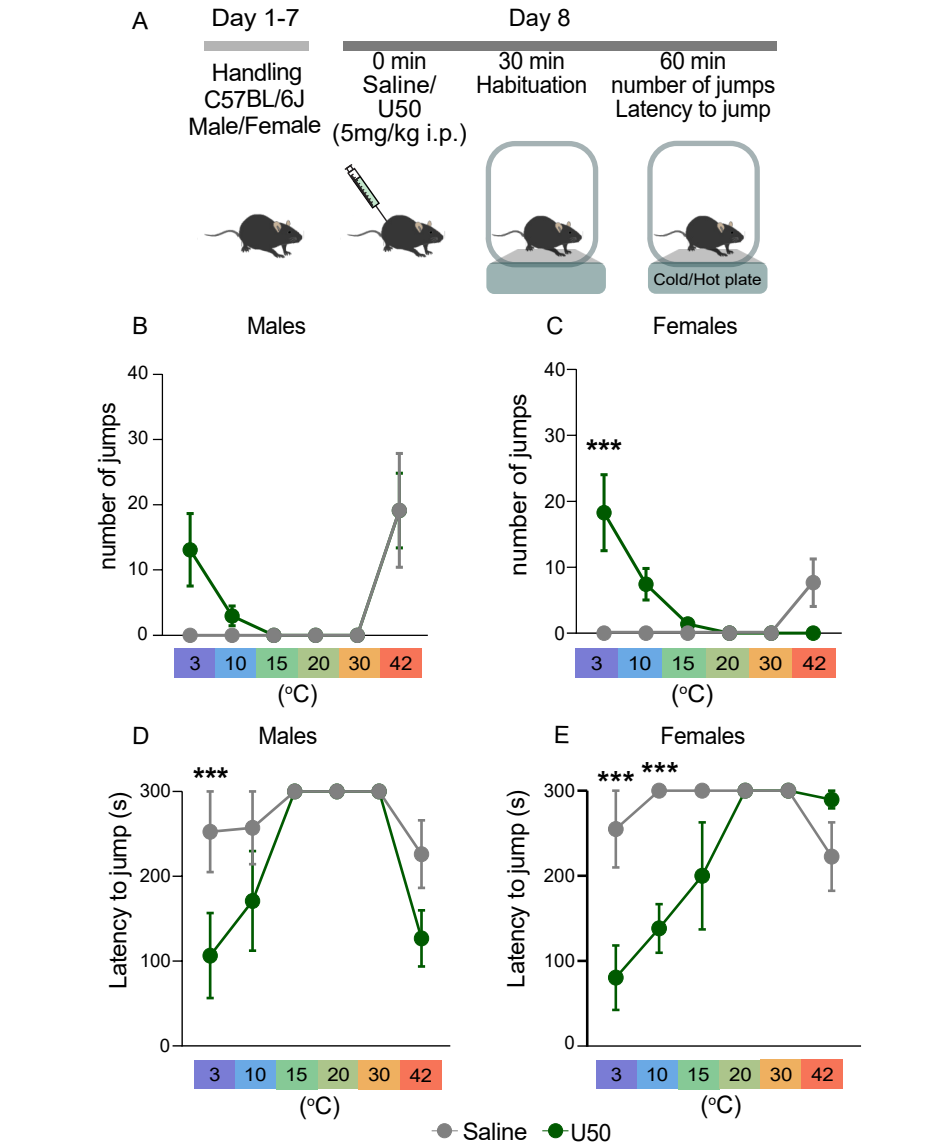
Figure 1


A
Day 1-7
Handling
C57BL/6J
Male/Female
Day 8
0 min
Saline/
U50
(5mg/kg i.p.)
30 min
Habituation
60 min
number of jumps
Latency to jump
Cold/Hot plate
B
Males
C
Females
number of jumps
***
3
10
15
20
30
42
(°C)
D
Males
E
Females
Latency to jump (s)
Saline
U50

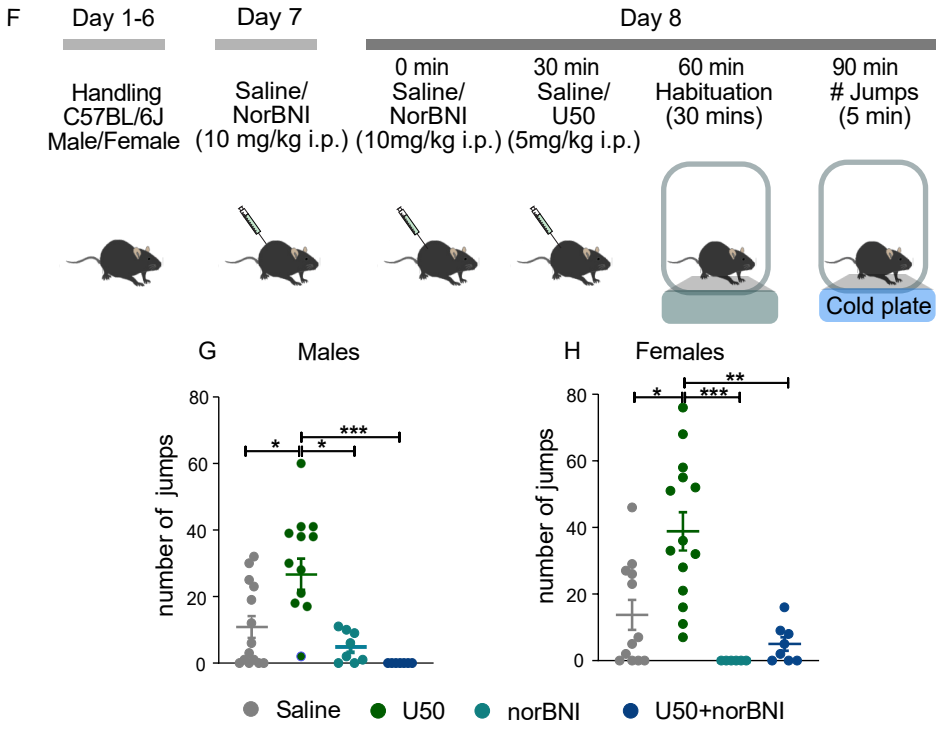

F
Day 1-6
Handling
C57BL/6J
Male/Female
Day 7
Saline/
NorBNI
(10 mg/kg i.p.)
Day 8
0 min
Saline/
NorBNI
(10mg/kg i.p.)
30 min
Saline/
U50
(5mg/kg i.p.)
60 min
Habituation
(30 mins)
90 min
Jumps
(5 min)
Cold plate
G
Males
H
Females
number of jumps
Saline
U50
norBNI
U50+norBNI

Figure 1: KOR-potentiated cold hypersensitivity is temperature-dependent

A) Outline of the experimental procedure. WT mice were injected with either saline or U50 (KOR agonist, 5 mg/kg, i.p.) 7days post-handling. Post injection mice were exposed to cold/hot plate assay across a range of temperatures 3°C, 10°C, 15°C, 20°C, 30°C, 42°C.

B) In males, (Data are presented as mean ± SEM; n = 5-7/group; Nonparametric mixed effects analysis (two way ANOVA) revealed temperature effect $F_{(5, 53)} = 8.476$ followed by Sidak's *post-hoc* test (p=0.06 vs respective saline treatment))

C) In females, KOR agonist U50 (5mg/kg i.p) significantly increased the number of jumps when compared to controls at 3°C. (Data are presented as mean ± SEM; n = 5-7/group; Nonparametric mixed effects analysis (two way ANOVA) revealed temperature effect $F_{(5, 57)} = 3.937$, treatment effect $F_{(1, 57)} = 4.956$ and a treatment X temperature $F_{(5, 57)} = 6.191$ followed by Sidak's *post-hoc* test (***p<0.001 vs respective saline treatment))

D) Latency to jump following KOR activation is decreased in males at 3°C only. (Data are presented as mean ± SEM; n = 5-7/group; Nonparametric mixed effects analysis (two way ANOVA) revealed temperature effect $F_{(5, 53)} = 4.792$ treatment effect $F_{(1, 53)} = 8.665$ and a treatment X temperature $F_{(5, 53)} = 2.446$ followed by Sidak's *post-hoc* test (***p<0.001 vs respective saline treatment))

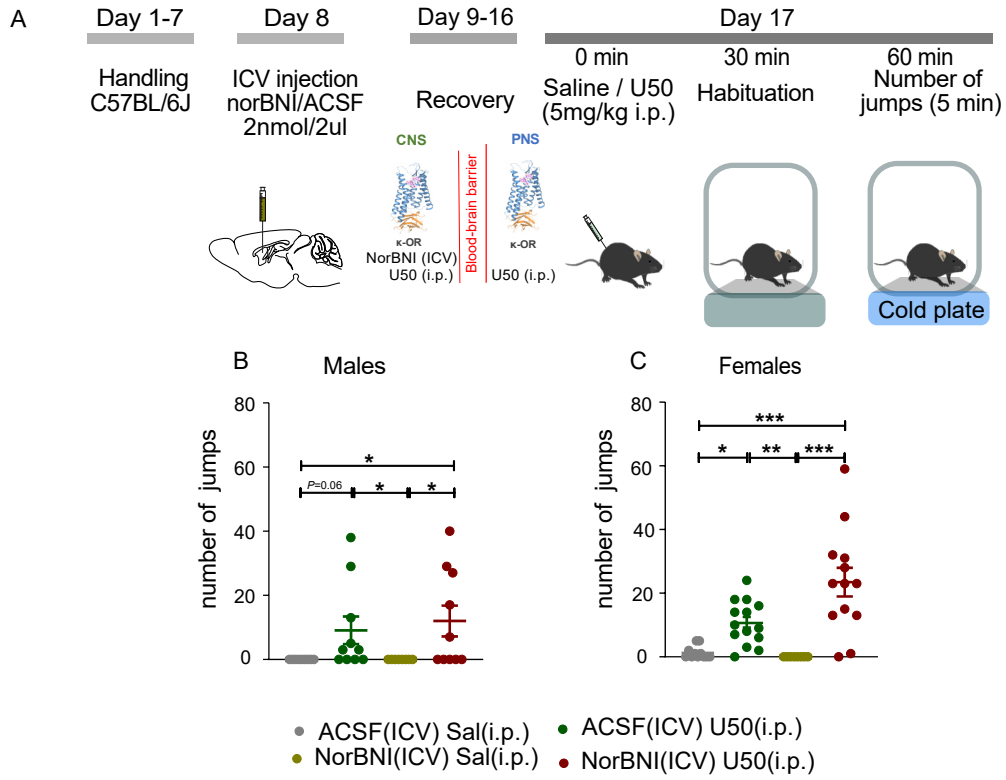
E) Latency to jump following KOR activation is decreased in females at 3°C and 10°C (data are presented as mean ± SEM; n = 5-7/group; Nonparametric mixed effects analysis (two way ANOVA) revealed temperature effect $F_{(5, 30)} = 5.917$, treatment effect $F_{(1, 29)} = 14.82$ and a treatment X temperature $F_{(5, 29)} = 6.722$ followed by Sidak's *post-hoc* test (***p<0.001 vs respective saline treatment))

F) Outline of experimental procedure. Timeline of the systemic injections of saline, U50, or norBNI, followed by habituation and cold plate assay at 3°C.

G) In males, activation of KOR by U50 significantly increased the number of jumps on a 3°C cold plate (data are presented as mean±SEM n=8-14/group; Kruskal-Wallis test revealed significance ***p<0.001 followed by Bonferroni *post-hoc* analysis. U50 vs. Saline *p<0.05). KOR antagonism with norBNI blocked cold hypersensitivity on a cold plate at 3°C. (U50 vs U50+norBNI***p<0.001).

H) In females, activation of KOR by U50 significantly increased the number of jumps on a 3°C cold plate (data are presented as mean±SEM n=8-14/group; Kruskal-Wallis test revealed significance ***p<0.001 followed by Bonferroni *post-hoc* analysis. U50 vs. Saline *p<0.05). KOR antagonism with norBNI blocked cold hypersensitivity on a cold plate at 3°C. (U50 vs U50+norBNI**p<0.01).

Figure 2


A
Day 1-7
Handling
C57BL/6J
Day 8
ICV injection
norBNI/ACSF
2nmol/2ul
Day 9-16
Recovery
Day 17
0 min
Saline / U50
(5mg/kg i.p.)
30 min
Habituation
60 min
Number of
jumps (5 min)
CNS
PNS
κ-OR
NorBNI (ICV)
U50 (i.p.)
Blood-brain barrier
κ-OR
U50 (i.p.)
Cold plate
B
Males
C
Females
number of jumps
P=0.06
ACSF(ICV) Sal(i.p.)
NorBNI(ICV) Sal(i.p.)
ACSF(ICV) U50(i.p.)
NorBNI(ICV) U50(i.p.)

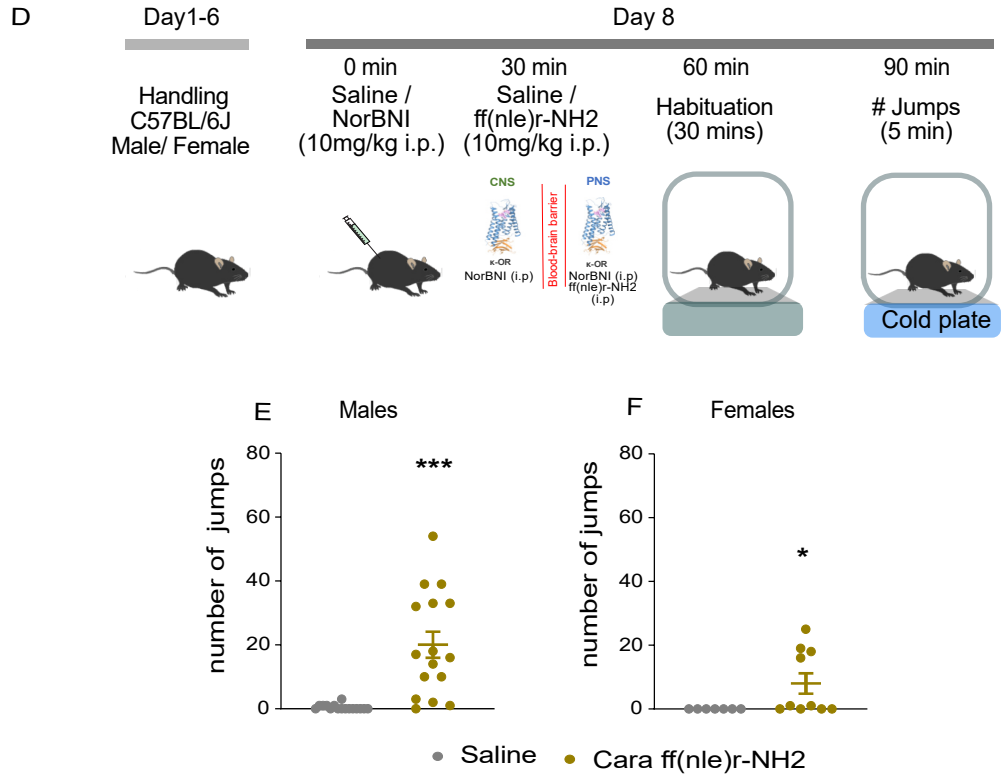

D
Day1-6
Handling
C57BL/6J
Male/ Female
Day 8
0 min
Saline /
NorBNI
(10mg/kg i.p.)
30 min
Saline /
ff(nle)r-NH2
(10mg/kg i.p.)
60 min
Habituation
(30 mins)
90 min
Jumps
(5 min)
CNS
PNS
κ-OR
NorBNI (i.p)
Blood-brain barrier
κ-OR
NorBNI (i.p)
ff(nle)r-NH2
(i.p)
Cold plate
E
Males
F
Females
number of jumps
Saline
Cara ff(nle)r-NH2

Figure 2: Peripheral activation of KORs is sufficient to drive cold hypersensitivity

A) Outline of the experimental procedure. Timeline of the ICV injections (NorBNI or aCSF), followed by habituation, systemic U50, or saline injections followed by cold plate assay at 3°C.

B) In males, mice infused with aCSF(ICV) and U50 (i.p.) showed cold hypersensitivity, as compared to the control group on 3°C cold plate assay. (Data are presented as mean±SEM n=8-14/group; Kruskal-Wallis test revealed the significance of $^{**}p<0.01$, followed by Bonferroni *post-hoc* analysis. aCSF (ICV)+U50 (i.p.) vs aCSF (ICV)+saline(i.p.) $p=0.06$)). 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.) $^{*}p<0.05$). Central KOR antagonism without peripheral activation has shown no cold hypersensitivity on a cold plate at 3°C (norBNI(ICV)+saline (i.p.) vs. norBNI (ICV)+U50(i.p.) $^{*}p<0.05$).

C) In females, mice infused with aCSF(ICV) and U50 (i.p.) showed cold hypersensitivity, as compared to the control group on 3°C cold plate assay. (data are presented as mean±SEM n=8-14/group; Kruskal-Wallis test revealed the significance of $^{***}p<0.001$, followed by Bonferroni *post-hoc* analysis. aCSF (ICV)+U50 (i.p.) vs aCSF (ICV)+saline(i.p.) $^{*}p<0.05$). Central KOR antagonism with norBNI, had no effect on cold hypersensitivity in females on a cold plate at 3°C (aCSF(ICV)+saline (i.p.) vs. norBNI (ICV)+U50(i.p.) $^{***}p<0.01$). Central KOR antagonism without peripheral activation has shown no cold hypersensitivity on a cold plate at 3°C (norBNI(ICV)+saline (i.p.) vs. norBNI (ICV)+U50(i.p.) $^{**}p<0.01$).

D) Outline of the experimental procedure. WT mice were injected with either saline or ff(nle)r-NH2 peripheral KOR agonist (10 mg/kg, i.p.) and subjected to cold plate assay at 3 ^{o}C. E and F) Peripherally restricted agonist ffir-NH2 (10mg/kg i.p.) significantly increased jumping on the cold plate at 3°C, compared to controls, in males (E) and females (F). (Males -saline (i.p.) vs. ff(nlr)r-NH2 (i.p.) $^{***}p<0.001$; females- saline (i.p.) vs. ff(nlr)r-NH2 (i.p.) $^{*}p<0.05$; n=7-16/group; Mann-Whitney U test). Data are expressed as mean±SEM.

Figure 3

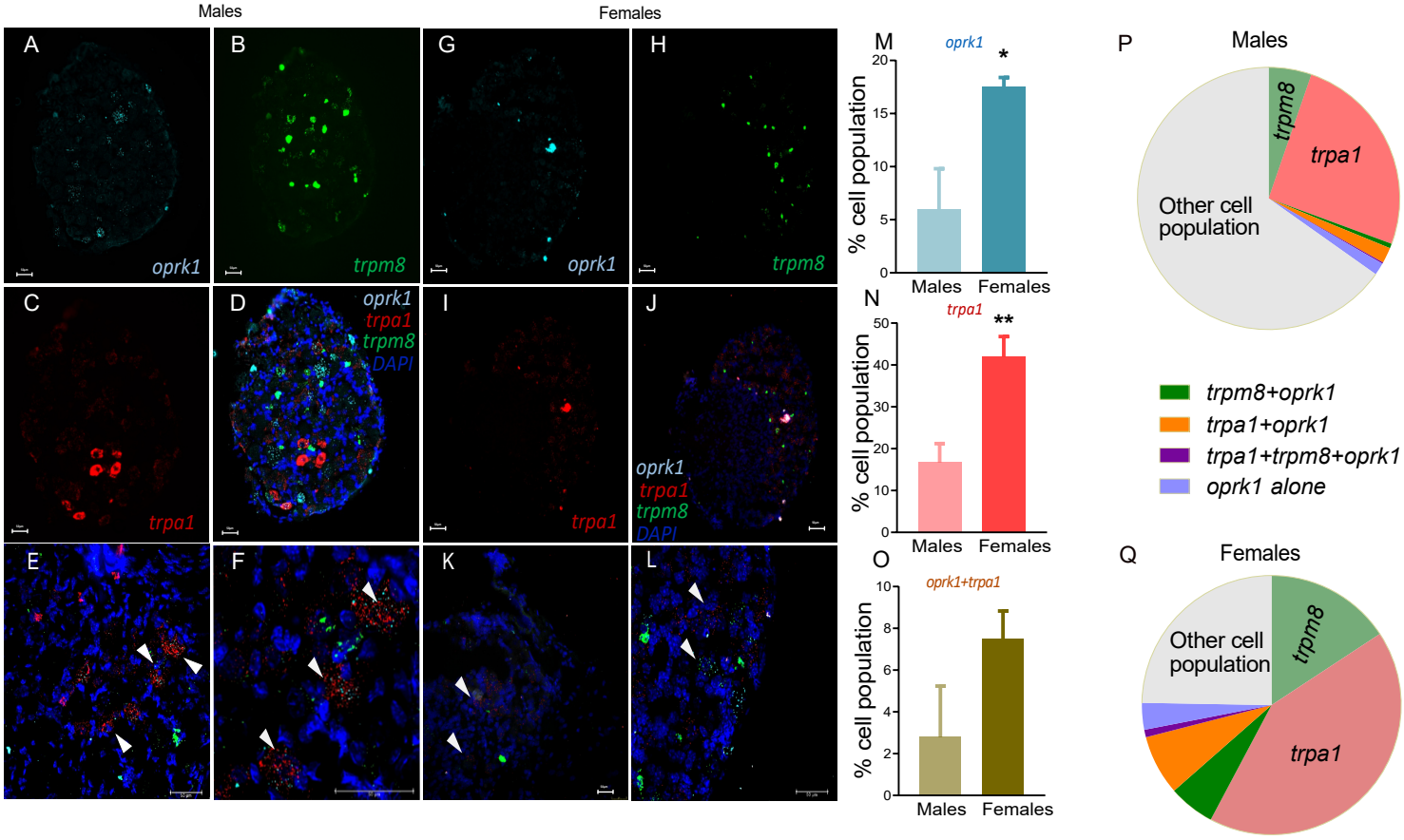

Males
Females
A
oprk1
B
trpm8
G
oprk1
H
trpm8
C
trpa1
D
oprk1
trpa1
trpm8
DAPI
I
trpa1
J
oprk1
trpa1
trpm8
DAPI
E
F
K
L
M
oprk1
*
% cell population
Males
Females
N
trpa1
**
% cell population
Males
Females
O
oprk1+trpa1
% cell population
Males
Females
P
Males
trpm8
trpa1
Other cell population
trpm8+oprk1
trpa1+oprk1
trpa1+trpm8+oprk1
oprk1 alone
Q
Females
Other cell population
trpm8
trpa1

Figure 3: Transcripts for KOR, TRPA1, and TRPM8 are differentially co-localized in mouse DRG neurons

A-F) representative data from males G-L) representative data from females. A) and G) Fluorescent *in situ* hybridization of mice DRG sections for *oprk1*, B) and H) Fluorescent *in situ* hybridization of mice DRG sections for *trpm8,* C) and I) Fluorescent *in situ* hybridization of mice DRG sections for *trpa1.* D)and J) Fluorescent *in situ* hybridization of mice DRG sections of all the three transcripts and DAPI. E), F), K), and L) show the colocalization of KOR with TRPM8 and TRPA1 at 40x. M) Females exhibited higher expression of *oprk1* expressing cells in female mice compared to the male mice in the DRG. (Data is represented as mean±SEM n=2-4; Mann-Whitney U test; females vs. males $*P<0.05$;) N) Females exhibited higher expression of *trpa1* expressing cells compared to the male mice in the DRG. (Data is represented as mean±SEM n=2-4; Mann-Whitney U test; females vs. males $**P<0.01$) O) No significant difference in expression of colocalization of *oprk1*+*trpa1* between males and females. P) Pie chart representing the cell population of *oprk1*, *trpm8, trpa1,* and co-expression of *trpa1* and *trpm8* with *oprk1* in males. Q) Pie chart representing the cell population of *oprk1*, *trpm8, trpa1,* and co-expression of *trpa1* and *trpm8* with *oprk1* in females.

Figure 4

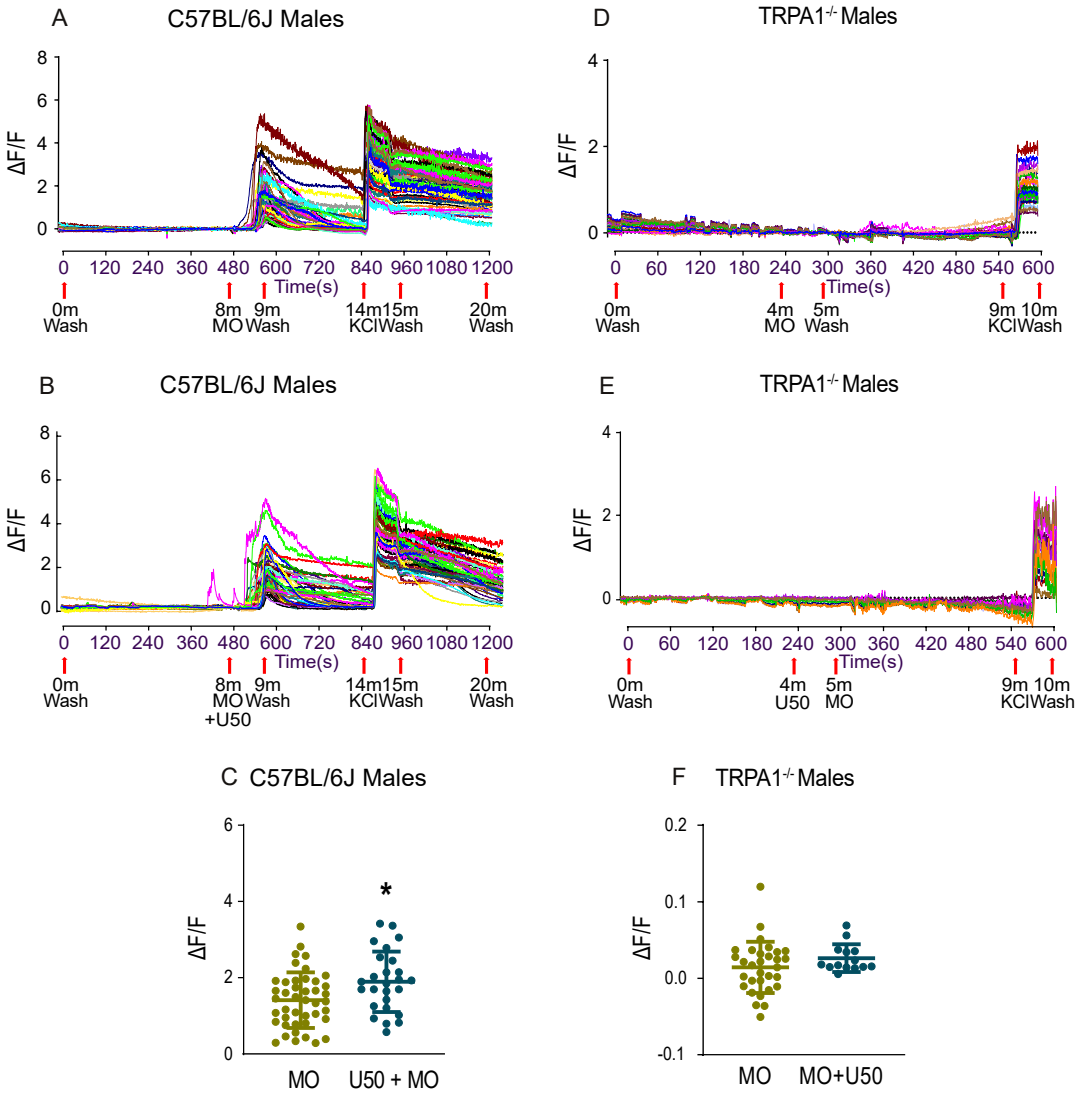

A
C57BL/6J Males
ΔF/F
Time(s)
0m Wash
8m MO
9m Wash
14m KCl
15m Wash
20m Wash
D
TRPA1-/- Males
4m MO
5m Wash
9m KCl
10m Wash
B
C57BL/6J Males
8m MO +U50
E
TRPA1-/- Males
4m U50
5m MO
C C57BL/6J Males
MO
U50 + MO
F TRPA1-/- Males
MO+U50

Figure 4: KOR activation potentiates TRPA1-dependent calcium signaling

A) Representative traces showing that mustard oil (MO) evokes calcium responses in male WT DRG cultures.

B) Representative traces showing that U50+MO also caused Ca^{+2}-mobilization in male WT DRG cultures.

C) Quantified data comparing peak intracellular concentration evoked by MO alone vs. U50 and MO together. U50+MO potentiated calcium responses significantly compared to MO alone (Data are expressed as mean±SEM; n= 26-43/group; two-tailed T-test; $*P < 0.05$;).

D) Representative traces showing that in male $Trpa1^{-/-}$ DRG cultures, MO application did not affect Ca^{+2}-mobilization

E) Representative traces showing that in $Trpa1^{-/-}$ DRG neuronal cultures, MO+U50 also had no effect in Ca^{2+} mobilization

F) Mean peak neuronal responses, MO vs. MO+U50 has no effect in $Trpa1^{-/-}$ DRG cultures (Data are expressed as mean±SEM; n = 15-30/group) suggesting the role of TRPA1 channels in mediating KOR-induced cold hypersensitivity.

Supplementary figures

Supp. Figure 1

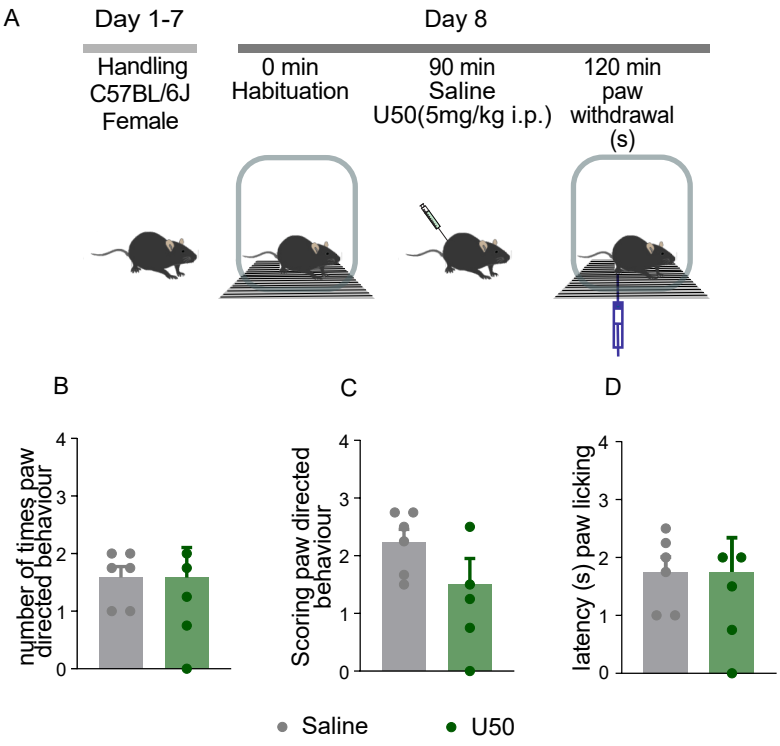


Figure S1: KOR activation does not alter behavior in the acetone evaporation test

A) Outline of the experimental procedure. Timeline of the systemic injections of U50, followed by habituation, and acetone evaporation assay. U50 had no effect on cold aversive behavior in females. The withdrawal paw behavior included B) quantifying the nocifensive responses C) scoring the severity of the nocifensive response D) duration of the nocifensive response. Data are expressed as mean± SEM (n=5-6/group).

Supp. Figure 2

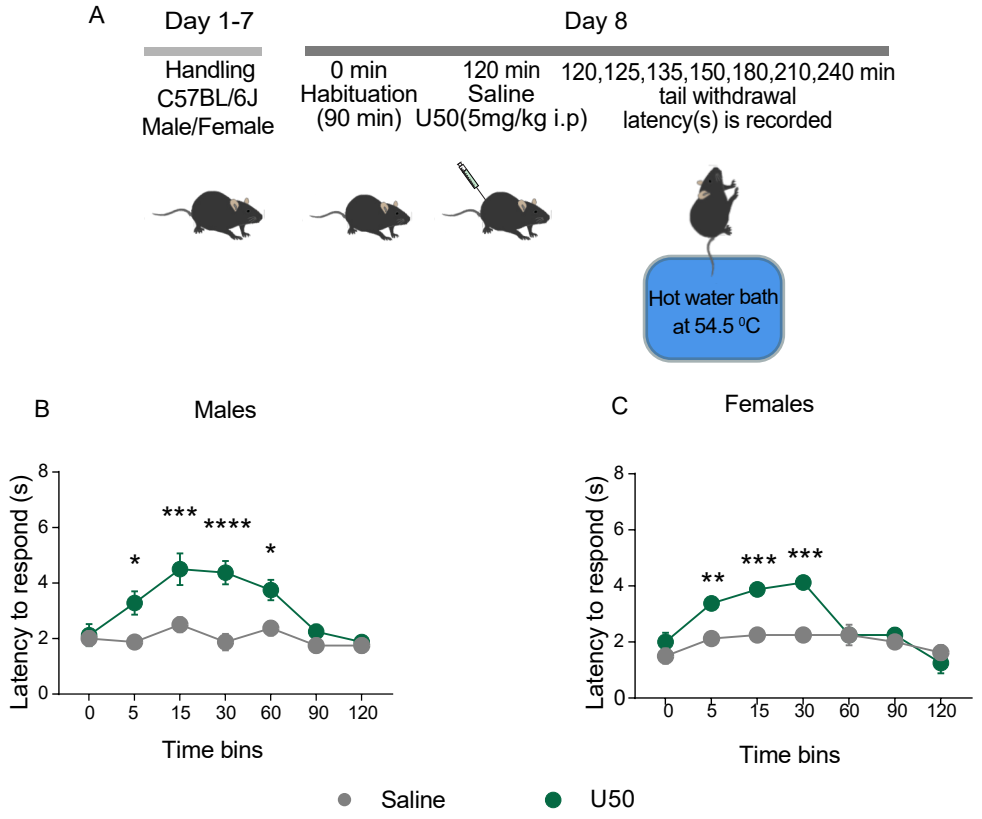


Figure S2: KOR activation is antinociceptive in the warm water tail withdrawal assay

A) Timeline of experimental procedures for tail withdrawal assay. The KOR activation mediated antinociception in B) males (Data are expressed as mean± SEM; (n=7-8/group; parametric repeated measures ANOVA (two 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's *post-hoc* test $^{*}P<0.05$, $^{**}P<0.01$ $^{***}P<0.001$ vs respective saline treatment) and C) females. (Data are expressed as mean± SEM; 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's *post-hoc* test. $^{**}P<0.01$ $^{***}P<0.001$ vs respective saline treatment).

Supp. Figure 3

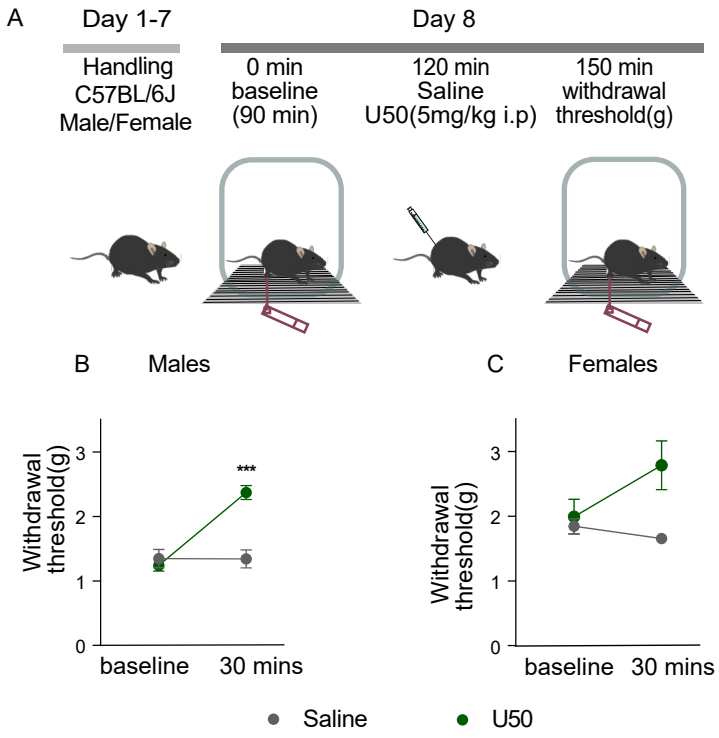


Figure S3: KOR activation is mechanically antinociceptive

A) Timeline of experimental procedures for von Frey assay (B) The KOR blocked mechanical sensitivity in B) males only (Data are expressed as mean± SEM (n=7-8/group parametric repeated measures ANOVA (two way ANOVA) revealed time effect $F_{(1, 15)} = 36$; dose-effect $F_{(1, 15)} = 10.96$ and time X dose-effect $F_{(1, 15)} = 36.54$ followed by Sidak's *post-hoc* test ***$P<0.001$ vs. respective saline treatment at 30 mins) but not in C) females.

Supp. Figure 4

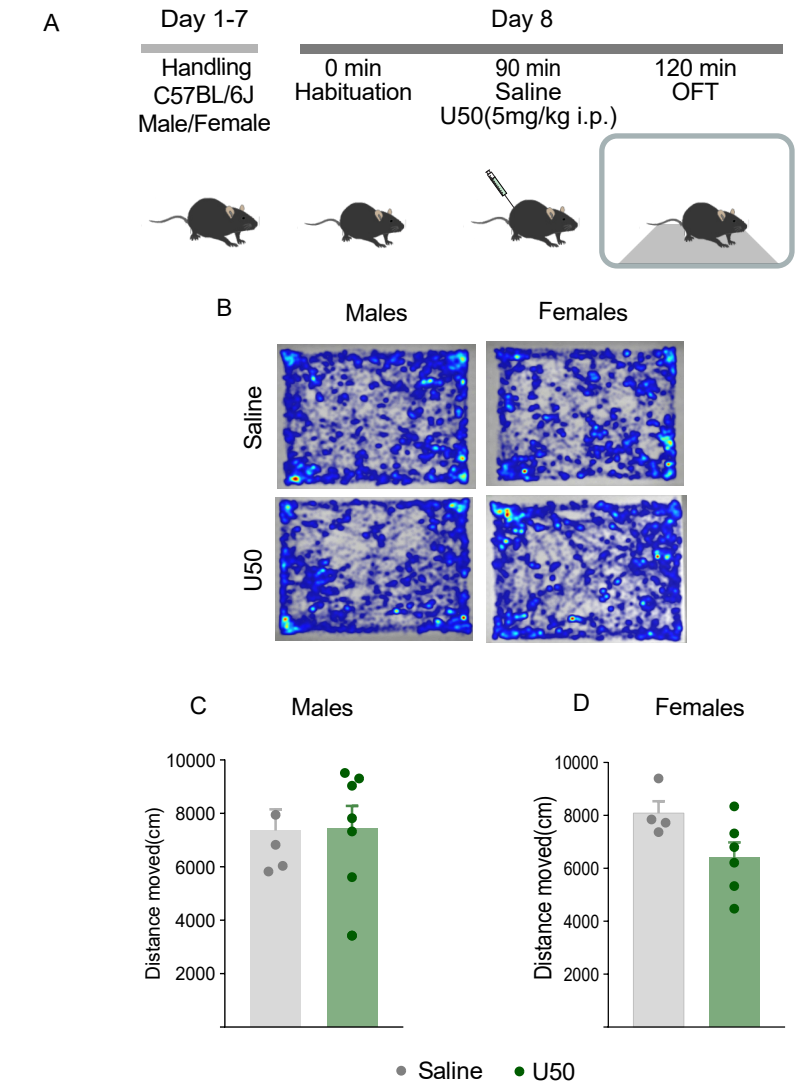


Figure S4: U50 (5 mg/kg) does not alter locomotor behavior

A) Outline of the experimental procedure. Timeline of the OFT assay, habituation followed systemic injections of saline or U50, and performing OFT. (B) Heat maps of treatment groups in OFT. U50 had no sedative effect in the open field test in C) males and D) females. Data are expressed as mean± SEM (n=5-7/group)

Supp. Figure 5

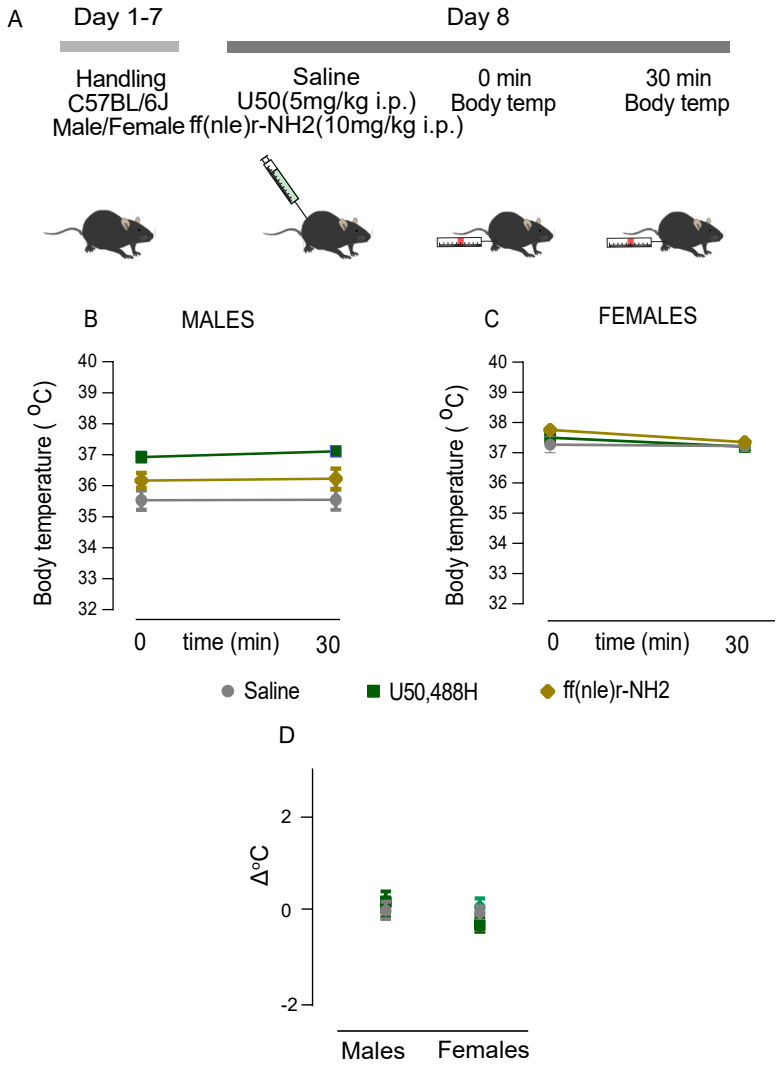


Figure S5: Systemic and peripheral activation of KOR does not alter core body temperature

A) Outline of the experimental procedure. Timeline of the assay, wherein mice are habituated, baseline rectal temperature is measured, followed by systemic injections of saline or U50 or ff(nle)r-NH2 and post-treatment temperatures were recorded. KOR agonists U50 and ff(nle)rNH2 did not affect core body temperature 30 mins post drug administration in B) males and C) females. Data are expressed as mean±SEM (n=8-10/group).

Supp. Figure 6

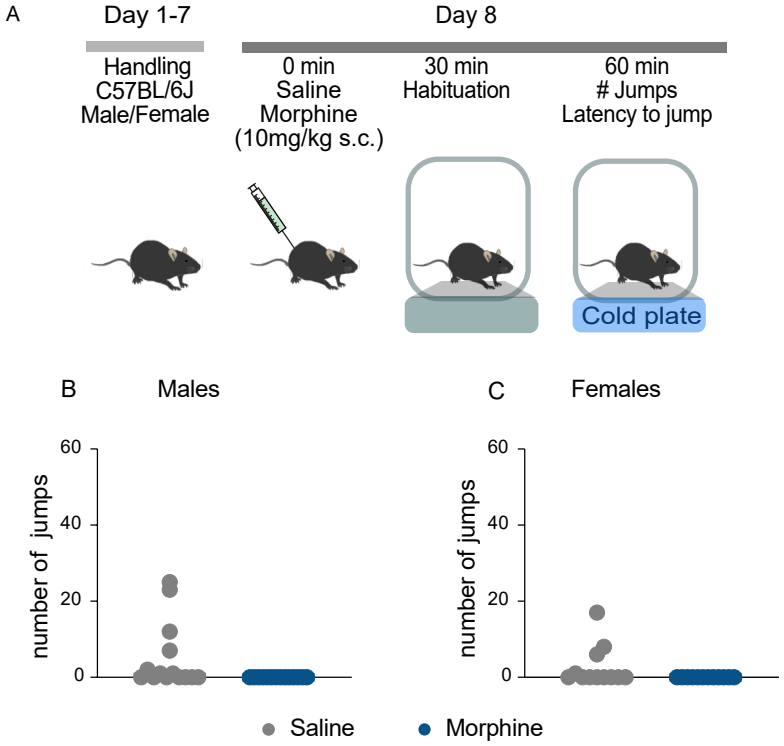


Figure S6: Morphine does not enhance cold sensitivity

A) Outline of the experimental procedure. Timeline of the systemic injections of morphine, followed by habituation, and cold plate assay at 3°C. Morphine did not affect cold sensitivity in both B) males and C) females. Data are expressed as mean±SEM (n=10-12)