Published in final edited form as:

Pharmacol Res. 2017 May ; 119: 153–168. doi:10.1016/j.phrs.2017.01.033.

Spinal or supraspinal phosphorylation deficiency at the MOR C-terminus does not affect morphine tolerance *in vivo*

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Abstract

The development of tolerance to morphine, one of the most potent analgesics, in the management of chronic pain is a significant clinical problem and its mechanisms are poorly understood. Morphine exerts its pharmacological effects via the μ -opioid receptor (MOR). Tolerance is highly connected to G-protein-coupled receptors (GPCR) phosphorylation and desensitization increase. Because morphine desensitization previously has been shown to be MOR phosphorylation- and β -arrestin2-independent (in contrast to agonists such as fentanyl), we examined the contribution of phosphorylation of the entire C-terminus to the development of antinociceptive tolerance to the partial (morphine) and full (fentanyl) MOR agonists *in vivo*. In MOR knockout (MORKO) mice, we delivered via lentivirus the genes encoding the wild-type MOR (WTMOR) or a phosphorylation-deficient MOR (Cterm(-S/T)MOR) in which all of the serine and threonine residues were mutated to alanine into the ventrolateral periaqueductal grey matter (vlPAG) or lumbar spinal cord (SC), structures that are involved in nociception. We compared the analgesic ED₅₀ in WTMOR- and Cterm(-S/T)MOR-expressing MORKO mice before and after morphine or fentanyl tolerance was induced. Morphine acute antinociception was partially restored in WTMOR- or Cterm(-S/T)MOR-transferred MORKO mice. Fentanyl acute antinociception was observed only in MORKO mice with the transgenes expressed in the SC. Morphine antinociceptive tolerance was not affected by expressing Cterm(-S/T)MOR in the vlPAG or SC of MORKO mice. Fentanyl-induced tolerance in MORKO mice expressing WTMOR or Cterm(-S/T)MOR, is greater than morphine-induced tolerance. Thus, MOR C-terminus phosphorylation does not appear to be critical for morphine tolerance *in vivo*.

Graphical Abstract

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Conflict of interest None.

Chemical compounds: Morphine sulfate: CID: 16051935; Fentanyl hydrochloride: CID: 83932; Ketamine: CID: 3821; Xylazine hydrochloride: CID: 68554; Isoflurane: CID: 3763; Pentobarbital sodium: CID: 23676152; Formaldehyde – Paraformaldehyde: CID: 712.

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Keywords

morphine; tolerance; mu-opioid receptor (MOR); C-terminus phosphorylation; fentanyl

1. Introduction

The analgesic efficacy of opioids in pain relief is unparalleled. However, the inability of opioids to maintain their effectiveness on the long-term highlights the need for more studies related to the molecular mechanisms of opioid tolerance [1]. Opioids used in the clinic exert their analgesic effects mainly through the activation of the μ -opioid receptor (MOR), a GPCR, as shown by the absence of antinociception in MOR-null mice [2,3]. Opioid receptor desensitization, which is defined as an agonist-dependent, progressive reduction in signal transduction after receptor activation [4], appears to be crucial for developing tolerance [5].

Desensitization occurs when serine/threonine protein kinases, such as G-protein receptor kinases (GRKs) specifically phosphorylate the agonist-occupied receptor to disrupt the coupling with its effectors and to increase its binding affinity to cytosolic proteins called β -arrestins. This leads to receptor internalization followed by recycling and resensitization [6]. The robust MOR desensitization evoked by full agonists like fentanyl was found to be GRK phosphorylation-dependent and was proposed to participate in the mechanism of tolerance to these opioids [7,8].

In the case of tolerance to morphine, the prototypic opioid widely used in clinical practice, desensitized MOR does not participate in the traditional pathway of internalization-recycling-reinsertion into the plasma membrane [4] because its lack of internalization [9,5,10]. Indeed, although the absence of morphine tolerance in β -arrestin2-null mice suggested a role for β -arrestin2 in this process [11], several other studies revealed that morphine-activated MOR does not desensitize via receptor phosphorylation and β -arrestin translocation [12–16]. More importantly, blocking MOR phosphorylation enabled high-efficacy opioids (fentanyl) to switch from the β -arrestin-dependent pathway used under normal conditions to the PKC epsilon (PKC ϵ) pathway that is primarily activated by morphine [17,18]. The ability to make high-efficacy opioids - that are known to produce less tolerance than morphine - to function like morphine by controlling the level of MOR phosphorylation suggests that MOR phosphorylation should be the main focus of studies aimed at suppressing tolerance mediated by partial agonists like morphine. MOR

phosphorylation is known to occur at multiple serine/threonine residues in the intracellular carboxyl-terminus (C-term) [19–22].

Systemic morphine acts simultaneously at multiple sites in the nervous system [23]. The spinal and supraspinal sites are known to interact in a supra-additive and synergetic way in morphine antinociception [24,25]. In our study, reinstating morphine antinociception by inserting MOR in the brains or/and spinal cords (SCs) of MOR knockout (MORKO) mice will allow us to test whether there is a difference in the degree of tolerance.

To definitely address the role of MOR C-term phosphorylation in morphine tolerance, a phosphorylation-deficient MOR (Cterm(-S/T)MOR) in which all of the serine and threonine residues in the C-term were mutated to alanine was incorporated into a lentivirus and microinjected into the ventro-lateral periaqueductal grey area (vlPAG) or the lumbar SC of MORKO mice, as these are specific nervous structures that are involved in nociception and pain. The role of C-term phosphorylation in tolerance to morphine and fentanyl was studied by comparing the analgesic effective dose in wild-type (WTMOR)- and mutant MOR-expressing MORKO mice.

2. Materials and methods

2.1. Cell culture

HEK293T cells were grown in advanced Dulbecco's modified Eagle's medium-reduced serum medium (Invitrogen, Carlsbad, CA, USA) with 5% fetal calf serum, 100 units/mL penicillin, and 100 µg/mL streptomycin in a 10% CO₂ incubator. For the lentiviral transductions, virus was applied at a multiplicity of infection of 500 when the cells were 25% confluent.

2.2. Construction and production of the lentiviral vector

The plasmid containing WTMOR was generously provided by Dr. Lee-Yuan Liu-Chen (Temple University, Philadelphia, PA, USA). The plasmid carrying the mutant Cterm(-S/T)MOR was constructed as described [19]. WTMOR and Cterm(-S/T)MOR were cloned into the EcoRI site of the pCDHGFP vector to generate pCDHGFP-WTMOR and pCDHGFP-Cterm(-S/T)MOR, respectively (System Biosciences, Mountain View, CA, USA). The correct gene orientation was confirmed by nucleotide sequencing. All of the plasmids were isolated using QIAGEN's Endotoxin-Free Plasmid Maxi Kit and maintained in 0.3 M sodium acetate/ethanol until ready for use (QIAGEN, Valencia, CA, USA). HEK293T cells were purchased from Open Biosystems. Fifteen-centimeter plates were coated with polylysine for 30 min at 37°C and washed thoroughly with sterilized distilled water. The cells (20 million) were plated on each 15-cm dish 24 h before transfection. On the day of transfection, the plasmids were ethanol-precipitated and dissolved in Tris-EDTA, pH 8.0. Invitrogen's ViraPower Lentivirus Expression System was used to produce lentivirus. Three microliters of Lipofectamine 2000 was used to transfect each microgram of the 4-plasmid mixture: pLP1, pLP2, pVSVG, and pCDHGFP (ratio 3:1:1.5:5). The supernatants were collected every 24 h for 4 days and stored at 4°C. The cell debris was cleared by centrifugation at 3000 rpm for 5 min and the supernatants were then filtered

through 0.45- μ m filters (SCHVU01RE; Millipore, Billerica, MA). To concentrate the lentivirus, 30 mL of the supernatant was transferred to sterilized ultracentrifuge tubes (Beckman Coulter, Brea, CA, USA), and 200 μ L of 60% iodixanol (Sigma-Aldrich) was layered on the bottom of the tube using a metal cannula. After ultracentrifugation for 2.5 h at 4°C, and 55000 g, the virus appeared as a visible band between the clear iodixanol and the red medium. All but the last 3 mL of the medium was then removed from the top. For larger volumes, the ultracentrifugation was repeated until the volume of the suspension was less than 30 mL, and then a final round of ultracentrifugation was carried out at 4°C and 75000 g for 180 min using only 30 μ L of an iodixanol underlayer. All but the last 200 μ L of the supernatant was subsequently removed very carefully [26]. The concentrated lentivirus solution was aliquoted and frozen at -80°C. The lentivirus was titered using HT-1080 cells, and flow cytometry was carried out according to the manufacturer's protocol (K4975-00; Invitrogen). All of the lentivirus used had a titer between 2 and 3 $\times 10^9$ transducing units/mL.

2.3. Animals

All animal care and experimental protocols complied with institutional and international standards (Principles of Laboratory Animal Care, National Institutes of Health), and were approved by the University of Minnesota Institutional Animal Care and Use (Minnesota, USA). Studies involving animals are reported in accordance with the ARRIVE guidelines [27,28]. A total of 156 animals were used in this study. Male wild-type C57Bl/6J mice (30–35 g) were purchased from Charles River. The homozygous MORKO mice were a generous gift from Dr. John Pintar (Robert Wood Johnson Medical School, Piscataway, NJ, USA) and were used for breeding. The mice were housed under a 12-h light/dark cycle at a temperature of 25 $\pm$ 2°C and humidity of 55%. A standard diet and water were provided *ad libitum*.

2.4. Experimental design

The experiments were performed according to a double-blind design.

Experiment 1—As shown in Fig. 1A, before stereotaxic or intrathecal (i.t.) injection of lentivirus, the antinociceptive effect of morphine (5 mg/kg, i.p.) was assessed using the tail-flick (TF) and hot-plate (HP) tests as pretest data. One day later, we delivered lentivirus that expressed WTMOR-GFP or Cterm(-S/T)MOR-GFP into the vIPAG by stereotaxic injection, or into the lumbar SC by direct transcutaneous i.t. injection in MORKO mice. The antinociceptive effects of morphine (5 mg/kg, i.p.) were tested 1 week and 2 weeks after the lentivirus transfer. These tests were designed to determine whether the WTMOR-GFP or Cterm(-S/T)MOR-GFP genes had been expressed enough to show responsiveness to morphine. The wild-type or mutant MOR-GFP-injected MORKO animals that showed significant antinociceptive effects of morphine were used in subsequent experiments. Three weeks after the transfer of WTMOR-GFP or Cterm(-S/T)MOR-GFP into the vIPAG or lumbar SC of MORKO mice, the ED₅₀ of morphine was measured using the up-and-down method that is described in a section below. Two days later, the evolution of morphine tolerance was studied in four groups of mice: wild-type mice (no transgenes) and GFP-, WTMOR-GFP-, or Cterm(-S/T)MOR-GFP-injected MORKO mice. Morphine tolerance was induced by implanting 75 mg morphine pellets into the four groups of mice for 6 days (1

pellet was implanted for the first 3 days and a second pellet was implanted for the last 3 days). The mice were assessed every day for their TF and HP response latencies for 6 days. On the last day of chronic morphine treatment nine hours after the pellets were removed, the morphine ED₅₀ was measured.

Experiment 2—To determine the effect of the phosphorylation-deficiency at the MOR C-term on fentanyl tolerance, we compared the ED₅₀ of fentanyl before and after fentanyl tolerance was induced in mice that had been injected with WTMOR-GFP or Cterm(-S/T)MOR-GFP in the vIPAG or lumbar SC (Fig. 1B). Tolerance was induced by i.p. injections of 60 µg/kg of fentanyl for 6 days. The antinociceptive effect of fentanyl was measured using the TF and HP tests.

2.5. Stereotaxic injections of the lentivector

For microinjections into the vIPAG, the mice (30–35 g) were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine. After mounting the mice on a Stoelting stereotaxic frame (Stoelting, Wood Dale, IL, USA), a midsagittal incision was made to expose the bregma and lambda. With the bregma as zero, the coordinates for the vIPAG were anteroposterior (AP) – 4.48 mm, mediolateral (ML) ± 0.57 mm, and dorsoventral (DV) – 3.30 mm [29]. One microliter of the lentiviral solution was injected (Quintessential Stereotaxic Injector; Stoelting, USA) on each side over a period of 5 min/injection and the cannula was kept in place for another 5 min before it was withdrawn to prevent backflow. The viral solution levels in the cannula tubing were marked before and after injection to confirm virus delivery. The mice received 5 mg/kg i.p. ketoprofen twice daily for antinociception and 20 mg/kg i.p. cefazolin three times daily for antimicrobial prophylaxis after the surgeries.

2.6. Direct transcutaneous intrathecal injection

The i.t. injections were performed as described [30–32]. The animals were first anesthetized for no more than 2 min with a mixture of 3% isoflurane in O₂. Then, a 30-G × 1/2" needle connected to a 50-µL Hamilton syringe (Hamilton, Reno, NV, USA) was inserted through the vertebral column into the subarachnoid space between vertebrae L4 and L5. Puncture of the dura was indicated by a stereotypical TF reflex. Five microliters of the lentivirus were injected.

2.7. Determination of morphine and fentanyl antinociception

Drug-induced antinociception was assessed using the TF and HP tests, which measure tail withdrawal (TF) and paw-licking/shaking and jumping (HP) latencies in response to radiant heat. The intensity of the TF test was set so that the control mice had an average tail withdrawal latency of 2.5–3.5 sec and the cut-off time was 10 sec. The HP was set to 56°C, and the cut off time was 30 sec. The area-under-the-time-response curve (AUC) [33] or the ED₅₀ value was used as an index of the antinociceptive effect of the drug(s). To determine the AUC, the TF or HP latency was recorded 30, 60, 90, 120, and 180 min after i.p. drug administration. The AUC value was obtained by calculating the area-under-the-time-response curve of the antinociceptive effect (test latency - basal latency) 0 to 180 min after the administration of the drugs. The ED₅₀ for morphine or fentanyl antinociception was determined using the up-and-down method described by Dixon [34]. The percent maximal

possible effect (%MPE) was calculated using the formula: (test latency - baseline latency) / (cut off time - baseline latency) $\times$ 100, where 50% MPE was considered to be a positive morphine antinociceptive response. This threshold was instituted because Dixon's up-and-down method to assess ED₅₀ required a binary outcome of positives and negatives. The threshold of 50% MPE was chosen because it represented robust morphine antinociception. This method requires the consecutive testing of mice using doses spaced at constant log intervals. A negative response elevates the dose for the next animal, and a positive result reduces the dose for the next mouse. The log interval was 0.15. The initial dose of morphine used on the first mouse in the sequence was selected based on an estimation of the ED₅₀ and the second dose was either a log interval higher or lower than the first dose depending on the response of the first rodent. Each sequence required between 6 and 9 animals to assess ED₅₀. All of the mice were used only once in each up-and-down sequence. After completing the sequence with 6 to 9 animals, the equation $X_f + k \times d$ was used to calculate the ED₅₀, where X_f was the value of the last dose injected, k was a tabular number obtained from a table reported by Dixon, and d was the interval in log scale [34]. The ED₅₀ of the morphine- or fentanyl-induced antinociception was determined for both morphine- or fentanyl-naïve animals and for mice chronically treated with morphine or fentanyl for 6 days.

2.8. Detection by immunofluorescence of the expression of the wild-type and mutant MOR

The mice were anesthetized with 100 mg/kg pentobarbital sodium and then perfused with Tyrode's solution (116 mM NaCl, 5.36 mM KCl, 2 mM MgCl₂.6H₂O, 0.406 mM MgSO₄.7H₂O, 1.23 mM NaH₂PO₄, 3 mM glucose, 26.2 mM NaHCO₃, pH 7.2), followed by 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (75 mM KH₂PO₄, 85 mM Na₂HPO₄.7H₂O). The brains and SC were post-fixed in 4% paraformaldehyde overnight at 4°C, immersed in 10% and 30% sucrose until they sank to the bottom, and frozen in OCT (Sakura Finetek, Tokyo, Japan). Transversal 30- μ m-thick sections were cut on a cryostat (Leica Microsystems, Wetzlar, Germany). The sections were blocked with 10% normal goat serum (Jackson Immuno-Research, West Grove, PA, USA) and 0.3% Triton X-100 (Sigma-Aldrich) in TBS (25 mM Tris, 3 mM KCl, 140 mM NaCl and 0.05% Tween 20, pH 7.4) for 1 h at room temperature. All of the primary antibodies were diluted in TBS containing 0.3% Triton X-100 and 0.5% BSA. GFP was stained with a 1:1000 dilution of mouse anti-GFP (Life Technologies, Carlsbad, CA, USA), and CGRP was stained with a 1:1000 dilution of rabbit anti-CGRP (GeneTex, Irvine, CA, USA). The sections were incubated with the primary antibodies overnight at 4°C and then washed three times for 10 min each. The secondary antibodies were goat anti-mouse Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) and goat anti-rabbit Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA) both diluted 1:200 in TBS containing 0.3% Triton X-100 and 0.5% BSA. The sections were incubated in secondary antibody for 1 h at room temperature and then washed three times for 10 min each before they were covered with a coverslip using Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Fluorescent images were captured with a Leica DMIRE2 microscope (Leica Microsystems, Wetzlar, Germany) connected to a BD CARVII confocal imager (BD Biosciences, San Jose, CA, USA) and a Hamamatsu EM CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) with laser light sources from Spectral Applied Research (green: 473 nm; red: 561 nm; Richmond Hill, ON, Canada). Successful lentivirus

stereotaxic and i.t. injections into the vIPAG and SC, respectively, were confirmed by immunohistochemical analysis after completion of the behavioral studies.

2.9. Quantitative RT-PCR (RT-qPCR)

RNA from the vIPAG and the lumbar SC was isolated using TRI Reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH, USA). RT-qPCR experiments were performed using QIAGEN's Quantitect SYBR Green RT-PCR kit and the Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA). RT-qPCR was performed according to the same procedure described by [35]. The specific primer sequences for MOR1 were: forward, 5'-AGAGGAAGAGGCTGGGGCG-3' and reverse, 5'-CATACATGACCAGGAAGTTTCCAAAG-3'. The primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: forward, 5'-GGTGAAGGTCGGTGTGAACG-3' and reverse, 5'-CTCGCTCCTGGAAGATGGTG-3' (Invitrogen, Carlsbad, CA, USA). A negative control without cDNA template (called water) was run simultaneously with every assay. To translate Ct values into absolute copy numbers, standard curves were obtained for MOR1 and GAPDH. Constructing the standard curve required a 10-fold serial dilution of templates. The 10-fold serial dilution of the templates was then amplified using the same primers for RT-qPCR MOR1 and GAPDH mRNA abundance analysis. Using the standard curve, Ct values for MOR1 in each sample was translated into absolute copy numbers, and the copy number was then normalized to the absolute quantity of GAPDH from the same sample. For each mRNA sample extracted from the vIPAG or lumbar SC, the RT-qPCR experiment was repeated four times.

2.10. Statistical Analysis

The results were analyzed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). The data are presented as means $\pm$ SEM. Statistical differences between values from 2 groups were determined using unpaired Student *t* tests, whereas statistical differences between 3 or more groups were analyzed using ANOVAs followed by Bonferroni's post hoc comparisons. A difference of $P < 0.05$ was considered statistically significant.

2.11. Chemicals

Morphine sulfate and fentanyl hydrochloride were obtained from the National Institute on Drug Abuse (Bethesda, MD, USA). Ketamine was purchased from Phoenix Pharmaceuticals (Belmont, CA, USA), xylazine was purchased from Lloyd Laboratories (Shenandoah, IA, USA) and isoflurane was purchased from Phoenix Pharmaceuticals. Triton X-100, bovine serum albumin, paraformaldehyde and iodixanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Advanced Dulbecco's modified Eagle's medium-reduced serum medium, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA).

3. Results

3.1. Lentiviral WTMOR-GFP and Cterm(-S/T)MOR-GFP gene transfer and expression in vivo

To monitor the transduction of the wild-type and mutant MOR, we constructed a lentiviral vector that expressed a GFP fusion protein conjugated to the C-term of WTMOR or Cterm(-S/T)MOR and delivered it into the vIPAG by stereotaxic injection, or into the lumbar SC by

direct transcutaneous i.t. injection. The presence of GFP fluorescence confirmed the transgene expression in the vIPAG and SC cells. In the immunofluorescence experiments using a GFP monoclonal antibody, immunoreactivity for GFP was detected bilaterally at the sites of injection into the vIPAG (Fig. 2H) and in the lumbar SC dorsal horn (DH) (Fig. 2A, C and D). To show that GFP is expressed in nociceptive neurons in the superficial layers of the SC DH at the L3-L5 level, GFP and calcitonin gene-related peptide (CGRP), the latter of which is a well-characterized marker for nociceptive neurons within the afferent fiber terminals [36], were double-labeled using a GFP monoclonal antibody and CGRP antiserum, respectively. Although CGRP has been assumed to be of primary sensory origin, since dorsal rhizotomy results in a decrease in CGRP-like immunoreactivity in the superficial layers of the ipsilateral DH, CGRP has also been previously demonstrated to be expressed in a population of DH neurons in the mouse lumbar SC after dorsal rhizotomy and local treatment with colchicine [36]. The colocalization of CGRP-positive cells and GFP immunofluorescence in a WTMOR-GFP-injected MORKO mouse, was observed in the superficial layers of the DH in the L3 spinal cord (Fig. 2C and D). The colocalization of MOR-GFP with CGRP shows that MOR is expressed within the sensory neurons associated with nociception. The stereotaxic and i.t. injection of the lentivirus into the vIPAG or the SC, respectively, resulted in the expression of WTMOR-GFP and Cterm(-S/T)MOR-GFP.

3.2. Morphine induced antinociception in MORKO mice injected with WTMOR-GFP or Cterm(-S/T)MOR-GFP into the vIPAG or lumbar SC

The acute antinociceptive effects of morphine in MORKO mice before and 1 week or 2 weeks after the stereotaxic or i.t. injection of WTMOR-GFP and Cterm(-S/T)MOR-GFP lentivirus are shown in Figs. 3 and 4. The AUC values were calculated from the area-under-the-time-response curve after acute morphine i.p. injection. If the lentivirus-mediated transfer of WTMOR-GFP or Cterm(-S/T)MOR-GFP results in expression in the nociceptive neurons of the vIPAG or the lumbar SC DH, the stimulation of the transferred receptors by morphine should lead to the inhibition of the TF or HP response. The acute basal nociceptive latency in stereotaxically and intrathecally saline-injected MORKO mice was similar to that in the GFP-injected mice as determined by the TF and HP response (Supplementary Information Figs. S1 and S2).

Before gene transfer, it was confirmed that morphine (5 mg/kg i.p.) did not elicit antinociception in terms of the TF ($AUC = 11.2 \pm 2.9 \text{ min} \times \text{sec}$) and HP ($AUC = 35.5 \pm 5.3 \text{ min} \times \text{sec}$) responses in MORKO mice destined to be injected with the lentivirus into the vIPAG. Beginning 1 week after the lentiviral transfer of WTMOR-GFP into the vIPAG, 47% (9/19) of the mice responded to morphine (5 mg/kg i.p.) with an average TF AUC of $87.2 \pm 23.4 \text{ min} \times \text{sec}$ (Fig. 3A) and an average HP AUC of $101.8 \pm 15.3 \text{ min} \times \text{sec}$ (Fig. 3B). One week after the injection of the Cterm(-S/T)MOR-GFP lentivirus into the vIPAG, 44% (8/18) of the mice responded to morphine (5 mg/kg i.p.) with an average TF AUC of $130.6 \pm 25.1 \text{ min} \times \text{sec}$ (Fig. 3C) and an average HP AUC of $85.7 \pm 28.1 \text{ min} \times \text{sec}$ (Fig. 3D). Two weeks after the transfer of the WTMOR-GFP gene into the vIPAG, the percentage of mice responding to morphine increased to 74% (14/19). The average AUCs also increased (Fig. 3A: $132.7 \pm 22.3 \text{ min} \times \text{sec}$; Fig. 3B: $145.7 \pm 32.6 \text{ min} \times \text{sec}$). Two weeks after the transfer of the Cterm(-S/T)MOR-GFP gene into the vIPAG, 12/18 mice displayed acute morphine

antinociception. The average TF (161.1 ± 24.8 min $\times$ sec) and HP (145.3 ± 31.4 min $\times$ sec) AUCs also increased (Fig. 3C and D). Compared to the averages before gene transfer, the AUC averages were significantly higher 1 and 2 weeks after gene transfer (Fig. 3E and F). This significant effect of the time-dependent gene expression on morphine antinociception is revealed by two-way ANOVAs (Fig. 3E: TF $F_{2,70} = 26.15$, $P < 0.001$; Fig. 3F: HP $F_{2,70} = 12.51$, $P < 0.001$). The average AUCs were not significantly different between the 1 and 2 week time points. No significant antinociceptive difference was observed when the average AUCs calculated from the MORKO mice expressing either WTMOR-GFP or Cterm(-S/T)MOR-GFP in the vIPAG were compared (Fig. 3E and F). Fig. 5A and B represent the time courses of the average baseline latencies as well as actual latencies in presence of acute morphine (5 mg/kg, i.p.) of the groups of mice whose AUC values were presented on Fig. 3, 2 weeks after gene transfer. However, only mice that display similar morphine antinociceptive responses (i.e., withdrawal latencies) during chronic morphine treatment (Fig. 6) were included into the analysis and were presented on Fig. 5A and B. Two-way ANOVAs on the withdrawal latencies of WT mice, saline, GFP-, WTMOR-GFP-, and Cterm(-S/T)MOR-GFP-injected MORKO mice revealed a significant “gene/saline transfer $\times$ time” interaction (in addition to significant main effects for “gene/saline transfer” and “time”) with the TF (Fig. 5A: $F_{20,235} = 10.33$, $P < 0.001$) and HP (Fig. 5B: $F_{20,235} = 5.75$, $P < 0.001$) tests. WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice showed significant antinociception in contrast to saline- and GFP-transferred MORKO mice 30 and 60 min after i.p. injection of morphine with the TF (Fig. 5A: $P < 0.001$) and 30 min after i.p. injection of morphine with the HP (Fig. 5B: $P < 0.01$ and $P < 0.05$, respectively). Small variations of the morphine antinociceptive responses were observed within the WTMOR-GFP- and Cterm(-S/T)MOR-GFP-injected groups of MORKO mice.

Before gene transfer, the lack of morphine-induced (5 mg/kg i.p.) antinociception in the MORKO mice destined to be intrathecally injected with lentivirus, was confirmed by the low average TF (9.3 ± 2.2 min $\times$ sec) and HP (29.9 ± 6.2 min $\times$ sec) AUC values (Fig. 4). One week after the i.t. injection of the WTMOR-GFP lentivirus, 52% (12/23) of the mice responded to morphine (5 mg/kg i.p.) as determined by both the TF (112.5 ± 22.9 min $\times$ sec) and HP (98.0 ± 21.8 min $\times$ sec) AUC values (Fig. 4A and B). One week after the i.t. injection of the Cterm(-S/T)MOR-GFP lentivirus, 48% (11/23) of the MORKO mice displayed both TF (average AUC = 103.8 ± 24.2 min $\times$ sec) and HP (average AUC = 121.6 ± 26.3 min $\times$ sec) responses (Fig. 4C and D). Two weeks after the i.t. injection of the WTMOR-GFP or Cterm(-S/T)MOR-GFP lentivirus, the number of mice responding to morphine increased to 73% (17/23) (Fig. 4A) and 87% (20/23) (Fig. 4C), respectively, if only the TF response was taken into consideration. However, the percentage of mice exhibiting both a TF and HP response was only 60% (14/23) in the WTMOR-GFP group (Fig. 4A and B) and 48% (11/23) in the Cterm(-S/T)MOR-GFP group (Fig. 4C and D). The presence of antinociceptive effects of morphine was evidenced by significant increase in the AUC averages in the two groups of mice 1 and 2 weeks after gene transfer into the SC and was confirmed by a significant main effect of “time-dependent gene expression” on the AUC values as shown by two-way ANOVAs (Fig. 4E: $F_{2,88} = 43.54$, $P < 0.001$) and (Fig. 4F: $F_{2,88} = 16.52$, $P < 0.001$). No significant antinociceptive difference was observed when the average of AUCs of the MORKO mice expressing either WTMOR-GFP or Cterm(-S/

T)MOR-GFP in the SC were compared (Fig. 4E and F). Fig. 5C and D represent the time courses of the average baseline latencies as well as actual latencies in presence of acute morphine (5 mg/kg, i.p.) of the groups of mice whose AUC values were presented on Fig. 4, 2 weeks after gene transfer. However only mice that display similar morphine antinociceptive responses (i.e., withdrawal latencies) during chronic morphine treatment (Fig. 7) were included into the analysis and were presented on Fig. 5C and D. Two-way ANOVAs on the withdrawal latencies of WT mice, saline-, GFP-, WTMOR-GFP-, and Cterm(-S/T)MOR-GFP-injected MORKO mice revealed a significant “gene/saline transfer $\times$ time” interaction (in addition to significant main effects for “gene/saline transfer” and “time”) with the TF (Fig. 5C: $F_{20,215} = 7.90$, $P < 0.001$) and HP (Fig. 5D: $F_{20,230} = 4.89$, $P < 0.001$) tests. WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice showed significant antinociception in contrast to saline- and GFP-transferred MORKO mice 30 min ($P < 0.001$) and 60 min ($P < 0.01$) after i.p. injection of morphine with the TF only (Fig. 5C). Small variations of the morphine antinociceptive responses were observed within the WTMOR-GFP- and Cterm(-S/T)MOR-GFP-injected groups of MORKO mice.

3.4. The mutation of MOR phosphorylation sites does not affect morphine tolerance when the transgene is expressed either in the vIPAG or lumbar SC

The evolution of morphine tolerance was studied in four groups of mice: wild-type mice (no transgenes) and GFP-, WTMOR-GFP-, or Cterm(-S/T)MOR-GFP-injected MORKO mice. Three weeks after the transfer of WTMOR-GFP or Cterm(-S/T)MOR-GFP into the vIPAG or lumbar SC, but before tolerance was induced, the ED₅₀ of morphine was measured using the up-and-down method and the threshold for a positive antinociceptive response was set at 50% MPE for both the TF and HP tests (Table 1). The numbers of animals in each group is reduced compared to previous experiments presented on Fig. 3 and 4, because MOR-transferred MORKO mice that did not show a morphine antinociceptive response (i.e., close to zero) or had latencies falling outside the average ranges represented on Fig. 5, were excluded. Morphine ED₅₀ was measured only with mice that displayed similar morphine antinociceptive responses (i.e., nociceptive latencies) (Fig. 5). Before the induction of morphine tolerance, the morphine ED₅₀ values were almost similar in the MORKO mice injected with WTMOR-GFP and in those injected with Cterm(-S/T)MOR-GFP into the vIPAG (Table 1A).

Morphine tolerance was induced by implanting 75 mg morphine pellets into the four groups of mice for 6 days. The mice were assessed every day for their TF and HP response latencies. The time-dependent effect of chronic morphine treatment in the 4 groups of mice was significant (Fig. 6A: $F_{18,228} = 21.66$, $P < 0.001$; Fig. 6B: $F_{18,228} = 7.84$, $P < 0.001$, two way ANOVAs). Although lower than in the wild-type mice, antinociception was observed in the WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice 1 and 2 days after morphine pellet implantation (Fig. 6A and B; $P < 0.001$ on day 1 and 2). Over the course of the next 4 days, the effect of morphine on thermal nociceptive latencies declined to reach the baseline of the control mice (GFP). Nine hours after the pellets were removed, the morphine ED₅₀ values for both the WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice increased about 3-fold and the values for the wild-type mice were increased about 11.5-fold with both the TF and HP (Table 1A). Chronic morphine exposure produced

significant tolerance in both the vIPAG WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice (Table 1A). However, tolerance in these mice was ~3.6- to 3.7-fold less than in the wild-type mice. The morphine ED₅₀ values were not significantly different between the WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice. MOR gene expression was similar between the WTMOR-GFP- and Cterm(-S/T)MOR-GFP-injected mice as verified by RT-qPCR (Fig. 6C). Thus, the absence of phosphorylation at the MOR C-term does not seem to affect morphine tolerance.

Before chronic morphine treatment, the MORKO mice expressing WTMOR-GFP and Cterm(-S/T)MOR-GFP in the lumbar SC had similar morphine ED₅₀ values (51.6 ± 2.9 mg/kg and 52.9 ± 3.4 mg/kg, Table 1B) which were ~22-fold higher than that of the wild-type mice (2.4 ± 0.1 mg/kg, Table 1B). On the first and second days of chronic treatment, morphine induced significant and similar antinociception in both the intrathecally WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice (Fig. 7A; $P < 0.001$) when the mice were tested for TF. However, when the HP test was employed, absence of antinociceptive responses to morphine was observed in the two groups of gene-transferred mice (Fig. 7B). The HP response involves a supraspinally-organized response [37]; thus the antinociceptive action of morphine only at the spinal level may not be enough to induce the same response as that observed in the TF test. After 6 days of chronic morphine treatment, the ED₅₀ of morphine increased about 1.6-fold in the MORKO mice injected with WTMOR-GFP and 1.3-fold in the MORKO mice injected with Cterm(-S/T)MOR-GFP (Table 1B). When comparing the WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice, there was no significant difference in the ED₅₀ of morphine. Equivalent MOR gene expression in mice injected with WTMOR-GFP and those injected with Cterm(-S/T)MOR-GFP was confirmed by RT-qPCR (Fig. 7C). This suggests that the absence of phosphorylation on C-term of MOR does not affect the development of morphine tolerance when the receptors are present in the lumbar SC.

3.5. Measurable fentanyl antinociception before and after tolerance may be mediated by the lumbar SC but not the vIPAG

Fentanyl is a highly efficient opioid that requires low receptor occupancy to exert its maximal analgesic effect [38]. Because fentanyl promotes the efficient GRK-dependent phosphorylation of MOR [8] and shows a reduced propensity to promote tolerance compared to morphine [39], we investigated whether the phosphorylation deficiency at the MOR C-term would exacerbate fentanyl-induced tolerance. As expected, the dose of the drug required to induce 50% MPE found in the wild-type mice was low (Table 2A and B). In the MORKO mice expressing the transgenes in the vIPAG, fentanyl-induced antinociceptive responses were not observed even after injection with a bolus dose of fentanyl (Table 2A).

When WTMOR-GFP and Cterm(-S/T)MOR-GFP were expressed in the SC of MORKO mice, fentanyl elicited measurable antinociception in the TF test (Table 2B). The threshold for a positive antinociceptive response was set at 50% of the MPE for the TF only, because it was observed that significantly higher fentanyl doses were required to reach 50% MPE when the mice were tested for the HP response. Thus, before tolerance was induced, the MORKO

mice injected with WTMOR-GFP displayed a fentanyl ED₅₀ that was slightly higher than the ED₅₀ measured in the Cterm(-S/T)MOR-GFP-transferred MORKO mice (Table 2B). Tolerance was induced by i.p. injections of 60 µg/kg of fentanyl once a day for 6 days. Percent of MPE was represented on the time-response curve because it takes account of the basal latency measured every day before fentanyl injection. The time-dependent effect of chronic fentanyl treatment in the 4 groups of mice was significant (Fig. 8A: $F_{18,120} = 6.65$, $P < 0.001$; Fig. 8B: $F_{18,120} = 7.67$, $P < 0.001$, two way ANOVAs). Although lower than in the wild-type mice, significant fentanyl antinociception was observed in the WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice only the first day of chronic treatment (Fig. 8A; $P < 0.001$ on day 1). On the seventh day, the ED₅₀ of fentanyl was measured. In the wild-type mice, the ED₅₀ increased 4.6-fold. As expected, the tolerance induced by fentanyl in wild-type mice was significantly less developed than that induced by morphine (11-fold increase). In MORKO mice expressing either the wild-type or mutant MOR in the SC, the fentanyl antinociceptive effect could only reach 30 % MPE after the injection of a bolus dose of fentanyl. At this high dose, it was difficult to assess the effect of the C-term MOR phosphorylation mutant on the ED₅₀ of fentanyl after inducing tolerance.

4. Discussion

The main goal of this study was to determine the *in vivo* contribution of MOR phosphorylation at all of the Ser/Thr sites located within the C-term domain of the receptor in morphine and fentanyl tolerance. Opioid tolerance is thought to be a complex process involving the interplay of multiple regulatory agonist-dependent mechanisms [4]. In contrast to what has been classically theorized, opioids tolerance does not result simplistically from only an imbalance of the desensitization-internalization-resensitization cycle that normally allows the renewal of the reactivable pool of receptors on the plasma membrane after a short drug exposure [38]. Tolerance is highly connected to increased MOR desensitization and phosphorylation. Because morphine-induced MOR phosphorylation was not observed in cultured cells expressing serine 363 (S363), threonine 370 (T370), and serine 375 (S375) phosphorylation-deficient MOR [16,17], these Ser/Thr residues in the C-term are logically thought to be the major phosphorylation sites. Morphine promotes minimal phosphorylation on S375, whereas full agonists, such as fentanyl, efficiently phosphorylate T370 and S375 located within the C-term of MOR [40,21]. S375 phosphorylation has been suggested to be important in promoting desensitization, β -arrestin binding, and MOR internalization [19,10]. However, a study that used knockin mice expressing a S375A phosphorylation-deficient MOR revealed that morphine tolerance occurs independently of S375 phosphorylation [41]. Because there is a basal level of S363 and T370 phosphorylation [19] that allows for β -arrestin binding in the absence of morphine, and because morphine can still desensitize in the absence of S363, T370, and S375 phosphorylation [16], the presence of the entire MOR C-term does not exclude the involvement of other residues in MOR phosphorylation/desensitization and β -arrestin binding.

In our study, mutating all of the phosphorylation sites in the C-term of MOR did not affect the development of morphine tolerance (Table 1). This implies that MOR phosphorylation at all of the Ser/Thr sites in the C-term is not critical for morphine tolerance *in vivo*. Because opioids can induce the translocation of the β -arrestin-GFP from the cytosol to the plasma

membrane when MOR is truncated at its C-term [42], it is possible that morphine-activated MOR recruits β -arrestin2 without being phosphorylated [43]. Also, because studies on GPCRs suggest that β -arrestins bind to the second and third intracellular loops [44,45,46], the role of other phosphorylated or unphosphorylated domains, beside the C-term in morphine desensitization needs to be considered. In our study, we could not decipher directly the role of β -arrestin2 in morphine tolerance. The use of double MOR/ β -arrestin2 knockout mice would have been more suitable.

The existence of data establishing that internalization is not necessary for dephosphorylation or recovery from MOR desensitization [47,21] raises questions regarding the importance of β -arrestin2, and consequently MOR internalization, in morphine tolerance. One study in which the short (<30 min) agonist treatment paradigm should minimize the roles of receptor internalization and resensitization in the desensitization process suggests that morphine-induced MOR desensitization is β -arrestin2-independent [16]. A PKC α , γ , ϵ -dependent mechanism that is different from that induced by full agonists has been proposed to mediate morphine desensitization and tolerance and to explain the differences in the degree of tolerance observed with different agonists [48]. If blocking MOR phosphorylation at S363, T370, and S375 after activation by full agonists can switch their signaling mechanism from a β -arrestin2-mediated pathway to a PKC ϵ -mediated pathway [17], then increasing MOR phosphorylation at these sites after morphine activation could be used as a tool to reduce morphine-induced tolerance. This also implies that MOR phosphorylation by PKC ϵ must occur at different residues other than S363, T370, and S375. Indeed, in contrast to what was observed following the inhibition of PKC ϵ [17], the transfection of a S363, T370, and S375 phosphorylation-deficient MOR did not affect morphine desensitization. Moreover, in our study, phosphorylation-deficiency at the MOR C-term did not alter morphine tolerance. These observations led to the hypothesis that different sites located outside of the MOR C-term may be directly or indirectly phosphorylated by PKC ϵ in order to achieve morphine desensitization. This was supported by the finding that the Gai2 protein subunit associated with MOR was phosphorylated by morphine-activated PKC ϵ [49].

Because fentanyl desensitization and tolerance are phosphorylation-dependent [8,17], we initially expected to observe an attenuated tolerance to fentanyl in MORKO mice spinally injected with the mutant MOR compared to those injected with WTMOR-GFP. However, the ED₅₀ could not be assessed after chronic treatment, because both groups equally exhibited 30% MPE with a bolus dose of fentanyl (Table 2B). Because our criterion was to compare the doses at at least 50% of the MPE, we cannot draw any conclusions regarding the effect of MOR mutation on the development of fentanyl tolerance. Also, if the fold of fentanyl tolerance was too great to measure, it might be because of the use of a high dose of fentanyl for chronic treatment. Using a lower dose of fentanyl to treat the mice chronically may provide data on the fold change of the ED₅₀ in fentanyl tolerant mice with the TF test. Surprisingly, before tolerance was induced, the injected mice displayed measurable acute fentanyl antinociception when the WTMOR-GFP and Cterm(-S/T)MOR-GFP genes were transferred only to the SC (Table 2). Microinjection of fentanyl into supraspinal structures such as the vIPAG is known to produce antinociceptive responses in the HP [50]. The absence of fentanyl antinociception observed when the MOR transgenes were expressed only in the vIPAG may be due to the pharmacokinetics of fentanyl. Because fentanyl is more

lipid-soluble than morphine, it crosses the blood-brain barrier faster. However, the duration of action of fentanyl in the nervous system after a single dose is significantly shorter than that of morphine due to its rapid redistribution from the brain to the body, rather than its rapid metabolism and excretion [51–54]. The intensity of the antinociceptive effect of fentanyl correlates with the concentration of the drug at the site of action in the nervous system, and not necessarily with the concentration of the drug in plasma. However, fentanyl accumulates after large or several smaller doses because redistribution is less effective in removing fentanyl from its site of action in the brain [55]. In our study, we observed a difference between spinal and supraspinal antinociceptive responses to fentanyl in WT naïve mice a difference that was not observed with morphine. Indeed, doses of fentanyl significantly higher than 60 µg/kg, i.p. were needed to determine the ED₅₀ (187 ± 9.0 µg/kg) in WT naïve mice subjected to the HP test to assess supraspinal-mediated antinociception, compared with the doses required to determine the ED₅₀ (13.0 ± 3.1 µg/kg) in mice subjected to the spinal antinociceptive TF test (Table 2C). Other studies reported that the fentanyl ED₅₀ (~182 µg/kg) in WT naïve C57BL/6J mice tested with the HP test was also significantly higher than that of mice which underwent the TF test (~24 µg/kg) [56,57]. Similar observations were made with the MORKO mice transferred with WT and mutant MOR genes to the SC. For these reasons, in this study we measured tolerance to fentanyl with the TF test only. However, we obtained the same ED₅₀ for morphine (2.4 ± 0.1 mg/kg, Table 1) with both the TF and HP tests. Interestingly, these observations imply that, depending on the dose, the systemic effect of fentanyl in WT individuals could be mostly at the spinal level. Because the doses of fentanyl necessary to determine the ED₅₀ with the HP test in WT mice were 8 to 14 times higher than those required with the TF test, and because MOR expression is only restricted to the vlPAG in MOR-transferred MORKO mice, extremely high doses of systemic fentanyl may be needed to accumulate at a small anatomical supraspinal structure, such as the vlPAG and to act on MOR to produce antinociception in MOR-transferred MORKO mice. Direct microinjection of fentanyl into the vlPAG expressing the MOR transgenes in MORKO mice could definitively address whether the rapid redistribution of fentanyl explains the absence of nociception in supraspinally MOR-transferred MORKO mice.

In the MORKO mice, the expression of wild-type and mutant MOR transgenes in one key nociceptive structure at a time, whether it is the vlPAG or the SC, did not fully restore morphine antinociception to the levels observed in the wild-type mice (Table 1). Indeed, before chronic treatment, the morphine ED₅₀ in the MORKO mice injected with WTMOR-GFP or Cterm(-S/T)MOR-GFP into the vlPAG was ~13–14-fold higher than in the wild-type mice, and the morphine ED₅₀ in the MORKO mice injected in the SC was ~22-fold higher. The ability of morphine to induce better antinociception when the receptor was expressed in the vlPAG compared to when it was expressed in the SC can be explained by the ability of the PAG to modulate the ascending nociceptive transmission and to exert a powerful descending inhibition of the spinal nociception [58]. Indeed, in normal conditions, the neurons of the PAG express opioid receptors that, when they are activated by endogenous opioids, stimulate the serotonergic neurons of the RVM that directly project to the spinal DH. Activation of the neurons in the descending pathway stimulates the release of serotonin and noradrenaline from their axons at the spinal level, which in turn induces secretion of

enkephalins from the interneurons of laminae I, II, and V [59,60,58]. These endogenous opioids will pre- and postsynaptically inhibit the primary afferents projecting to the DH via opioid receptors, resulting in analgesia [59,61,58]. Thus, the action of morphine on MOR in the vIPAG can inhibit both spinal and supraspinal nociception and is more efficient than when MOR is expressed in the SC. The injection of MOR into both the vIPAG and lumbar SC sites was not sufficient to fully restore normal morphine antinociception in the gene-transferred MORKO mice (Supplementary Material Figs. S3). This is not surprising because, in contrast to wild-type mice, the MORKO mice did not have MOR delivered and expressed in a cell type-specific manner in all the other parts of the ascending and descending pathways in the SC or in supraspinal regions such as the rostral ventromedial medulla (RVM), cerebral cortex, striatum, the locus coeruleus and the peripheral sensory systems [59]. The involvement of nervous regions other than the vIPAG and DH in morphine antinociception could also explain the difference in the degree of tolerance induced by chronic morphine treatment between WT mice and MORKO mice injected with the transgenes (Table 1). The degree of tolerance was 11.3-fold higher in the WT mice, whereas it was only 3.1-fold higher in MORKO mice when WTMOR-GFP or Cterm(-S/T)MOR-GFP were expressed in the vIPAG, and 1.3- to 1.6-fold higher when the transgenes were expressed in the lumbar SC (Table 1). Compared with the WT mice, tolerance was less developed in both the WTMOR-GFP- and mutant MOR-transferred MORKO mice. However, it should be noted that morphine antinociception was already low in the WTMORGFP- and Cterm(-S/T)MOR-GFP- transferred MORKO mice compared with the WT mice, as indicated by higher ED₅₀ values in the MOR-transferred MORKO mice before chronic morphine treatment (Table 1). In addition, the time courses of Figs. 6A and B indicate that the MPE of morphine was 2.1- to 2.4-fold lower in WTMORGFP- (i.e., $42.2 \pm 6.4\%$ or 6.2 ± 0.4 sec with the TF test) and mutant MOR-transferred (i.e., $37.3 \pm 3.5\%$ or 5.6 ± 0.3 sec with the TF test) MORKO mice after one day of chronic morphine treatment compared with WT mice (i.e., $89.2 \pm 6.9\%$ or 9.3 ± 0.5 sec with the TF). Similar results were observed with the HP test (Fig. 6B). Because of this reduced morphine antinociception, the nociceptive sensitivity of MOR-transferred MORKO mice reached the baseline faster than the WT mice, after 3 days of morphine sustained-release (Fig. 6A). Because the ED₅₀ values were already high, the window for the antinociceptive effects of morphine was narrowed in the MORKO mice injected with the transgenes. Because MOR expression occurs at one anatomical site, saturation of the available receptors by the drug could occur faster; the degree of tolerance in MOR-transferred MORKO mice should only reflect what is happening at that site. Furthermore, we cannot exclude the role of C-term phosphorylation of MOR in opioid tolerance when regions other than the vIPAG and DH are involved.

When the transgenes were expressed in the lumbar SC, the degree of tolerance induced by chronic morphine treatment in the MORKO mice was significantly less developed (i.e., 1.3- to 1.6-fold) than in the WT mice (i.e., 11.3-fold) (Table 1B), while the tolerance induced by chronic fentanyl treatment was significantly greater (Table 2B). This suggests that tolerance to systemic morphine in WT mice may mainly occur in the descending pathways from supraspinal regions in addition of the vIPAG, while tolerance to systemic fentanyl may mainly involve spinal mechanisms or the ascending pain pathways. As stipulated above, fentanyl antinociception was partially restored only after intrathecal injection of MOR and

not after injection into the vIPAG. And chronic fentanyl treatment eliminated fentanyl antinociceptive activity. This reinforces the hypothesis of the SC being the critical anatomical structure for systemic fentanyl antinociception. Also, regardless of the phosphorylation state of the receptor, fentanyl tolerance will develop. This supports the notion that β -arrestin2 can be recruited upon fentanyl treatment and is the key for fentanyl tolerance.

5. Conclusions

Besides the fact that differential opioid tolerance may occur in the ascending and descending pain pathways, our study suggests that morphine tolerance may not require the phosphorylation of MOR at all of the Ser/Thr of the C-term domain at the vIPAG and lumbar SC *in vivo*. Studying the effect of C-term phosphorylation-deficient MOR in the development of morphine tolerance in double MOR/ β -arrestin2 knockout cells or mice could definitely address the role of β -arrestin2 in morphine desensitization and tolerance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institutes of Health DA011806 and DA031442, USA.

Abbreviations

MOR	μ -opioid receptor
C-term	C-terminus

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Fig. 1.

Experimental design. Tests for the antinociceptive effects of (A) morphine and (B) fentanyl. TF, tail-flick test; HP, hot-plate test.

Fig. 2.

GFP expression in the vlPAG and its colocalization with CGRP in the SC of gene-transferred MORKO mice. (A–G) Representative fluorescent micrographs of SC transverse sections 1 week after the intrathecal injection of WTMOR-GFP lentivirus (A–D) or saline (control) (E–G) are shown at $\times 20$ magnification. (A) Photomicrograph of the DH superficial layers labeled with a monoclonal GFP antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG in a WTMOR-GFP-expressing mouse. (B) Photomicrograph of the same mouse and section in (A) labeled with CGRP antiserum and Alexa Fluor 594-conjugated goat anti-rabbit IgG. (C) Merge of the two images in (A and B) showing the colocalization of GFP and CGRP in neurons in the DH of a WTMOR-GFP-expressing mouse (arrows). (D) High-magnification ($\times 60$) photomicrograph of the same mouse and section in (C) showing the colocalization of GFP and CGRP in neurons of the laminae I–III of the DH (arrows). (E) Photomicrograph of the DH superficial layers showing an absence of labeling by a

monoclonal GFP antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG in a saline-injected mouse. (F) Photomicrograph of the same mouse and section in (E) labeled with CGRP antiserum and Alexa Fluor 594-conjugated goat anti-rabbit IgG. (G) Merge of the two images in (E, F) showing no colocalization between GFP and CGRP in neurons in the DH of a saline-injected mouse. (A–G) Scale bars: 30 μm . (H) Representative fluorescent micrographs of the viPAG 1 week after local injection of the GFP lentivirus are shown at $\times 5$ magnification. Scale bar: 200 μm .

Fig. 3.

Acute antinociceptive effect of morphine before and after the transfer of the WTMOR-GFP and Cterm(-S/T)MOR-GFP genes to the vIPAG of MORKO mice. The AUC evolution for morphine (5 mg/kg i.p.) was measured using the TF (A) and HP (B) tests before, and 1 or 2 weeks after, the stereotaxic injection of WTMOR-GFP into the vIPAG. The AUC evolution for morphine (5 mg/kg i.p.) was measured using the TF (C) and HP (D) tests before, and 1 week or 2 weeks after, the stereotaxic injection of Cterm(-S/T)MOR-GFP into the vIPAG. A comparison of the average AUC values in the WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice before, and 1 or 2 weeks after, stereotaxic injection into the vIPAG showed no differences between the two groups when morphine antinociception was assessed using the TF (E) and HP (F) tests. Significant differences relative to before gene transfer were determined using one-way ANOVAs, followed by Bonferroni's post hoc comparisons. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ vs before gene transfer in the

WTMOR-GFP group; ### $P < 0.001$ and ## $P < 0.01$ vs before gene transfer in the Cterm(-S/T)MOR-GFP group.

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Fig. 4.

Acute antinociceptive effect of morphine before and after the transfer of the WTMOR-GFP and Cterm(-S/T)MOR-GFP genes to the lumbar SC of MORKO mice. The AUC evolution for morphine (5 mg/kg i.p.) was measured using the TF (A) and HP (B) tests before, and 1 or 2 weeks after the i.t. injection of WTMOR-GFP into the lumbar SC. The AUC evolution for morphine (5 mg/kg i.p.) was measured using the TF (C) and HP (D) tests before, and 1 or 2 weeks after the intrathecal injection of Cterm(-S/T)MOR-GFP into the lumbar SC. A comparison of the average AUC values in the WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice before, and 1 or 2 weeks after intrathecal injection into the lumbar SC showed no differences between the two groups when morphine antinociception was assessed using the TF (E) and HP (F) tests. Significant differences relative to before gene transfer were determined using one-way ANOVAs, followed by Bonferroni's post hoc comparisons. *** $P < 0.001$ and * $P < 0.05$ vs before gene transfer in the WTMOR-GFP group; ### $P < 0.001$ and ## $P < 0.01$ vs before gene transfer in the Cterm(-S/T)MOR-GFP group.

Fig. 5.

Time courses of the effect of acute i.p. injection of morphine (5 mg/kg) on the nociceptive response latencies 2 weeks after the transfer of the WTMOR-GFP and Cterm(-S/T)MOR-GFP genes to the vIPAG (A, B) and lumbar SC (C, D) of MORKO mice. The nociceptive response latencies were assessed using the TF (A, C) and HP (B, D). Points and whiskers are means $\pm$ SEMs. Significant differences relative to the baseline were determined using one-way ANOVAs, followed by Bonferroni's post hoc comparisons. *** $P < 0.001$ and ** $P < 0.01$ vs baseline in the WTMOR-GFP group; ### $P < 0.001$ and # $P < 0.05$ vs baseline in the Cterm(-S/T)MOR-GFP group; and ††† $P < 0.001$ and † $P < 0.05$ vs baseline in the WT group.

Fig. 6.

(A, B) Development of morphine tolerance in WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice 3 weeks after stereotaxic injection into the vlPAG. The points and whiskers represent the means $\pm$ SEMs. A time course was carried out to determine the morphine antinociceptive responses during 6 days of chronic treatment with morphine (75 mg pellet every 3 days) in four groups of mice: wild-type (WT, no transgenes, n = 6) and GFP- (n = 9), WTMOR-GFP- (n = 14) and Cterm(-S/T)MOR-GFP- (n = 13) transferred MORKO mice. The morphine antinociceptive responses were measured using the TF (A) and HP (B) tests. Significant differences relative to before chronic morphine treatment were determined using one-way ANOVAs, followed by Bonferroni's post hoc comparisons. *** $P < 0.001$ vs before chronic treatment in the WTMOR-GFP group; ### $P < 0.001$ vs before chronic treatment in the Cterm(-S/T)MOR-GFP group; and ††† $P < 0.001$ and †† $P < 0.01$ vs before chronic treatment in the WT group. (C) RT-qPCR showing the level of MOR1 gene

expression in the vIPAG of wild-type (WT) naïve, MORKO naïve, GFP-transferred MORKO (MORKO GFP), WTMOR-GFP-transferred, and Cterm(-S/T)MOR-GFP-transferred MORKO mice. MOR1 mRNA levels are expressed relative to GAPDH mRNA levels in the vIPAG. Each value represents the mean $\pm$ SEM of three independent experiments. *** $P < 0.001$ vs WT naïve.

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Fig. 7.

(A, B) Development of morphine tolerance in WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice 3 weeks after i.t. injection into the lumbar SC. The points and whiskers represent the means $\pm$ SEMs. A time course was carried out to determine the morphine antinociceptive responses during 6 days of chronic treatment with morphine (75 mg pellet every 3 days) in four groups of mice: wild-type (WT, no transgenes, n = 6) and GFP- (n = 6), WTMOR-GFP- (n = 14), and Cterm(-S/T)MOR-GFP- (n = 16) transferred MORKO mice. The morphine antinociceptive responses were measured using the TF (A) and HP (B) tests. Significant differences relative to before chronic morphine treatment were determined using one-way ANOVAs, followed by Bonferroni's post hoc comparisons. *** $P < 0.001$ vs before chronic treatment in the WTMOR-GFP group; ### $P < 0.001$ vs before chronic treatment in the Cterm(-S/T)MOR-GFP group; and ††† $P < 0.001$ and †† $P < 0.01$ vs before chronic treatment in the WT group. (C) RT-qPCR showing the level of MOR1 gene expression in the lumbar SC of wild-type (WT) naïve, MORKO naïve, GFP-transferred MORKO (MORKO GFP), WTMOR-GFP-transferred, and Cterm(-S/T)MOR-GFP-

transferred MORKO mice. MOR1 mRNA levels are expressed relative to GAPDH mRNA levels in the lumbar SC. Each value represents the mean $\pm$ SEM of three independent experiments. *** $P < 0.001$ vs WT naïve.

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Fig. 8.

(A, B) Development of fentanyl tolerance in WTMOR-GFP- and Cterm(-S/T)MOR-GFP-transferred MORKO mice after i.t. injection into the lumbar SC. The points and whiskers represent the means of %MPE $\pm$ SEMs. A time course was carried out to determine the fentanyl antinociceptive responses during 6 days of chronic treatment with fentanyl (60 μ g/kg/day i.p.) in four groups of mice: wild-type (WT, no transgenes, n = 6) and GFP- (n = 6), WTMOR-GFP- (n = 6), and Cterm(-S/T)MOR-GFP- (n = 6) transferred MORKO mice. The fentanyl antinociceptive responses were measured using the TF (A) and HP (B) tests. Significant differences relative to before chronic fentanyl treatment were determined using one-way ANOVAs, followed by Bonferroni's post hoc comparisons. *** $P < 0.001$ vs before chronic treatment in the WTMOR-GFP group; ### $P < 0.001$ vs before chronic treatment in the Cterm(-S/T)MOR-GFP group; and ††† $P < 0.001$ and †† $P < 0.01$ vs before chronic treatment in the WT group.

Table 1

Tolerance induced by morphine in mice injected with the wild-type or mutant MOR-expressing lentiviruses. Three weeks after WTMOR-GFP or Cterm(-S/T)MOR-GFP expression in the vIPAG (A) and lumbar SC (B) of MORKO mice, the ED₅₀ values of morphine required to inhibit the TF and HP responses were determined by the up-and-down method before and after chronic treatment with morphine (75 mg pellet every 3 days for 6 days). The values represent the mean $\pm$ SEM (n = 3 batches of 6–7 mice).

A	Tail-flick and Hot-plate	
	ED ₅₀ of morphine (mg/kg)	
	Before treatment	After treatment
WT mice (no transgene)	2.4 $\pm$ 0.1	27.2 $\pm$ 2.2 (11.3-fold) ^{†††}
MORKO mice WTMOR in vIPAG	31.8 $\pm$ 2.6	ns { 98.6 $\pm$ 7.2 (3.1-fold) ^{***}
MORKO mice Cterm(-S/T)MOR in vIPAG	33.4 $\pm$ 2.1	
		102.1 $\pm$ 10.6 (3.1-fold) ^{###}
B	Tail-flick only	
	ED ₅₀ of morphine (mg/kg)	
	Before treatment	After treatment
WT mice (no transgene)	2.4 $\pm$ 0.1	27.2 $\pm$ 2.2 (11.3-fold) ^{†††}
MORKO mice WTMOR in SC	51.6 $\pm$ 2.9	ns { 80.7 $\pm$ 5.4 (1.6-fold) ^{***}
MORKO mice Cterm(-S/T)MOR in SC	52.9 $\pm$ 3.4	
		70.0 $\pm$ 4.4 (1.3-fold) ^{##}

^{***} $P < 0.001$ vs before tolerance in the WTMOR-GFP group;

^{###} $P < 0.001$ and

^{##} $P < 0.01$ vs before tolerance in the Cterm(-S/T)MOR-GFP group; and

^{†††} $P < 0.001$ vs before tolerance in the WT group.

Table 2

(A, B) Tolerance induced by fentanyl in mice injected with the wild-type and mutant MOR-expressing lentiviruses. Three weeks after WTMOR-GFP or Cterm(-S/T)MOR-GFP expression in the vIPAG (A) or lumbar SC (B) of MORKO mice, the ED₅₀ values of fentanyl required to inhibit the TF responses were determined by the up-and-down method before and after chronic treatment with fentanyl (60 µg/kg/day i.p. for 6 days). (C) Comparison of the ED₅₀ values of fentanyl measured with the TF versus HP in naïve WT mice: the fentanyl ED₅₀ assessed with the HP is 14.4-fold higher than the fentanyl ED₅₀ measured with the TF. The values represent the mean ± SEM (n = 6).

A	Tail-flick only	
	ED ₅₀ of fentanyl (µg/kg)	
	Before treatment	After treatment
WT mice (no transgene)	13.0 ± 3.1	59.5 ± 3.2 (4.6-fold) ^{†††}
MORKO mice WTMOR in vIPAG	> 3364	> 3364
MORKO mice Cterm(-S/T)MOR in vIPAG	> 3364	> 3364

B	Tail-flick only	
	ED ₅₀ of fentanyl (µg/kg)	
	Before treatment	After treatment
WT mice (no transgene)	13.0 ± 3.1	59.5 ± 3.2 (4.6-fold) ^{†††}
MORKO mice WTMOR in SC	30.0 ± 2.0	> 3364 (~ 30 % MPE)
MORKO mice Cterm(-S/T)MOR in SC	23.6 ± 1.3	> 3364 (~ 30 % MPE)

C	ED ₅₀ of fentanyl (µg/kg)	
	Tail-flick	Hot-plate
WT mice (naïve no transgene)	13.0 ± 3.1	187.5 ± 9.0 (14.4-fold) ^{†††}

^{†††} $P < 0.001$ vs before tolerance in the WT group.