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Mu-opioid receptors in nociceptive afferents produce a sustained suppression of hyperalgesia in chronic pain

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Abstract

The latent sensitization model of chronic pain reveals that recovery from some types of long-term hyperalgesia is an altered state in which nociceptive sensitization persists but is suppressed by the ongoing activity of analgesic receptors such as μ -opioid receptors (MORs). To determine whether these MORs are the ones present in nociceptive afferents, we bred mice expressing Cre-recombinase under the Nav1.8 channel promoter (Nav1.8cre) with MOR-floxed mice (fMOR). These Nav1.8cre/fMOR mice had reduced MOR expression in primary afferents, as revealed by quantitative PCR, *in situ* hybridization and immunofluorescence colocalization with the neuropeptide CGRP. We then studied the recovery from chronic pain of these mice and their fMOR littermates. When Nav1.8cre/fMOR mice were injected in the paw with complete Freund's adjuvant they developed mechanical hyperalgesia that persisted for over two months, whereas the responses of fMOR mice returned to baseline after three weeks. We then used the inverse agonist naltrexone to assess ongoing MOR activity. Naltrexone produced a robust reinstatement of hyperalgesia in control fMOR mice, but produced no effect in the Nav1.8/fMOR males and a weak reinstatement of hyperalgesia in Nav1.8/fMOR females. Naltrexone also reinstated swelling of the hind paw in fMOR mice and female Nav1.8cre/fMOR mice, but not male Nav1.8cre/fMOR mice. The MOR agonist DAMGO inhibited substance P release in fMOR

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mice but not Nav1.8cre/fMOR mice, demonstrating a loss of MOR function at the central terminals of primary afferents. We conclude that MORs in nociceptive afferents mediate an ongoing suppression of hyperalgesia to produce remission from chronic pain.

Keywords

chronic pain; C-fiber; complete Freund's adjuvant; inflammation; latent sensitization; opioid receptor; primary afferent; substance P

1. Introduction

Chronic pain disorders are not a mere exacerbation of acute pain but entail a switch of pain-processing mechanisms to a pathological state that may last indefinitely. Insight into the nature of this altered state has been provided by latent sensitization, a phenomenon observed in chronic pain studied in rodents [3,11,30,43] that may also extend to humans [34,35]. The key discovery of this conceptual model is that the apparent recovery that follows the hyperalgesia produced by certain injuries is not a return to normal pain transmission, but an altered state in which hyperalgesia persists but is suppressed by opioid receptor activation to produce remission [11,43]. Thus, if opioid inverse agonists are administered during this state there is a reinstatement of hyperalgesia lasting several hours. In contrast, opioid inverse agonists do not produce hyperalgesia in animals without latent sensitization. This resembles some forms of chronic pain in which pain appears in episodes. Several types of injury induce latent sensitization, including paw incision [3,37], complete Freund's adjuvant (CFA) [11], carrageenan [19] and nerve injury [39]. The fact that reinstatement of hyperalgesia by opioid antagonists can be repeated over months [3,11] indicates that latent sensitization lasts indefinitely.

The molecular mechanisms of latent sensitization are being actively investigated. Maintenance of hyperalgesia involves NMDA receptors [4,11], substance P [38] and adenylyl cyclase [11]. Suppression of hyperalgesia is primarily mediated by μ -opioid receptors (MORs), with additional contributions of δ and κ opioid, α_{2A} adrenergic and neuropeptide Y receptors [3,11,39,43]. Mice with global MOR knockout (KO) do not fully recover from CFA-induced hyperalgesia [43], showing that MORs suppress hyperalgesia in the remission phase of latent sensitization. However, the location of these receptors has not been determined. The objective of this study was to determine whether the MORs that suppress hyperalgesia in the remission phase of latent sensitization are present in nociceptive primary afferents. Mice expressing Cre-recombinase under Nav1.8 channel promoter control (Nav1.8cre mice), which selectively express Cre in all nociceptive primary afferents [42], were bred with mice in which the *oprm1* gene is flanked by loxP sequences (fMOR mice) [44] to selectively eliminate MORs in nociceptors. We demonstrate that these receptors are crucial for the suppression of hyperalgesia during latent sensitization in a sexually dimorphic manner. MORs in primary afferents have important functions at their peripheral terminals and their central synapses. We also investigated the effect of MOR deletion at these two locations by studying NTX-induced peripheral inflammation and MOR inhibition of substance P release at the central synapses.

2. Methods

2.1 Animals

All animal procedures were approved by the Animal Research Committee of UCLA and the Institutional Animal Care and Use Committee of the Veteran Affairs Greater Los Angeles Healthcare System, and conform to NIH guidelines. Experiments were designed to minimize the number of animals used and their suffering. Animals were provided food and water *ad libitum* and group-housed in 12-hour light-dark cycle.

Mice with exons 2 and 3 of the *oprm1* gene flanked by the LoxP cassette (fIMOR), with a genetic background of 75:25% of C57BL/6:129 [44], were provided by Drs. Kieffer and Gaveriaux-Ruff. Mice expressing one allele of Cre recombinase driven by the Nav1.8 promoter (Nav1.8cre), with C57BL/6 genetic background, were provided by Dr. John N. Wood [44]. Mice with fIMOR on both alleles were bred with mice expressing one allele of Nav1.8cre in the Animal Breeding Core of the Center for the Study of Opioid Receptors and Drugs of Abuse, UCLA. Genotyping was done using samples of ear tissue by Transnetyx, Inc. (Memphis, TN) by two independent assays for 1) fIMOR on none, one or both alleles and 2) the presence or absence of Cre recombinase, using proprietary primer sequences.

2.2 Quantitative RT-PCR

Quantitative RT-PCR (RT-qPCR) was performed as described [44] in Nav1.8Cre/fIMOR mice and fIMOR mice. Mice were euthanized with 5% isoflurane followed by cervical dislocation and cortical tissue and pooled dorsal root ganglia from T13-L6 were dissected from individual mice into RNA Later solution (cat. no. AM7020, Invitrogen, Carlsbad, CA). RNA was purified with PureLink™ RNA Mini Kit (cat. no. 12183018A, Invitrogen) and quantified with Qubit™ RNA Broad Range Assay kit and Qubit™ fluorometer (cat. no. Q10210, Invitrogen). RNA (1 µg) from each DRG pool was reverse-transcribed in a final volume of 20 µl using qScript cDNA supermix (cat. no. 95048, Quantabio, Beverly, MA). Quantitative PCR was performed on cDNA in quadruplicate (two 96-well plates containing duplicates were used) using PerfeCTa® SYBR® Green SuperMix Low Rox (cat. no. 95056, Quantabio) and QuantStudio3 (Applied Biosystems, Waltham, MA). PCR was performed with an initial 2-minute denaturation at 95°C followed by 45 cycles of PCR (15 seconds at 95°C, 45 seconds at 55°C). Melt curve analysis performed at the end of PCR amplification reproducibly showed a single peak for each gene in each sample. Relative expression ratios (Nav1.8Cre/fIMOR vs fIMOR) were calculated by using β-actin as reference gene and the 2^{-Ct} method to evaluate differential expression levels.

Primer sequences:

5'-TGTGCACTTTTATTGGTCTC-3' (*β-actin* forward)

5'-GATGTATGAAGGCTTTGGTC-3' (*β-actin* reverse)

5'-TCAAGGCCCTGGATTCCGTACCC-3' (*oprm1* forward)

5'-CGGGCAGACCAATGGCAGAAGAGA-3' (*oprm1* reverse)

2.3 RNAscope *in situ* hybridization

Mice were euthanized with 5% isoflurane followed by cervical dislocation. Tissue was collected from the L2-L6 dorsal root ganglia and snap-frozen in liquid nitrogen and embedded in OCT (Fisher HealthCare, Carlsbad, CA). Tissue was sectioned at 16 μ m and directly applied to gelatin-coated slides, prepared as previously described by submerging slides into a solution of 5 g of porcine gelatin (cat. no. 900-70-8, EMS brand, Hatfield, PA) dissolved at 45°C in 1 L deionized H₂O with 0.5 g chromium potassium sulfate dodecahydrate (CrK(SO₄)₂·12H₂O) (Fisher Scientific, Carlsbad, CA). Tissue was fixed to the slides for 15 minutes in fresh 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) at 4°C. Slides were dehydrated in a series of ethanol gradients at room temperature for 5 minutes each (50%, 70% and 100%). The workflow was then performed in accordance to the manufacturer's protocol for the RNAscope® Multiplex Fluorescent Assay (Advanced Cell Diagnostics, Newark, CA). Riboprobes 426011-C2 and 315841 selectively bind to sequences contained within the murine *scn10a* and *oprm1* genes, respectively. The *oprm1* and *scn10a* riboprobes were labeled with Atto550 and Alexa Fluor 488 fluorophores, respectively.

2.4 Immunohistochemistry

Mice were euthanized with pentobarbital (100 mg/kg) and fixed by aortic perfusion of 100 ml phosphate buffer (0.1 M sodium phosphate, pH 7.4) with 0.01% heparin, followed by 200 ml of ice-cold fixative (4% paraformaldehyde, 0.18% picric acid in phosphate buffer). A segment of the lumbar spinal cord (L3-L4) or spinal cord slices were fixed with 4% paraformaldehyde, cryoprotected in 20% sucrose, embedded in Tissue-Tek (Sakura Finetek USA, Inc., Torrance, CA) and frozen onto coverslips on dry ice. Free-floating transversal sections (25 μ m thick) were cut with a cryostat (Leica Biosystems, Buffalo Grove, IL). Sections were washed twice with phosphate-buffered saline (PBS) and twice with PBS containing 0.3% Triton X-100 (PBS/Triton) and 1% normal serum (goat or donkey, Jackson ImmunoResearch, West Grove, PA). Sections were incubated overnight in PBS/Triton containing 10% normal serum with primary antibodies (see below). Isolectin B4 (IB4)-biotin (5 μ g/mL, Sigma-Aldrich) was included in some experiments and detected using streptavidin-Alexa Fluor 488 (Invitrogen-Molecular Probes, Eugene, OR). After three washes with PBS, sections were incubated for two hours with secondary antibodies (1:2,000): Alexa Fluor-488 goat anti-guinea pig IgG (Invitrogen-Molecular Probes), Alexa Fluor-568 goat anti-rabbit IgG (Invitrogen-Molecular Probes) and Alexa Fluor-594 donkey anti-guinea pig IgG (Jackson ImmunoResearch). After four more PBS washes, sections were mounted in Prolong Gold (Invitrogen-Molecular Probes). All incubations and washes were done at room temperature.

Primary antibodies:

CGRP (dilution 1:10,000) [27]: Polyclonal rabbit antibody against rat CGRP₂₃₋₂₇, VKDNFVPTNVGSEAF (Dr. Catia Sternini, Division of Digestive Diseases, UCLA).

MOR (dilution 1:1,000) [7,40]: Affinity purified guinea pig antibody against the peptide sequence NHQLENLEAETAPLP, corresponding to amino acids 384-398 of the C-terminus of human MOR (GP10106, Neuromics, Edina, MN).

NK1R (dilution 1:3,000) [8,9,14]: Rabbit antiserum against amino acids 385-407 at the C-terminus of the rat NK1R (AB5060, EDM Millipore, Billerica, MA).

2.5 Substance P release measured as NK1R internalization

Spinal cord slices were prepared as previously described in rats [1,29]. The spinal cord was extracted from adult mice under pentobarbital anesthesia (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). Coronal slices (400 μ m, 3-4 per mouse) were cut from the lumbar spinal cord (L2-L4) with a Leica VT1000S vibratome (Buffalo Grove, IL). Slices were kept in artificial cerebrospinal fluid (aCSF), which contained (in mM) 124 NaCl, 1.9 KCl, 26 NaHCO₃, 1.2 KH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂ and 10 glucose, oxygenated with 95% O₂/5% CO₂. Vibratome cutting was done in sucrose-aCSF, which was the same aCSF medium with 5 mM KCl and 215 mM sucrose instead of NaCl. Slices were left to recover in oxygenated aCSF at 35°C for 1 hr and then were incubated with drugs at 35 °C in 5 ml of aCSF. At the end of the experiment, slices were fixed in ice-cold fixative and processed for NK1R immunohistochemistry.

NK1R internalization was used as a measure *in situ* of substance P release, a method extensively validated [1,2,15,26,29]. NK1R neurons in lamina I were visually counted while classifying them as with or without internalization, using a Zeiss Axio-Imager A1 (Carl Zeiss, Inc., Thornwood, NY) fluorescence microscope with a 63 $\times$ (1.40 numerical aperture) objective. The criterion for having internalization was the presence of ten or more NK1R endosomes. The person counting the neurons was blinded to the treatment and genotype. Results were expressed as the percentage of the NK1R neurons in lamina I with NK1R internalization.

2.6 Confocal microscopy

Confocal images were acquired using a Zeiss LSM 710 microscope (Carl Zeiss, Inc., Thornwood, NY), with objectives of 20 $\times$ (numerical aperture 0.8) and 63 $\times$ oil (numerical aperture 1.4). Laser excitation lines and emission windows were: Alexa Fluor 488, excitation 488 nm, emission 500–540 nm; Alexa Fluor 568, excitation 561 nm, emission 580–630 nm; Alexa Fluor 594, excitation 561 nm, emission 500–677 nm; Atto 550, excitation 561 nm, emission 570–648. The pinhole was set to 1.0 Airy unit for each objective and fluorophore. The separation between optical sections was 0.85 μ m or 0.99 μ m for the 20 $\times$ objective and 0.38 μ m for the 63 $\times$ objective, as determined by the microscope software using the Nyquist formula. Imaris 6.1.5 (Bitplane AG, Zurich, Switzerland) was used to crop the image stacks. Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA) was used to adjust the gamma and to assemble the multi-panel figures.

2.7 Image analysis

In situ hybridization: Three DRG histological sections per mouse were imaged with the confocal microscope using the 20 $\times$ objective, obtaining stacks of 3 optical sections separated 0.99 μ m. Since there were 4 groups of mice with $n = 4$ mice per group, a total of 48 confocal stacks were obtained. Fiji/ImageJ (1.51n, Wayne Rasband, National Institutes of Health) with a cell counter plugin (Kurt De Vos, University of Sheffield, UK) was used to count cell profiles in the middle optical section of each stack. Cells marked with the *scn1a* or *oprm1*

probes were counted first, and then those labeled with both probes. The percentage of *scn1a* cells positive for *oprm1* was then calculated. Data from the three histological sections from each mouse were then averaged and taken as the value for that mouse. The investigator taking the confocal images and counting the cells was blinded towards genotype and sex corresponding to each sample.

Intensity of MOR immunofluorescence: A single optical section was chosen in the middle of the confocal stack and MOR staining was measured as the integrated intensity using Fiji/ImageJ. For images taken at 20 $\times$, measures were taken in a region of interest drawn by hand to encompass the entire superficial dorsal horn (laminae I-II). In images at 63 $\times$, measures were taken on the entire optical section. Measures of integrated intensity were taken for pixels over an intensity threshold value of 50 (20 $\times$) or 40 (63 $\times$) in a scale of pixel intensity of 0-255.

Colocalization of two immunofluorescence labels in the dorsal horn: This was measured in whole confocal stacks (voxel-by-voxel) using Imaris, as previously described [27]. Briefly, threshold pixel intensity values were set for each label (40 for MOR, 20 for CGRP, 20 for IB4) and colocalization was measured as the Pearson correlation coefficient of the pixels above this threshold.

2.8 Complete Freund's Adjuvant (CFA) injection

CFA (5 μ l, undiluted, Sigma Aldrich, St. Louis, MO) was injected subcutaneously in the right hind paw using a 50 μ l Hamilton syringe and a 26 G needle, as described [30,43]. The needle was inserted at an oblique angle from the heel in the middle of the paw, near the base of the third toe. It was held in place for 15 s and then gently withdrawn.

2.9 Mechanical allodynia measurements

As an indicator of the hyperalgesia produced by CFA [6,16] or its reinstatement by antagonists [39], we measured mechanical allodynia using von Frey filaments [5]. After 3 days of habituation to the tester and the testing room, mice were habituated for 30 min to acrylic enclosures on an elevated metal grid (10 $\times$ 10 $\times$ 12 cm, IITC Life Sciences). A set of calibrated plastic von Frey filaments ('Touch-Test', North Coast Medical, Inc., San Jose, CA) was then used to measure allodynia by the up-down method. Data were analyzed using the Dixon algorithm [12] to obtain the 50% paw withdrawal threshold (PWT) in grams. Baseline PWT measures were taken for 5 days prior to CFA injection, as well as the day of injection. Then the PWT was measured at days 1, 3, 7, 14, 21, 28, 35, 40, and 52 after CFA [43]. To assess reinstatement of allodynia, mice were given 3 mg/kg s.c. naltrexone (Sigma-Aldrich, St. Louis, MO) or 1 mg/kg s.c. BRL4408 (Tocris Bioscience, Bristol, UK) and PWT were measured at 20, 40, 60, 90 min and 24 h.

The study consisted of two groups: Nav1.8cre/flMOR mice (male, $n=8$; female, $n=6$) or their flMOR littermates (male, $n=7$; female, $n=8$). Sample size was targeted at 16 mice per genotype based on a power analysis of previous data [43], indicating that $n=14$ is needed for 0.8 power and that $n=18$ yields 0.9 power. To study sex differences, each of these two groups was subdivided into males and females. Two cohorts of mice, each consisting of approximately the same number of mice from each group and sex, were ran at different

times. The mice in each cohort were matched by age (30–40 days) and sex. The experimenter was blinded regarding the group to which each mouse belonged.

2.10 Measures of paw edema

The mouse was gently restrained by one investigator while another measured the dorsal-plantar distance of the hind paw at the metatarsal level using digital calipers (Vinca, 0–150 mm, 0.1 mm precision) [32]. Paw edema reported as paw thickness (mm) was assessed at days 0, 1, 3, 7 after CFA injection, and at times 0, 20, 40, 60, 90 and 120 min after NTX injection.

2.11 Data analysis

Data were analyzed using Prism 7.03 and 8.00 (GraphPad Software, San Diego, CA) and expressed as mean $\pm$ standard error. Statistical significance was set at 0.05. Experiments with one variable were analyzed by t-test or one-way ANOVA, as appropriate. Other statistical analyses consisted of repeated measures two-way ANOVA (Table 1) with Holm-Sidak's post-hoc tests and repeated measures three-way ANOVA (Table 2), assuming sphericity.

3. Results

3.1 Decrease in MOR mRNA in dorsal root ganglion (DRG) neurons: RT-qPCR

To determine the decrease in expression of MOR mRNA in nociceptive primary afferents we performed RT-qPCR for *oprm1* (MOR) mRNA [44] in DRG (T13–L6, pooled) and brain cortex of fMOR mice ($n=8$) and Nav1.8cre/fMOR mice ($n=9$). Expression of β -actin was used as the reference. We found that expression of *oprm1* significantly decreased in the DRG of Nav1.8cre/fMOR mice compared with fMOR mice, whereas no differences between these genotypes were found in the cortex (Fig. 1).

3.2 Decrease in MOR mRNA in DRG neurons expressing Nav1.8 channels: *in situ* hybridization

A second measure of the decrease in expression of MORs in nociceptive primary afferents in the Nav1.8cre/fMOR mice relative to their fMOR littermates was *in situ* hybridization. Since the elimination of MORs was targeted to DRG neurons expressing Nav1.8 channels we used *oprm1* (MOR) and *scn10a* (Nav1.8) riboprobes. There was a large number of cell bodies expressing both *scn10a* and *oprm1* in the L2–L6 DRG of the fMOR mice (Fig. 2A), which markedly decreased in the Nav1.8cre/fMOR mice (Fig. 2B). To quantify this decrease and study possible sex differences, DRG (T13–L6) were harvested from four male and four female mice of each genotype. Confocal images were obtained from three DRG histological sections from each mouse. The percentage of cells expressing both *scn10a* and *oprm1* relative to the total number of cells expressing *scn10a* was obtained for each DRG section. Results from the three sections from each mouse were averaged and taken as a value for that mouse. The resulting data were then analyzed by two-way ANOVA (Table 1 row 1). There was a large decrease in the percentage of DRG cells expressing *scn10a* that also expressed *oprm1* in the Nav1.8cre/fMOR mice relative to their fMOR littermates (Fig. 2C). However, a few DRG cells expressing both mRNAs remained in the Nav1.8cre/fMOR mice.

No sex differences were observed, indicating that the elimination of MORs in nociceptive afferents was similar in both sexes.

3.3 Decrease in MORs in primary afferents in the dorsal horn: immunohistochemistry

A guinea pig antibody against MORs (Neuromics GP10106) was used to assess changes in MOR immunoreactivity in the dorsal horn of these mice. This antibody produced a distinct staining of cells and fibers in laminae I and II (Fig. 2) similar to that found in rats [7,40,41]. However, this and other MOR antibodies tested produced artefactual staining of mouse DRG neurons that persisted in global MOR KO mice (data not shown). To assess the elimination of MORs in primary afferents, we studied the colocalization of MORs with CGRP and isolectin B4 (IB4), which label two populations of primary afferents encompassing most nociceptors [42]. Images of the whole dorsal horn taken with a 20× objective showed MOR staining throughout laminae I and II in the fMOR mice (Fig. 2A), whereas in the Nav1.8cre/fMOR mice there was a decrease in MOR staining in a band corresponding to outer lamina II (Fig. 2B). At this magnification, however, MOR integrated intensity in laminae I-II was not significantly different between Nav1.8cre/fMOR mice and their fMOR littermates (Fig. 2C). The difference in MOR staining became conspicuous when using a 63× objective (Fig. 2D, E), with a statistically significant decrease in MOR integrated intensity in Nav1.8cre/fMOR mice compared with fMOR mice (Fig. 2F).

To determine whether the decrease in MOR immunoreactivity occurred in the primary afferents, we measured the colocalization of MOR with CGRP and IB4 in confocal stacks taken at 63×. Qualitatively, there was a decrease in puncta positive for both MOR and CGRP in the superficial dorsal horn of Nav1.8cre/fMOR mice (Fig. 2D, E). Quantitatively, CGRP/MOR colocalization was measured as the Pearson correlation coefficient, which showed a positive colocalization and decreased significantly in Nav1.8cre/fMOR mice compared to their fMOR littermates (Fig. 2G). In contrast, there was no colocalization of MOR and IB4 staining (Fig. 2H, I), with a Pearson correlation coefficient of -0.0004 ± 0.0064 ($n=6$ images from 3 fMOR mice, 2 images/mice). We concluded that in the Nav1.8cre/fMOR mice there is a selective decrease of MORs in CGRP-containing primary afferent fibers in the dorsal horn.

3.4 Mice lacking MORs in nociceptors do not recover from mechanical hyperalgesia

Once we established that MORs were deleted from nociceptors in the Nav1.8cre/fMOR mice, we studied how these mice recovered from CFA-induced hyperalgesia. Nav1.8cre/fMOR mice and their fMOR littermates were injected with CFA in one hind paw. To study sex differences each genotype was subdivided by sex, resulting in four groups of $n=8$. Two female Nav1.8cre/fMOR mice and one male fMOR mouse died during the experiment, reducing the n of these groups to 6 and 7, respectively. Baseline responses to von Frey filaments were measured daily for 5 days. No differences between genotypes and sexes and no drift in the baseline was observed (Fig. 4A, Table 1 row 1). Following injection of CFA (5 μ l s.c., undiluted) in one hind paw all mice developed strong mechanical hyperalgesia. Whereas in the fMOR mice the hyperalgesia subsided back to baseline levels by day 21, in the Nav1.8cre/fMOR mice the recovery was incomplete, with substantial levels of hyperalgesia remaining 52 days post-CFA (Fig. 3A). Two-way ANOVA yielded significant

effects of time, genotype and their interaction, either when data of both sexes were combined (Table 1 row 2), or when data from male and females were analyzed separately (Table 1 rows 3 and 4). To detect any sex differences, the data were separated by sex and analyzed by three-way ANOVA (Table 2 row 1), which yielded no significant effect of sex or any significant interaction of sex with the other two variables, time and genotype. Although PWT measures seemed consistently lower in male relative to female Nav1.8cre/flMOR mice (Fig. 3a), area under the curve analyses indicated no significant sex differences: male 18.6 ± 1.5 ($n=8$), female 26.0 ± 3.7 ($n=6$); t-test, $p=0.063$, $t=2.05$, $df=12$. We concluded that deleting MORs deletion from nociceptive afferents prevents the full recovery from CFA-induced hyperalgesia.

There was, however, a partial recovery from mechanical hyperalgesia in the Nav1.8cre/flMOR mice. To determine whether it was mediated by MORs other than the ones present in nociceptors, mice of both genotypes were injected with the MOR inverse agonist naltrexone (NTX, 3 mg/kg s.c.) on day 40 after CFA. In the flMOR mice, either male (Fig. 3B) or female (Fig. 3C), NTX produced a marked hyperalgesia that disappeared after 24 h. In the Nav1.8cre/flMOR mice, NTX produced no significant effect in the males (Fig. 3B) and a small but statistically significant hyperalgesia in the females (Fig. 3C). When sexes were combined, the difference in the effect of NTX between genotypes was highly significant (Table 1 row 6). Two-way ANOVAs separating sexes yielded significant effect of genotype for males (Table 1 row 7) but not for females (Table 1 row 8). Sex differences were studied using three-way ANOVA (Table 2 row 2), which yielded very significant effects of time, genotype and their interaction. Although there was no significant effect sex, the interaction of sex with genotype and of the three variables was significant. We concluded that in the male Nav1.8cre/flMOR mice the partial recovery from hyperalgesia (Fig. 3A) is not mediated by MORs, whereas in the female Nav1.8cre/flMOR mice this recovery from hyperalgesia could be mediated by MORs located in places other than primary afferents, possibly in dorsal horn neurons or the brain.

To examine the possibility that the partial recovery from hyperalgesia in the Nav1.8cre/flMOR mice is mediated by α_{2A} adrenergic receptors [43], we determined whether it was eliminated by the selective α_{2A} adrenergic antagonist BRL44408 [21,33]. Indeed, BRL44408 (1 mg/kg s.c.) induced a marked increase of hyperalgesia in both the flMOR and the Nav1.8cre/flMOR mice, male and female (Fig. 3D, E). The effect of BRL44408 was significant both when results were combined by sex (Table 1 row 9) or segregated by sex (Table 1 rows 10 and 11). Three-way ANOVA showed that there are no sex differences in the effect of BRL44408 (Table 2 row 3).

3.5 Reinstatement of paw swelling by NTX

The initial injection of CFA in the hind paw produced a notable swelling, which was maximal on day 1 and decreased gradually afterwards (Fig. 5A). This edema was measured as an increase in the dorsoventral thickness of the paw. ANOVA (Table 1 row 12) revealed a significant effect of CFA but not of genotype (flMOR vs. Nav1.8cre/flMOR) or their interaction.

When NTX was injected without a prior injection of CFA, it did not produce any edema in either the fIMOR or the Nav1.8cre/fIMOR mice (Fig. 5B). ANOVA yielded no significant effects of NTX, genotype or their interaction (Table 1 row 13).

However, when NTX was injected to fIMOR mice previously injected with CFA there was a marked edema of the injected hind paw (Fig. 5C, D). This effect occurred a long time after the injection of CFA (up to 80 days), which suggests that in mice with latent sensitization MORs suppress not only hyperalgesia but also swelling of the peripheral tissues. In Nav1.8cre/fIMOR mice there was a remarkable sex difference in the effect of NTX after CFA. NTX produced no edema in the males (Fig. 5C), whereas in the females it produced edema like in the fIMOR mice (Fig. 5D). Two-way ANOVA of the data from male mice (Table 1 row 14) yielded no significant effect of genotype but a significant interaction of genotype with the effect of NTX, whereas in female mice (Table 1 row 15) there was a significant effect of NTX but not of genotype or their interaction.

To confirm this sex difference in the NTX-induced edema, we increased the sample size by adding new cohorts to these four groups of mice. Edema was measured before NTX (0 min) and 60 min after NTX, and data were combined with the same time points of Fig. 5C-D. This confirmed that NTX increased paw thickness in the fIMOR mice, male and female, and in the Nav1.8cre/fIMOR female mice, but not in the Nav1.8cre/fIMOR male mice (Fig. 5E). Results were analyzed by three-way ANOVA (Table 2 row 4), which yielded significant effects of the variables time (effect of NTX) and sex, but not genotype. However, there was a significant three-way interaction between the variables, indicating that the sex difference was related to genotype. Two-way interactions were not significant. In conclusion, NTX-induced edema disappeared in the male but not the female Nav1.8cre/fIMOR mice.

3.6 MOR inhibition of substance P release was eliminated in Nav1.8cre/fIMOR mice

To study the effect of MOR depletion in nociceptors on their central synapses, we evaluated the inhibition of substance P release by the MOR agonist DAMGO [8,18]. Substance P release was evoked by incubating spinal cord slices for 2 min with 10 μ M NMDA + 10 μ M D-serine (D-Ser), in order to activate NMDA receptors in primary afferents that induce substance P release [8,9,23,25,28,31]. Three spinal cord slices per mouse were obtained from fIMOR mice ($n=7$, 3 male, 4 female) and Nav1.8/fIMOR mice ($n=9$, 4 male, 5 female). Mice were sacrificed 53-57 days after CFA injection in the hind paw. Of the three slices obtained from each mouse, one was used as a control, another was incubated for 2 min with NMDA and D-Ser (both 10 μ M), and the third was incubated for 60 min with 1 μ M DAMGO and then for 2 min with NMDA+D-Ser. NK1R internalization in lamina I-II neurons was used as a measure *in situ* of substance P release [8,9,23,26]. In the control slices, NK1R internalization was not significantly different between fIMOR mice and Nav1.8cre/fIMOR (Fig. 6A, $p=0.064$ in the Holm-Sidak's post-hoc test). In the slices incubated with NMDA+D-Ser there was a similar increase in NK1R internalization in both genotypes (Fig. 6A). In the slices preincubated with DAMGO, NMDA-induced NK1R internalization decreased in the fIMOR mice but not in the Nav1.8cre/fIMOR mice (Fig. 6A; two-way ANOVA in Table 1 row 16). Representative images of NK1R-positive dorsal horn neurons illustrating the absence and presence of NK1R internalization are showed in Fig.

6B-G. We concluded that MOR inhibition of substance P release was lost in the Nav1.8cre/flMOR mice.

4. Discussion

We found that mice with a selective depletion of MORs in nociceptive primary afferents do not recover from CFA-induced hyperalgesia. Therefore, this MOR population is required for the suppression of hyperalgesia in the chronic pain state, as represented by the latent sensitization model.

4.1 Validation of the MOR KO in nociceptive primary afferents

Evidence from multiple experimental approaches confirms the selective deletion of MORs in nociceptive afferents in the Nav1.8cre/flMOR mice, as shown by Weibel et al. [44] in a Nav1.8cre/flMOR line generated independently. First, real-time RT-qPCR showed that *oprm1* mRNA was decreased in the DRG but not in the brain cortex of these mice. Second, RNAscope *in situ* hybridization showed that in these mice *oprm1* mRNA was depleted from DRG neurons expressing *scn10a* mRNA for Nav1.8 channels, which are expressed by all nociceptive primary afferents [42]. We found that 14% of the *scn10a* positive DRG neurons also expressed *oprm1* in the Nav1.8cre/flMOR mice, down from 33% in the flMOR mice, indicating a predominant but not total MOR KO in Nav1.8-expressing primary afferents. Third, immunofluorescence double-labeling in the dorsal horn showed a substantial decrease of MOR immunoreactivity in fibers containing the neuropeptide CGRP, which is expressed by peptidergic (PEP1 and PEP2) as well as some non-peptidergic (NP2) primary afferents [42]. In contrast, MOR immunoreactivity in dorsal horn neurons was not changed in the Nav1.8cre/flMOR mice, showing that MOR elimination is specific to the primary afferents. We found that MORs do not colocalize with IB4 staining, which is consistent with reports that MOR immunoreactivity and function is lower in IB4-binding primary afferents [17,46].

In another study [10] MORs were eliminated from nociceptive primary afferents by using TRPV1cre mice instead of the Nav1.8cre mice used by Weibel et al. [44] and by us. Both lines were crossed with the same flMOR mice [44]. Like the Nav1.8cre/flMOR mice, the TRPV1cre/flMOR mice showed a marked reduction of MOR immunoreactivity in CGRP-containing primary afferents. However, the distribution of MOR immunoreactivity in the dorsal horn was somewhat different in these two lines: the TRPV1cre/flMOR mice show a large decrease in MORs in lamina I that we did not find in the Nav1.8cre/flMOR mice. Hence, these two mouse lines do not seem to deplete MORs from the exact same populations of primary afferents.

4.2 Effect of MOR KO in nociceptors on CFA-induced hyperalgesia

The main finding of this study is that mice with the selective MOR depletion in nociceptors do not recover from the mechanical hyperalgesia induced by CFA injection. Although there was a partial recovery, almost two months post-CFA these mice had only recovered to half their baseline withdrawal thresholds. These results are similar to those that we obtained using mice with global MOR KO [43], showing that the MORs that suppress hyperalgesia in latent sensitization are in nociceptive afferents. However, unlike global MOR KO mice,

Nav1.8cre/fMOR mice showed a sex-dependent (in females but not in males) induction of hyperalgesia by NTX, which is used to define the presence of constitutively active MORs [11,43]. This sex difference was not caused by a different extent of deletion of MORs in nociceptive afferents, which was shown to be the same in males and females by *in situ* hybridization. Therefore, we concluded that in male mice the MORs that suppress hyperalgesia are located in nociceptive afferents, whereas in females there is some contribution of MORs located in dorsal horn neurons or supraspinally. More complex phenomena, such as different heterodimerization of MORs and κ -opioid receptors in males and females [24], could also explain our observations.

In contrast to NTX, the α_{2A} adrenergic antagonist BRL44408 produced a robust reinstatement of hyperalgesia in both male and female Nav1.8cre/fMOR mice, confirming that these mice had latent sensitization. Together with a previous study [43], these results indicate that the partial suppression of hyperalgesia in the Nav1.8cre/fMOR mice is mediated by α_{2A} receptors, likely in addition to δ and κ opioid receptors.

4.3 Paw swelling produced by NTX

Another important finding is that NTX induces swelling of the hind paw long after it was injected with CFA. This shows that latent sensitization not only manifests itself as a reinstatement of hyperalgesia but also as reappearance of inflammation. This could be caused by inhibition by NTX of MORs present in peripheral nerve terminals and immune cells in the paw that suppress inflammation. Alternatively, blockade by NTX of MORs in the central terminals of primary afferents could suppress their disinhibition and trigger retrograde signals that produce neurogenic inflammation [20,22]. Both effects could take place: studies of migraine headache show that MORs block distension of meningeal blood vessels by both peripheral and central mechanisms [45]. Interestingly, we found that while NTX produced edema in female Nav1.8cre/fMOR mice with latent sensitization, it did not produce any edema in the males. This indicates that the MORs that suppress inflammation are located in primary afferents in males but not in females. It is possible that in Nav1.8cre/fMOR male mice the inflammation-suppressing role of MORs has been taken over by other receptors [43].

4.4 Effect of MORs in the central synapse of primary afferents: substance P release

A well-known effect of MORs at the central synapse of primary afferents is to inhibit neurotransmitter release [13], in particular that of substance P [8,18,47]. This effect is mediated by the inhibition of voltage-gated Ca^{2+} channels and NMDA receptors in the presynaptic terminals [8,48]. Here we show that MOR inhibition of NMDA receptor-induced substance P release is absent in the Nav1.8cre/fMOR mice. This demonstrates that in these mice there is a loss of MOR function in the central synapse of peptidergic primary afferents, which is likely to contribute to the loss of MOR suppression of hyperalgesia. Thus, maintenance of hyperalgesia in latent sensitization requires the activation of spinal NMDA receptors [11,36] and neurokinin 1 receptors [38]. Furthermore, MORs in primary afferent terminals inhibit NMDA receptors by inhibiting the adenylyl cyclase-protein kinase A pathway [8], which is also essential for latent sensitization [11]. Therefore, the central terminals of nociceptive afferents may be the location of the molecular mechanisms for both

the maintenance of hyperalgesia and its suppression by opioid receptors in latent sensitization.

4.5 Physiological and therapeutic implications

The most important conclusion from this study is that MORs in primary afferents play a crucial role in suppressing hyperalgesia in latent sensitization. If we assume that latent sensitization is a valid model of chronic pain, then these MORs are essential for keeping pain in check in chronic pain disorders. Therefore, using peripherally-restricted MOR antagonists to avoid the development of MOR tolerance and opioid-induced hyperalgesia in patients with chronic pain, as recently suggested [10], may result in an increase in pain. However, this would depend on whether MORs on nociceptors produce this pain-suppressing effect at their central or their peripheral terminals. If pain suppression occurs only at the central terminals, which are protected by the blood-brain barrier, then peripherally-restricted MOR antagonists would not affect it. In fact, Corder et al. [11] found that systemic administration of the peripherally-restricted MOR inverse agonist naltrexone methobromide did not reinstate hyperalgesia in mice with CFA-induced latent sensitization. Together with our results, this indicates that the pain-suppressing effect of MORs occur at the central synapse of primary afferents and not at their peripheral terminals. This seems to be inconsistent with the analgesic role of MORs in peripheral terminals reported by Weibel et al. [44], who found that the peripherally-restricted MOR agonist loperamide alleviated inflammatory hypersensitivity and that this was blocked by the peripheral opioid antagonist naloxone methiodide. However, the study by Weibel et al. was done two days after CFA injection and activated MORs with loperamide, whereas the study by Corder et al. [11] was done in mice with latent sensitization, weeks after CFA injection and without agonist activation of MORs because they are in a constitutively active state [11,43]. Taken together, these observations suggest that MORs in the central synapse of primary afferents are the ones that suppress hyperalgesia in latent sensitization by becoming constitutively active, whereas MORs in the peripheral terminal contribute to the analgesic effect of opiates in the inflammatory state. The neuronal and molecular mechanisms that maintain MORs in the constitutively activated state are still unknown, but locating them at the central primary afferent terminals will facilitate their study.

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Figure 1. RT-PCR shows decreased expression of MORs in DRG neurons from Nav1.8cre/fIMOR mice

RT-PCR with *opmr1* primers was performed with brain cortex (left) and DRG (right) from Nav1.8cre/fIMOR mice and their fIMOR littermates. Data points are different mice, $n=8$ for fIMOR, $n=9$ for Nav1.8/fIMOR. Bars are mean $\pm$ standard error. T-tests (unpaired, two-tailed): DRG, $p=0.0243$, $t=2.503$, $df=15$; cortex, $p=0.6057$, $t=0.527$, $df=15$.

Figure 2. *In situ* hybridization shows that Nav1.8cre/fIMOR mice have decreased expression of MORs in DRG neurons that express Nav1.8 channels

A, B: DRG sections from fIMOR (**A**) or Nav1.8cre/fIMOR mice (**B**) were labeled using RNAscope *in situ* hybridization with *opmr1*-Atto 550 (red) and *scn10a*-Alexa Fluor 488 (green) riboprobes. Confocal images are 7 optical sections at 20 $\times$. Arrows indicate cell bodies positive for both *opmr1* and *scn10a*. **C:** The colocalization of the expression of the *opmr1* and *scn10a* genes was quantified in DGR sections from Nav1.8cre/fIMOR mice and their fIMOR littermates, male and female, 4 mice/group, 3 DRG section/mouse. Data are the number of cells positive to both *opmr1* and *scn10a* divided by the number of *scn10a* positive cells. Results of a two-way ANOVA are in Table 1; p values in the legend are from a Holm-Sidak's post-hoc test comparing genotypes within each sex.

63×, MOR integrated intensity significantly decreased in the Nav1.8cre/flMOR mice. T-tests (unpaired, two-tailed): $p=0.0040$, $t=3.27$, $df=19$. **G:** CGRP/MOR colocalization measured as the Pearson correlation coefficient decreased in the Nav1.8cre/flMOR mice. Values above 0.1 (dotted line) are considered positive colocalization. T-tests (unpaired, two-tailed): $p=0.0007$, $t=4.64$, $df=11$. Data points are images taken from 3 mice of each genotype, 2 images/mice. Bars are mean $\pm$ standard error. **H, I:** MOR and IB4 labeling (63×, single optical sections) of central laminae I-II; there is no visible colocalization. Asterisks (*) indicate MOR neurons.

Figure 4. CFA-induced latent sensitization in Nav1.8cre/flMOR mice

Nav1.8cre/flMOR mice (male, $n=8$; female, $n=6$) or their flMOR littermates (male, $n=7$; female, $n=8$) were injected with 5 μ l CFA in the hind paw. Mechanical hyperalgesia was measured with von Frey filaments and indicated as 50% paw withdrawal threshold (PWT). **A:** Baseline responses were measured daily for 5 days, then CFA was injected (day 0), and responses measured at the times indicated. **B, C:** On day 40, the MOR inverse agonist naltrexone (NTX, 3 mg/kg s.c.) was injected. **D, E:** On day 52, the α_{2A} receptor antagonist BRL44408 (1 mg/kg s.c.) was injected. Two-way ANOVA results for each panel are in Table

1. Holm-Sidak's post-hoc tests: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with fIMOR mice of the same sex (**A**) or with baseline measures (0 min) (**B-E**). Three-way ANOVA results for panel A and combined panels B-C and D-E are in Table 2.

Figure 5. Paw edema produced by NTX in mice with CFA-induced latent sensitization

A: Paw edema (ipsilateral hind paw) produced by injecting CFA to fIMOR mice ($n = 6$, 4 males, 2 females) and Nav1.8cre/fIMOR mice ($n = 6$, 2 males, 4 females). **B:** NTX (3 mg/kg s.c.) did not produce edema in Nav1.8cre/fIMOR mice ($n = 7$) and fIMOR mice ($n = 7$) not injected with CFA in the hind paw. **C:** Male Nav1.8cre/fIMOR mice ($n=8$) and fIMOR mice ($n=7$) were injected with NTX (3 mg/kg s.c.) 53-57 days after CFA and the resulting edema in the ipsilateral hind paw was measured at the times indicated. **D:** Female Nav1.8cre/fIMOR mice ($n=6$) and fIMOR mice ($n=8$) were injected with NTX (3 mg/kg s.c.) 53-57

days after CFA and the edema in the ipsilateral hind paw was measured. The dotted lines indicates average baseline paw thickness. **E:** Other cohorts of flMOR and Nav1.8cre/flMOR mice, male and female, were injected with CFA in the hind paw and then with NTX (3 mg/kg s.c.) 35 days later. Paw thickness measures were taken just before NTX (0 min) and 60 min later, and then aggregated with measures at the same times from panels C and D. Each point is from a different mouse and numbers in the bars are number of mice (*n*). Two-way ANOVA results are in Table 1. Holm-Sidak's post-hoc tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with time 0 days or 0 min of mice of the same genotype. Three-way ANOVA of panel E is in Table 2 row 4.

Figure 6. Loss of MOR inhibition of substance P release in Nav1.8cre/fMOR mice
 Spinal cord slices were prepared from fMOR mice ($n=7$, 3 males, 4 females) and Nav1.8cre/fMOR mice ($n=9$, 4 males, 5 females). Three slices were obtained from each mouse and were assigned one of the following treatments: none (control); 10 μ M NMDA + 10 μ M D-Ser for 2 min (NMDA); 1 μ M DAMGO for 60 min, then NMDA + D-Ser for 2 min (DAMGO+NMDA). **A**. Percentage of neurons in laminae I-II with NK1R receptor internalization. Bars are mean $\pm$ standard error. Each point correspond to a slice from a different mouse. Holm-Sidak's post-hoc tests of repeated measured two-way ANOVA (in

Table 1 row 16): * $p<0.05$, *** $p<0.001$ compared to control for the same genotype, ### $p<0.001$ compared to NMDA for the same genotype. **B-G.** Confocal images from representative histological sections from the slices. Images are 3 optical sections. Main panels were taken with a 20× objective and insets with a 63× objective; scale bars are 100 μm and 10 μm , respectively. Cells with and without NK1R internalization are labeled * and o, respectively.

Table 1

Data analyses by two-way ANOVA

row	Fig.	Data analyzed	groups (n)	variable 1	variable 2	interaction	subject matching
1	2C	<i>In situ</i> hybridization	4 (4, 4, 4, 4)	genotype $p < 0.0001$ $F_{1,12} = 54$	sex $p = 0.757$ $F_{1,12} = 0.10$	$p = 0.670$ $F_{1,12} = 0.19$	(no RM)
2	4A	baseline von Frey (days -4 to 0), all groups	4 (8, 7, 6, 8)	time (CFA) $p = 0.386$ $F_{4,100} = 1.0$	genotype & sex $p = 0.876$ $F_{3,25} = 0.23$	$p = 0.880$ $F_{12,100} = 0.54$	$p = 0.026$ $F_{25,100} = 1.76$
3		CFA von Frey, combining sexes	2 (14, 15)	time (CFA) $p < 0.0001$ $F_{13,351} = 66$	genotype $p < 0.0001$ $F_{1,27} = 47$	$p < 0.0001$ $F_{13,351} = 13$	$p = 0.001$ $F_{27,351} = 2.9$
4		CFA von Frey, male	2 (8, 7)	time (CFA) $p < 0.0001$ $F_{13,169} = 37$	genotype $p < 0.0001$ $F_{1,13} = 44$	$p < 0.0001$ $F_{13,169} = 9.4$	$p = 0.038$ $F_{13,169} = 1.9$
5		CFA, female	2 (6, 8)	time (CFA) $p < 0.0001$ $F_{13,156} = 31$	genotype $p = 0.0047$ $F_{1,12} = 12$	$p < 0.0001$ $F_{13,156} = 4.7$	$p < 0.0001$ $F_{12,156} = 4.6$
6	4B-C	NTX von Frey, combining sexes	2 (14, 15)	time (NTX) $p < 0.0001$ $F_{6,162} = 65$	genotype $p < 0.0003$ $F_{1,27} = 17$	$p < 0.0001$ $F_{6,162} = 21$	$p = 0.018$ $F_{27,162} = 1.8$
7	4B	NTX von Frey, male	2 (8, 7)	time (NTX) $p < 0.0001$ $F_{6,78} = 54$	genotype $p < 0.0006$ $F_{1,13} = 21$	$p < 0.0001$ $F_{6,78} = 27$	$p = 0.013$ $F_{13,78} = 2.3$
8	4C	NTX von Frey, female	2 (6, 8)	time (NTX) $p < 0.0001$ $F_{6,72} = 25$	genotype $p = 0.19$ $F_{1,12} = 1.9$	$p = 0.0022$ $F_{6,72} = 3.8$	$p = 0.34$ $F_{12,72} = 1.14$
9	4D-E	BRL44408 von Frey, combining sexes	2 (14, 15)	time (BRL44408) $p < 0.0001$ $F_{6,162} = 72$	genotype $p < 0.0001$ $F_{1,27} = 104$	$p = 0.0004$ $F_{6,162} = 4.4$	$p = 0.37$ $F_{27,162} = 1.08$
10	4D	BRL44408 von Frey, male	2 (8, 7)	time (BRL44408) $p < 0.0001$ $F_{6,78} = 36$	genotype $p < 0.0001$ $F_{1,13} = 60$	$p = 0.011$ $F_{6,78} = 3.0$	$p = 0.23$ $F_{13,78} = 1.3$
11	4E	BRL44408 von Frey, female	2 (6, 8)	time (BRL44408) $p < 0.0001$ $F_{6,72} = 38$	genotype $p < 0.0001$ $F_{1,12} = 38$	$p = 0.18$ $F_{6,72} = 1.5$	$p = 0.37$ $F_{12,72} = 1.1$
12	5A	CFA-induced edema	2 (6, 6)	time (CFA) $p < 0.0001$ $F_{3,30} = 18$	genotype $p = 0.55$ $F_{1,10} = 0.55$	$p = 0.289$ $F_{3,30} = 1.3$	$p = 0.015$ $F_{10,30} = 4.0$

row	Fig.	Data analyzed	groups (n)	variable 1	variable 2	interaction	subject matching
13	5B	NTX-induced edema (no CFA)	2 (7, 7)	time (NTX) $p=0.382$ $F_{5, 60} = 1.08$	genotype $p=0.117$ $F_{1, 12} = 2.86$	$p=0.838$ $F_{5, 60} = 0.41$	$p<0.0001$ $F_{1, 2, 60} = 6.0$
14	5C	NTX-induced edema after CFA, male	2 (7, 8)	time (NTX) $p=0.0004$ $F_{5, 65} = 5.2$	genotype $p=0.115$ $F_{1, 13} = 2.8$	$p=0.0271$ $F_{5, 65} = 2.7$	$p=0.0008$ $F_{1, 3, 65} = 3.2$
15	5D	NTX-induced edema after CFA, female	2 (8, 6)	time (NTX) $p<0.0001$ $F_{5, 60} = 6.9$	genotype $p=0.161$ $F_{1, 12} = 2.2$	$p=0.732$ $F_{5, 60} = 0.56$	$p=0.042$ $F_{1, 2, 60} = 2.0$
16	6A	NK1R internalization	2 (7, 10)	drugs $p<0.0001$ $F_{2, 28} = 38$	genotype $p=0.0203$ $F_{1, 14} = 6.8$	$p=0.0679$ $F_{2, 28} = 2.96$	$p=0.026$ $F_{1, 4, 28} = 2.4$

ANOVAs were repeated measures (RM) for the variable 'time', except for row 1 (no RM). Details on group composition and sample sizes (n) are given in the corresponding figure legend.

Table 2

Data analyses by three-way ANOVA

row	Fig.	Data analyzed	groups (n)	variables & interactions	p	F	DFn, DfD
1	4A	CFA von Frey, male and female	4 (7, 8, 8, 6)	time (CFA)	<0.0001	66	13, 325
				sex	0.66	0.20	1, 25
				genotype	<0.0001	43	1, 25
				time × sex	0.38	1.07	13, 325
				time × genotype	<0.0001	13	13, 325
2	4B-C	NTX von Frey, male and female	4 (7, 8, 8, 6)	sex × genotype	0.55	0.36	1, 25
				time × sex × genotype	0.20	1.32	13, 325
				time (NTX)	<0.0001	70	6, 150
				sex	0.17	1.95	1, 25
				genotype	0.0003	17	1, 25
3	4D-E	BRL44408 von Frey, male and female	4 (7, 8, 8, 6)	time × sex	0.78	0.54	6, 150
				time × genotype	<0.0001	21	6, 150
				sex × genotype	0.035	5.0	1, 25
				time × sex × genotype	0.017	2.69	6, 150
				time (BRL44408)	<0.0001	73	6, 150
4	5E	NTX-induced edema after CFA, male and female	4 (11, 14, 13, 12)	sex	0.89	0.019	1, 25
				genotype	<0.0001	95	1, 25
				time × sex	0.035	2.32	6, 150
				time × genotype	0.001	3.98	6, 150
				sex × genotype	0.77	0.084	1, 25
				time × sex × genotype	0.96	0.25	6, 150
				time (NTX)	<0.0001	22	1, 50
				sex	0.0013	12	1, 42
				genotype	0.30	1.09	1, 50
				time × sex	0.30	1.10	1, 42
				time × genotype	0.35	0.90	1, 50

row	Fig.	Data analyzed	groups (n)	variables & interactions	p	F	DFn, DFd
				sex × genotype	0.60	0.27	1, 42
				time × sex × genotype	0.0198	5.87	1, 42

ANOVAs were repeated measures for the variable 'time'. Sphericity was assumed. DFn: degrees of freedom numerator; DFd: degrees of freedom denominator.