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Sustained morphine administration induces TRPM8 dependent cold hyperalgesia

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Abstract

It is not uncommon for patients chronically treated with opioids to exhibit opioid induced hyperalgesia (OIH), and this has been widely reported both clinically and experimentally. The molecular substrate for this hyperalgesia is multifaceted, and associated with a complex neural reorganization even in the periphery. For instance, we have recently shown that chronic morphine-induced heat hyperalgesia is associated with an increased expression of GluN2B containing N-methyl-D-aspartate (NMDA) receptors, as well as of the neuronal excitatory amino acid transporter 3/excitatory amino acid carrier 1, in small diameter primary sensory neurons only. Cold allodynia is also a common complaint of patients chronically treated with opioids, yet its molecular mechanisms remain to be understood. Here we present evidence that the cold sensor TRPM8 channel is involved in OIH. After 7 days of morphine administration, we observed an upregulation of TRPM8 channels both by patch clamp recording on sensory neurons and western blot on dorsal root ganglia (DRGs). The selective TRPM8 antagonist RQ-00203078 blocked cold hyperalgesia in morphine treated rats. Also, TRPM8 knockout mice (KO) failed to develop cold hyperalgesia after chronic administration of morphine. Our results demonstrate that chronic morphine upregulates TRPM8 channels, which is in contrast with the previous finding that acute morphine triggers TRPM8 internalization.

Perspective—Patients receiving chronic opioid are sensitive to cold. We now show in mice and rats that sustained morphine administration induces cold hyperalgesia and an up-regulation of TRPM8. Knockout or selectively blocking TRPM8 reduces morphine induced cold hyperalgesia suggesting TRPM8 is regulated by opioids.

Keywords

Cold receptor; whole cell recording; menthol; cold plate; paw lift; 5°C

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Disclosures

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Introduction

Opioids are ideal for controlling acute pain, but poor at resolving chronic pain because of induced neural plasticity. Signs and symptoms of neural plasticity are first noticed when tolerance and opiate induced hyperalgesia (OIH) appear, usually within a few days.^{53, 59} OIH is a paradoxical increase in pain that persists as long as the opiate is administered, even when upping the dose. Animal studies have linked OIH to specific molecular changes involving the N-methyl-D-aspartate (NMDA) receptor, glutamate transporters, adenosine-A1 receptor, serotonin receptor, K⁺/Cl⁻ co-transporter, transient receptor potential vanilloid 1 (TRPV1), ephrinB receptor, intracellular messenger molecules including mammalian target of rapamycin (mTOR), protein kinase A (PKA) and calcium/calmodulin-dependent protein kinase II (CaMKII), as well as epigenetic mechanisms.^{2, 3, 35, 54, 76}

We postulated that the cold channel TRPM8 is also involved in OIH given that cold intolerance is a consistent finding in patients chronically taking opioids as well as during opioid withdrawal.^{13, 18, 37, 39, 75} Here too plasticity must be involved since the first few doses of an opioid produce cold analgesia^{43, 51} and decreased TRPM8 currents,⁷⁹ whereas during withdrawal cold sensitivity and increased TRPM8 currents are observed.^{37, 79} A small dysregulation of TRPM8 could explain an opiate induced cold hypersensitivity given that even small changes in TRPM8 activity will be felt as a change in temperature.⁸⁵

To investigate a possible contribution of TRPM8 to OIH, we monitored behavioral and electrophysiological responses to pharmacologic stimulation and inhibition. TRPM8 KO mice were also used in the current study. Furthermore we determined the protein expression of TRPM8 once OIH was established. Patch clamp recordings were conducted on small diameter neurons in whole DRGs of the rats (*ex vivo* preparation), because most of primary sensory neurons expressing TRPM8 are of small diameters (i.e. < 30 microns).²³

Materials and Methods

Animals

Male Sprague-Dawley rats (180–200 g, Charles River), male TRPM8 knockout mice (Stock NO. 008198, Jackson Laboratory), and wild type mice (Stock No. 000664 C57BL/6J, Jackson Laboratory) were used in the experiments. All animals were housed on a 12-hr light–dark cycle and given food and water *ad libitum*. For each group, 6–25 animals were used based on the experimental procedures. Behavioral analyses were conducted on both rats and mice. Patch clamp recordings and western blots were conducted on rats only. Behavioral testing on the cold plate was done on both rats and mice.

Ethics

Procedures for the maintenance and use of the experimental animals conformed to the regulations of UCSF Committees on Animal Research and were carried out in accordance with the guidelines of the NIH regulations on animal use and care (Publication 85–23, Revised 1996). The UCSF Institutional Animal Care and Use Committee approved the protocols used in this study.

Drugs

Morphine was administered continuously for 7 days using osmotic mini-pumps (2ML1 for rats, and 1007D for mice, Alzet, CA, USA). Briefly, under anesthesia with isoflurane and using sterile techniques, an incision was made on the mid back and a pump was inserted subcutaneously. The pumps delivered sterile morphine sulfate (15 mg/ml, West Ward Pharmaceutical, Illinois, USA) at a rate of 10 μ l/hr in rats or 0.5 μ l/hr in mice. On the 7th day, rats and mice were euthanized and DRGs were collected. The pumps were taken out, and the remaining morphine was measured to ensure that the correct amount had been delivered and that morphine was still being dispensed, i.e. that the pumps were not yet empty and that animals were not in opiate withdrawal at the time of euthanasia. There were no events of empty pumps in the study. Control groups received the same procedures but the osmotic pumps were filled with sterile saline.

The TRPM8 selective antagonist RQ-00203078 (Tocris, USA) was dissolved in DMSO (50 mg/ml) and then further diluted in physiological saline, reaching a final concentration of 3 mg/ml⁷¹. Rats received 3 mg/kg of RQ-00203078 (i.p.) on day 6 immediately following cold plate testing. Rats were tested again on the cold plate 2 hr later as the drug reaches its maximal effect 2 hr after administration.⁷¹

Behavioral studies

An investigator blind to the treatment groups, meaning that the person was unaware of which drug each animal received, conducted the behavioral studies.

We chose the cold plate assay to measure responses to a noxious cold stimulus. This assay was reported almost two decades ago⁴² and has since been used as originally described or with variations.^{44, 46, 47, 70, 84, 86, 87, 93} On each of the testing days, animals were brought into the testing room for 1 hr in order to acclimate them to the environment. The room temperature was kept at $24 \pm 1^\circ\text{C}$. The plate was cooled down to $5 \pm 0.1^\circ\text{C}$. Rats and mice were then placed on the plate for 5 min and scored. This was repeated three times, with a 10 min interval between each test during which they were returned to their cages. While on the plate, normal animals ambulated and explored, but they rarely lifted or shook a paw.^{42, 47} Also, as long as they were acclimated to the cold plate, which took 7 to 10 days, they did not show signs of anxiety (defecating or urinating), or try to escape by attempting to climb on the Plexiglas® walls. The number of paw lifts quantified the response to cold during an observation period of 5 min. After each test, the cold plate was cleaned thoroughly.

Rats and mice freely walking on the 5°C cold plate for 5 min did not exhibit any signs of skin injury, given that this was a mild nociceptive stimulus. In fact, animals could be left 20 min on the plate with no signs of injury.²⁹ To further ensure that this protocol did not cause tissue injury, immediately after 5 min on the 5°C plate we assessed the surface temperature of the hind paws with an infrared thermometer (Fisher Scientific™ Traceable™ Noncontact Infrared Thermometers) in rats (n = 9) and mice (n = 6).

For measuring the mechanical threshold, rats were brought into the testing room and then placed on a grid for 1 hr in order to acclimate. An electronic von Frey anesthesiometer (IITC Life Science) was used to measure the mechanical withdrawal threshold. Briefly, a rigid von

Frey tip was attached to an electronic probe and the weight in grams (g) at which the rodent retracted from the tip was obtained. Each rat was tested 3 times and the mean value was used as the withdrawal threshold. The testing was repeated again 2 hr after RQ-00203078 administration.

Patch clamp recordings

The methods for intact DRG recordings have been described before.^{35, 36} Briefly, rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.). A lumbar laminectomy was performed; the L4 and L5 DRGs were removed and placed into carbogenized artificial cerebrospinal fluid (aCSF). The aCSF contained: 124 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4 , 1.0 mM MgCl_2 , 2.0 mM CaCl_2 , 25 mM NaHCO_3 and 10 mM glucose. The connective tissue surrounding each DRG was carefully removed and ganglia were transferred to a recording chamber, which was perfused with aCSF at a rate of 2–3 ml/min. The temperature of aCSF was maintained at $32 \pm 1^\circ\text{C}$. A fine mesh anchor (SHD-22L, Harvard, USA) was used to stabilize the DRGs during recordings. The surface of each DRG was further locally digested with the enzyme mix LiberaseTM (Sigma-Aldrich, St-Louis, USA) to expose the neurons. The local digestion usually took 20 to 40 min. Patch clamp recordings were then conducted on DRGs within 6 hr after euthanasia.

DRG neurons were visualized with a 40 $\times$ water-immersion objective using a standard microscope (FN-600; Nikon, Japan) equipped with infrared differential interference contrast optics. The image was captured with an infrared-sensitive CCD (IR-1000, Dage MTI, USA) and displayed on a black-white video monitor. Currents were recorded with an Axon 200B amplifier (Molecular Devices, USA) connected to a Digidata interface (Digidata 1322A, Molecular Devices, USA) and low-pass filtered at 5 kHz, sampled at 1 kHz, digitized, and stored using pCLAMP 10.2 software (Molecular Devices, USA). Patch pipettes were pulled from borosilicate glass capillary tubing (BF150-86-10, Sutter, USA) with a P97 puller (Sutter, USA). The resistance of the pipette was 4–5 M Ω when filled with the recording solution, which contained: 140 mM KCl, 2 mM MgCl_2 , 10 mM HEPES, 2 mM Mg-ATP, 0.5 mM Na_2GTP , pH 7.4. The osmolality was adjusted to 290–300 mOsm. After a gigaseal was established on a neuron, the membrane was broken and the cell was selected for further study if it had a resting membrane potential less than -50 mV. The access resistance was 10–20 M Ω and continuously monitored. Data were discarded if the access resistance changed more than 15% during an experiment. Given that most DRG neurons expressing TRPM8 were of small diameter (< 30 μm),²³ small DRG neurons were exclusively selected for patch clamp recording. The size of the each neuron was determined directly by measuring the diameter on the screen.

All drugs were purchased from Sigma-Aldrich except if noted otherwise. Menthol was dissolved in DMSO to reach the concentration of 1M and stored as a stock solution in small vials. The stock solution was diluted to the desired concentration with aCSF immediately before use. L-menthol (100 μM) was applied focally by pressure ejection via a micropipette connected to a Picrospitzer II (puff at 1–2 psi, General Valve, USA) using nitrogen as a gas. The pipette tip was located approximately 50 μm from the recorded neuron so that drugs reached all parts of the neuron. The neurons were clamped at -70 mV to record menthol-

induced currents. Changes in currents greater than 20% of baseline were determined to be inward currents induced by the agonist. To confirm the specificity of menthol induced currents, a second puff of menthol was applied after a highly selective TRPM8 antagonist (RQ-00203078, 100 nM) was bath applied on the same neuron for 10 min. The interval of 10 min was chosen to avoid desensitization of TRPM8 induced by repeated menthol application. The concentration of RQ-00203078 (100 nM) was based on the previous report showing that its IC₅₀ is 5.8 nM for TRPM8, and larger than 10 μ M for TRPA1.⁷¹ The initial menthol induced currents were considered the full response of the TRPM8 channels. The channel inhibition after adding RQ-00203078 and then menthol again was reported as a percentage of the initial menthol current.

Western blots

The L1 to L6 DRGs from each rat were homogenized in lysis buffer (30 mM Tris HCl, 1 mM EGTA, 0.1 mM Na₃VO₄, 10 mM Na₄P₂O₇, 10 mM NaF, pH 7.4) containing the complete protease inhibitor mini EDTA-free (cat #04693159001, Roche Diagnostics) and phosphatase inhibitor cocktail (cat # P0044, Sigma-Aldrich). Tissue homogenates were centrifuged at 23,000 g for 15 min at 4°C.

The bicinchoninic acid (BCA) assay was performed to determine the protein concentration in the cell lysate fraction. For western blot analysis, 20 μ g of protein was separated on a 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked in LI-COR Odyssey Blocking Buffer (LI-COR Biosciences) for 1 hr at room temperature and incubated overnight at 4°C with primary antibodies: monoclonal mouse anti- β -actin (1:10,000, cat #: A2228, Sigma-Aldrich) and rabbit polyclonal anti-TRPM8 (1: 200, cat #: ACC-049, Alomone Lab, Israel). After washing 3 times with phosphate buffered saline containing Tween 20 (PBST), the membranes were incubated for 1 hr at room temperature with a fluorescent secondary antibody (1:15,000, IRDye 680RD polyclonal goat anti-mouse IgG cat #926-68072, or IRDye 800CW polyclonal goat anti-rabbit IgG cat #926-32211, LI-COR, USA). After washing 3 times with phosphate buffer saline (PBS), the membranes were imaged and quantified using the LI-COR Odyssey Infrared Imaging System. For each animal, the expression of TRPM8 was normalized to beta-actin, and then the ratio was compared between control and morphine groups.

Statistical analysis

All results were presented as the mean $\pm$ SEM. For the analysis of cold plate data, repeated-measures one-way ANOVA followed by Bonferroni post hoc or Student's t-test was used. For menthol induced inward currents and western blot analysis, the Student's t-test was used. The Chi-square test was used to compare the responsive percentage of neurons to menthol, as well as the inhibitory rate of RQ-00203078 on menthol-induced currents. Statistical significance was determined at $p < 0.05$.

Results

Morphine administration for 1 week induces cold hyperalgesia

On the cold plate the average number of paw lifts per 5 min prior to treatment was 4.1 ± 0.5 and 3.7 ± 0.4 for the control ($n = 8$) and morphine ($n = 25$) groups respectively. Twenty-four hours (day 1 or D1) after starting the morphine infusion, rats showed a reduced response to the cold plate stimulus, with almost negligible lifts compared to baseline and the saline group ($p < 0.01$, Fig. 1). These data showed that initially morphine had an antinociceptive effect on cold behavior. On days 2 and 3, the number of cold induced paw lifts was back to baseline in all animals, i.e. morphine treatment was no more associated with an antinociceptive effect for the cold stimulus. Starting on day 4, rats receiving morphine showed an increased number of paw lifts (Fig. 1). Over the next few days, the cold pain score continued to increase (Fig. 1). On day 7, in morphine treated rats the average number of paw lifts was up to 13.1 ± 2.5 , significantly more than in the control group (2.6 ± 0.6 , $p < 0.001$, Fig. 1). In control rats, during the 7-day testing period, the number of paw lifts showed a tendency to decrease, although this did not reach significance ($p > 0.05$). The constant response to the cold stimulus in the latter group excludes the possibility that repetitive testing might have induced hyperalgesia.

For all the tested animals, once returned to their cages, none exhibited spontaneous pain behaviors suggestive of tissue damage, such as paw shaking and licking, or limping. To assess if tissue damage could occur during the testing, we measured the temperature of hindpaws in naïve rats after 5 min on the 5°C cold plate. The average hindpaw temperature was $13.7 \pm 0.7^{\circ}\text{C}$ ($n = 9$), which is unlikely to be associated with tissue damage. By comparison, the cold pressor test in humans, which consists of a complete immersion of a hand or forearm in cold water at 0°C to 5°C for 3 to 5 min,⁶³ does not cause any injury even in children after an immersion time of 4 min.^{19, 20, 28}

TRPM8 expression is upregulated in morphine treated rats

Several lines of evidence support that TRPM8 expressing primary sensory neurons are critically involved in cold hyperalgesia in models of somatic or visceral pain.^{48, 81, 92} We therefore did patch clamp recordings of intact DRGs to investigate possible changes of TRPM8 responses in morphine tolerant rats. Small diameter neurons ($< 30 \mu\text{m}$) were selected since TRPM8 mainly localizes in these cells.²³

Inward currents were induced by puff application of L-menthol ($100 \mu\text{M}$, 1s). Menthol is commonly used to stimulate TRPM8 in patch clamp recordings.^{61, 62, 92} TRPM8 channels mediated these currents since they were reduced by $95.1 \pm 3.8\%$ in the morphine group and by $92.7 \pm 2.9\%$ in the control group following bath application of the TRPM8 selective antagonist RQ-00203078 (supplementary Fig. S1, $n = 4$ for each group). The average diameter of responsive small neurons in the saline treated control group was $25.1 \pm 2.7 \mu\text{m}$, while in the morphine group it was $23.9 \pm 3.1 \mu\text{m}$. This difference in diameter between the two groups was not statistically significant ($p > 0.05$). In the morphine treated group, menthol induced currents had an average amplitude of $24.5 \pm 7.1 \text{ pA/pF}$, whereas in the control group the pooled value for inward currents was only $2.7 \pm 0.3 \text{ pA/pF}$ ($p < 0.01$, Fig.

2A). The number of neurons showing inward currents following menthol application in the morphine group was 19 out of 85 (22.4%) recorded neurons (from 11 of 20 morphine treated rats)(Fig. 2B). In the control group, the number of menthol responsive neurons was similar, 12 out of 57 (21.1%) recorded neurons (from 7 of 17 saline treated rats). The Chi square test did not reveal a difference between the two groups ($p > 0.05$). These results indicate that TRPM8 channels are up-regulated following sustained morphine administration, but their expression appears to remain limited to the same sub-population of DRG neurons.

To complement the results obtained with patch clamp recordings, western blots on DRGs were conducted to measure TRPM8 expression at the protein level. On the 7th day, the relative expression level of TRPM8 in the DRGs was increased to 0.029 ± 0.003 in morphine treated rats, which was significantly higher than in the control rats (0.018 ± 0.002 , $p < 0.05$, $n = 7$, Fig. 2C).

Inhibition of TRPM8 blocked morphine induced cold hyperalgesia

To verify that the TRPM8 channel mediated morphine induced cold hyperalgesia, we administered RQ-00203078, a selective antagonist of TRPM8, to both morphine treated and saline control rats. A dose of 3 mg/kg (i.p) RQ-00203078 blocked the cold plate induced paw lifts in both groups. In the morphine treated group, the antagonist reduced the number of paw lifts from 13.1 ± 2.5 to 2.2 ± 0.9 ($p < 0.01$, $n = 8$, Fig. 3). In the saline treated group, the number of paw lifts was reduced from 2.9 ± 1.1 to 0.6 ± 0.3 ($p < 0.01$, $n = 9$).

Sustained morphine administration also induced mechanical hyperalgesia as determined by the response to von Frey hair stimulus ($p < 0.01$, $n = 7$, supplementary Fig. S2). We therefore used the mechanical threshold to determine if RQ-00203078 blocked behaviors other than the cold behavior. In saline treated rats, administration of RQ-00203078 did not affect withdrawal from the mechanical stimulus, which was 25.4 ± 2.1 g (pre-administration) and 26.9 ± 2.2 g (post-administration) respectively ($p > 0.05$). Similarly, administration of RQ-00203078 did not have any significant effect on mechanosensitivity in morphine treated rats. The threshold was 16.9 ± 2.4 g before RQ-00203078 and 16.1 ± 2.5 g after RQ-00203078 (supplementary Fig. S2). Therefore, RQ-00203078 selectively blocked cold hyperalgesia without affecting mechanical hyperalgesia in morphine treated rats.

TRPM8 knock out mice did not develop cold hyperalgesia after sustained morphine administration

TRPM8 KO mice were also tested on the cold plate with wild type (WT) littermates serving as controls. KO and WT mice ($n = 8$ for each group) were implanted with morphine filled mini-osmotic pumps. At baseline (i.e. before morphine was administered), KO mice showed a tendency for reduced nociceptive cold behavior compared with WT, but the difference did not reach significance ($p > 0.05$). The average number of paw lifts on day 1 for KO mice was 0.4 ± 0.2 while that of WT was 1 ± 0.5 (Fig. 4). From days 2 to 7, the average number of paw lift did not change in the KO mice. In contrast, WT mice showed obvious cold hyperalgesia with the number of paw lifts increasing from 2.7 ± 1 to 14.8 ± 1.6 from D2 to D7 (Fig. 4). Starting from D3 until D7, the WT mice showed obvious increased responses to cold plate compared with KO ($p < 0.01$ or 0.001). We also measured the temperature of

hindpaws of the naïve mice after spending 5 min on the 5°C cold plate. The average hindpaw temperature was $16.3 \pm 0.9^\circ\text{C}$ ($n = 6$), implying that just as in rats the cold plate did not cause the mice skin temperature to fall within the range of tissue damaging temperatures. In conclusion, data from knockout mice and pharmacological inhibition indicate that TRPM8 channels are necessary for the appearance and maintenance of cold hyperalgesia during sustained morphine administration.

Discussion

In summary, our data show that chronic opiate induced cold hyperalgesia can be modeled in mice and rats. This behavioral change depends on the cold sensing TRPM8 channel because it failed to develop in KO mice, and menthol consistently induced larger inward currents in sensory neurons from morphine treated rats. A non-selective action of menthol on other channels is unlikely given that its effect was completely blocked by the TRPM8 antagonist RQ-00203078. Finally, the increase of TRPM8 seven days after morphine was started is a further evidence for the involvement of this cold sensor in OIH.

After 5 min the skin temperature of the hindpaws of rats and mice was measured to be $13.7 \pm 0.7^\circ\text{C}$ and $16.3 \pm 0.9^\circ\text{C}$ respectively, within the range of temperatures that activate TRPM8.⁶⁰ In humans when a temperature of -5°C is applied continuously to the skin for 30 sec, the subcutaneous temperatures drop to only about 17°C .⁶⁵ Given that TRPM8 is maximally activated at temperatures between 2 and 32°C ,^{7, 16, 24, 48} it is likely that the 5°C cold plate mainly activated TRPM8.

Activation of TRPM8 channels on primary sensory neurons is critical to sensing cool and noxious cold temperatures, and mediating cold-induced analgesia.⁴⁹ TRPM8 is not alone, as several other ion channels, such as other TRPs and NaV1.9, contribute to cold sensation.^{52, 58} TRPA1 detects noxious cold, and as such TRPA1 KO mice have reduced responses to a temperature of 0°C .⁵² Given that a 5°C stimulus was used here, the TRPA1 channel is less likely to be involved in the observed behavioral changes. The TRPC5 channel is also involved in cold sensation, but mainly activated in non-noxious range ($25\text{--}37^\circ\text{C}$),¹⁰⁰ making it unlikely that it is involved in our model. NaV1.9, a tetrodotoxin (TTX) resistant sodium channel is involved in cold sensation,⁵⁸ and TTX resistant channels, including NaV1.9, are upregulated by morphine or morphine's metabolite M3G.^{11, 27} The response of the TRPM8 channel, however, would not depend on the presence of NaV1.9, since the NaV1.9 KO mice still respond to TRPM8/TRPA1 blockers.⁵⁸ Finally, we can speculate that a downregulation of the TASK-3 leak potassium channel in a subpopulation of TRPM8 neurons⁶⁴ might also be involved in the increased cold sensitivity in morphine treated rats.

It remains that the greater expression of the TRPM8 channels, within a few days of starting morphine, is likely to contribute significantly to the observed cold hyperalgesia. An increase in TRPM8 is documented in different pain models, including models of chemotherapy-induced neuropathy,^{34, 45} experimental colitis,⁴¹ diabetic neuropathy,⁶⁸ and mechanical nerve injury.⁷⁴ The increase in TRPM8 seen after chronic morphine is unique in that it does not involve any injury, therefore the underlying cellular mechanism might be easier to isolate. Data obtained in neoplastic cells, such as prostate and breast cancer, suggest that sex

steroids are involved in TRPM8 expression. In prostate cancer the presence of a presumed androgen response element was demonstrated in the promoter region of TRPM8. Also, putative binding sites for p53 were exposed in the TRPM8 promoter, and over-expression of p53 up-regulates the expression of TRPM8 mRNA.⁶ In breast cancer, TRPM8 expression appears regulated by the estrogen receptor alpha.¹² In addition, several gene transcription factors such as c-Myc, CREB, and Mef-2 bind to the TRPM8 promoter regions.⁹⁹ In a model of chemotherapy induced cold hyperalgesia, the c-Myc inhibitor 10058-F4 blocked the appearance of the abnormal cold sensitivity and the concomitant increase in TRPM8 mRNA. How chronic morphine leads to an increase in TRPM8 expression will have to be determined in future experiments.

The increased menthol induced currents in morphine treated animals could involve greater TRPM8 channel opening time, in addition to an upregulation of TRPM8. Besides being stimulated by cooling and menthol,^{4, 5, 98} the TRPM8 channel is regulated by second messengers. PIP2 in particular, is an allosteric enhancer of TRPM8 at the cytoplasmic surface of the cell membrane.^{25, 40, 56, 69, 77, 78, 83} The association of PIP2 and TRPM8 increases the potency of cold and menthol by several-fold.^{96, 97} How much PIP2 is available at the membrane depends on the amount produced vs. the amount metabolized, which is determined by the activity of PIP2 related enzymes. Morphine might increase PIP2 through a phospholipase D (PLD) pathway⁵⁰ after activating the mu-opiate receptor (MOR). MOR could form a molecular complex with TRPM8 to enable the activation of PLD, similarly to other G protein-coupled receptors (GPCRs),^{15, 32, 72} as was recently shown for 5HT1b and TRPM8.⁸⁹ MOR is critical to OIH⁸² and presumed to be also key to morphine induced cold hyperalgesia. MOR and TRPM8 are mainly expressed in small sized DRG neurons.^{49, 90} One would expect both receptors to co-exist in the same neurons, yet this has never been verified due to lack of commercially specific TRPM8 antibodies. Indirect evidence includes a reported MOR and TRPM8 interaction when both receptors are transfected in HEK293 cells.⁷⁹

Given that TRPM8 mediates cold hyperalgesia during morphine withdrawal,⁷⁹ one could ask if this is the driving mechanism behind the current findings. It is very unlikely though that our animals were undergoing withdrawal given that morphine was constantly administered through osmotic pumps. Another potentially confounding variable in our protocol would be that the daily exposure to the cold plate would itself induce a cold hypersensitivity. Actually, we found that over time control animals appeared to desensitize to the cold stimulus (Fig. 1), ruling out this possibility.

Our behavioral data show that rats quickly became tolerant to the analgesic effect of morphine. On day 1 after being implanted with a morphine pump, rats were unresponsive to the cold stimulus, while on days 2 and 3 they responded similarly to vehicle treated animals. Then, starting on day 4, OIH developed, with both rats and mice showing a progressive increase in the number of paw lifts in response to the 5°C stimulus. The initial antinociceptive effect of morphine on cold behavior is consistent with earlier studies showing that acutely morphine blocks cold pain and induces TRPM8 internalization,⁷⁹ which explains the initial decreased response to cold. In contrast, when given chronically, morphine appears to have the opposite effect, as it induced an upregulation of TRPM8 at the

membrane. This would explain the progressive increase in cold sensitivity. The time difference between the morphine tolerance (day 2) and cold hyperalgesia (day 4) seen here is in accord with the previous suggestion that tolerance and OIH are separate phenomena.¹⁴

Relevance

The CDC guideline for prescribing to chronic pain patients has recently changed in favor of a much more conservative use of opioids.²⁶ It remains that many of the issues encountered after prolonged administration of opioids are also met after short-term administration. For instance, tolerance and OIH appear within days, usually when they are prescribed for post-op or posttrauma pain.^{9, 21, 38, 88} Hence, there is a need to develop new drugs not just to replace but also to complement opioids. TRPM8 agonists, amongst other drug candidates, have the potential to become standard analgesics for neuropathic, cancer, and inflammatory pain.^{17, 22, 33, 55, 73, 80, 91} Yet, patients chronically taking opioids have increased responses to the cold pressor test.^{13, 53} Does this mean that TRPM8 agonists would worsen OIH? TRPM8 mediated increased cold sensitivity might not be an issue since cold allodynia was not reported in clinical trials testing the analgesic effects of menthol.^{17, 80, 91} On the other hand, TRPM8 is associated with pain conditions such as migraine^{8, 10} visceral⁶⁶ and bladder pain,³¹ suggesting that stimulation of this channel could be pro-nociceptive in OIH. Obviously, further studies are needed. Some of the challenges might be to develop peripherally acting (non-brain penetrant) selective ligands for TRPM8 channels, as well as strategies to prevent the appearance of ligand-induced TRPM8 channel desensitization.

Conclusion

Because opioid induced TRPM8 plasticity is a simple and reproducible model, it might open the door to understanding the plasticity associated with other pain conditions in which cold allodynia/hyperalgesia and TRPM8 involvement are reported. Such conditions include complex regional pain syndrome,³⁰ chemotherapy-induced neuropathies,⁹⁵ and painful bladder syndrome.⁶⁷ More broadly, TRPM8 plasticity is of interest to the understanding of a number of diseases with demonstrated upregulation of this channel, including prostate, breast, skin, and primary brain tumors, inflammatory disorders, and pulmonary hypertension.^{1, 57, 94}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sustained morphine administration induces cold hyperalgesia.
- TRPM8 is upregulated in DRGs following sustained morphine administration.
- TRPM8 knockout or pharmacologically blocking TRPM8 reduces cold hyperalgesia.

Fig. 1.

Sustained morphine administration induces cold hyperalgesia. Rats treated with morphine showed analgesia to the cold stimulus on day 1 (D1), but from day 4 (D4) onwards they started showing increased paw lifts in response to nociceptive cold (i.e. hyperalgesia). This increase lasted until the final testing day (D7). ** $p < 0.01$, *** $p < 0.001$. $n = 25$ for morphine group, and $n = 8$ for saline group.

Fig. 2.

Sustained morphine administration induces an upregulation of TRPM8 receptors in DRGs after 7 days. **A.** Patch clamp recordings of small diameter DRG neurons ($< 30 \mu\text{m}$) from whole DRGs reveal that menthol application ($100 \mu\text{M}$, 1s) induces larger inward currents in the morphine treated group. *Upper panel:* representative current traces show menthol induced inward currents in small DRG neurons from the saline (Sal) and morphine (Mor) treated group. *Lower panel:* scatter plot of pooled inward currents. **B.** Bar graph showing no significant difference in the percentage of menthol responsive neurons between saline and morphine treated groups. **C.** Western blot analysis revealed an upregulation of TRPM8 channels in DRGs following sustained morphine administration. *Upper panel:* immunoblots from saline and morphine treated animals. *Lower panel:* statistical analysis shows that the relative expression of TRPM8 in the morphine group is significantly higher compared with saline group. β -actin serves as an internal control. Numbers in the columns stand for sample numbers. *, $p < 0.05$, and **, $p < 0.01$.

Fig. 3.

In vivo administration of the TRPM8 selective antagonist RQ-00203078 (3 mg/kg) inhibits paw lifts during the cold plate test in both saline and morphine treated rats. * $p < 0.05$, ** $p < 0.01$. $n = 8$ for each group.

Fig. 4. TRPM8 knock out (KO) mice receiving sustained morphine administration did not develop cold hyperalgesia. In contrast, wild type (WT) mice receiving morphine developed obvious cold hyperalgesia. WT mice receiving saline serving as control did not develop cold hyperalgesia. ** $p < 0.01$, *** $p < 0.001$. $n = 8$ for each group.