ANESTHESIOLOGY

Cross-talk between Human Spinal Cord μ-opioid Receptor 1Y Isoform and Gastrin-releasing Peptide Receptor Mediates Opioid-induced Scratching Behavior

Xian-Yu Liu, Ph.D., Yehuda Ginosar, M.B.B.S., Joseph Yazdi, M.D., Alexander Hincker, M.D., Zhou-Feng Chen, Ph.D.

ANESTHESIOLOGY 2019; 131:381–91

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- The spinal administration of opioids can cause intense pruritis
- Interactions between specific μ-opioid receptor isoforms and the gastrin releasing peptide receptor in spinal tissues likely mediate morphine-induced pruritus

What This Article Tells Us That Is New

- Human spinal cord tissue expresses the 1Y isoform of the μ-opioid receptor, and that isoform functionally interacts with the gastrin releasing peptide receptor to cause cellular calcium influx
- Blocking interactions between the 1Y isoform and the gastrin releasing peptide receptor does not reduce opioid analgesia
- Eliminating interactions between the 1Y isoform and the gastrin releasing peptide receptor or reducing 1Y isoform activation may reduce opioid-induced pruritis

ABSTRACT

Background: Although spinal opioids are safe and effective, pruritus is common and distressing. The authors previously demonstrated in mouse spinal cord that interactions between μ-opioid receptor isoform 1D and gastrin releasing peptide receptor mediate morphine-induced scratch. The C-terminal of 1D inhibits morphine-induced scratch without affecting analgesia. The authors hypothesize that human spinal cord also contains itch-specific μ-opioid receptor isoforms which interact with gastrin releasing peptide receptor.

Methods: Reverse transcription polymerase chain reaction was performed on human spinal cord complimentary DNA from two human cadavers. Calcium responses to morphine (1 μM) were examined using calcium imaging microscopy on human cells (HEK293) coexpressing gastrin releasing peptide receptor and different human μ-opioid receptor isoforms. The authors assessed morphine-induced scratching behavior and thermal analgesia in mice following intrathecal injection of morphine (0.3 nmol) and a transactivator of transcription peptide designed from C-terminal sequences of 1Y isoform (0, 0.1, and 0.4 nmol).

Results: The authors demonstrated 1Y expression in the spinal cord dorsal horn. Morphine administration evoked a calcium response (mean ± SD) (57 ± 13 nM) in cells coexpressing both gastrin releasing peptide receptor and the 1Y isomer. This was blocked by 10 μM naltrexone (0.7 ± 0.4 nM; $P < 0.0001$), 1 μM gastrin-releasing peptide receptor antagonist (3 ± 2 nM; $P < 0.0001$), or 200 μM 1Y-peptide (2 + 2 nM; $P < 0.0001$). In mice, 0.4 nmol 1Y-peptide significantly attenuated morphine-induced scratching behaviors (scratching bouts, vehicle *vs.* 1Y-peptide) (92 ± 31 *vs.* 38 ± 29; $P = 0.011$; n = 6 to 7 mice per group), without affecting morphine antinociception in warm water tail immersion test (% of maximum possible effect) (70 ± 21 *vs.* 67 ± 22; $P = 0.80$; n = 6 mice per group).

Conclusions: Human μ-opioid receptor 1Y isomer is a C-terminal splicing variant of *Oprm1* gene identified in human spinal cord. Cross-talk between 1Y and gastrin releasing peptide receptor is required for mediating opioid-induced pruritus. Disrupting the cross talk may have implications for therapeutic uncoupling of desired analgesic effects from side effects of opioids.

(ANESTHESIOLOGY 2019; 131:381–91)

It is more than 40 yr since Snyder's landmark discovery of spinal cord opioid receptors in rats,[1] followed within 3 yr by the clinical description of spinal opioids to treat pain in humans.[2] Spinal opioids have become a mainstay of clinical pain management in labor,[3–5] in perioperative care,[6–11] and in chronic pain management.[12,13] Spinal opioids are frequently coadministered with local anesthetics to provide analgesia requiring lower doses of each agent, thus minimizing most undesirable side effects.[14,15] Most opioid-induced side effects such as nausea, vomiting, and respiratory depression are more commonly observed following systemic administration.[8] By contrast, pruritus is

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). The work presented in this article has been presented at the 50th Annual Meeting of the Society of Obstetric Anesthesia and Perinatology in Miami, Florida, on May 9 to 13, 2018. X.-Y.L. and Y.G. contributed equally to this article.

Submitted for publication August 26, 2018. Accepted for publication March 26, 2019. From the Center for the Study of Itch, Departments of Anesthesiology, Psychiatry and Developmental Biology (X.-Y.L., Z.-F.C.), the Division of Obstetric Anesthesiology, Department of Anesthesiology, Barnes Jewish Hospital (Y.G., A.H.), Washington University School of Medicine, St. Louis, Missouri; the Mother and Child Anesthesia Unit, Department of Anesthesiology, Hadassah Hebrew University Medical Center, Jerusalem, Israel (Y.G.); and SpineMore Surgical Associates, St. Louis, Missouri (J.Y.).

 Anesthesiology 2019; 131:381–91. DOI: 10.1097/ALN.0000000000002776

<zdoi;. DOI: 10.1097/ALN.0000000000002776>

more commonly observed following intrathecal or epidural administration,[16,17] particularly in obstetric patients.[18,19] Spinal opioid-induced pruritus is commonly described for all spinal opioids and for both intrathecal and epidural routes. It is one of the most irritating side effects following spinal opioid administration and markedly decreases patient satisfaction. Nevertheless, the mechanisms by which opioids induce pruritus in human remain poorly understood.

The *Oprm1* gene that encodes the μ-opioid receptor (MOR) comprises dozen of alternative splicing variants or isoforms in rodents and humans, and some might be responsible for physiologic function unrelated to analgesia[20–22] and for individual variability in sensitivity to opioid drugs.[23,24] Most *Oprm1* isoforms differ in the structurally distinct C-terminals, which may underlie their functional differences.[22] Gastrin-releasing peptide receptor (GRPR) is a G_q protein-coupled receptor; it is expressed in the dorsal spinal cord, whereas its endogenous neuropeptide ligand, gastrin-releasing peptide, is expressed in sensory neurons, but not in the spinal cord.[25–27] GRPR is a pivotal receptor in the spinal cord for relaying nonhistaminergic itch transmission from the periphery to the brain.[28–30] Unlike *Oprm1* isoforms, no GRPR splicing variant has been reported in mice or in humans. In mice, we demonstrated that spinal opioids induce pruritus by activating the MOR1D isoform which cross-activates GRPR in the spinal cord GRPR neurons.[31] By contrast, opioids induced analgesia through the MOR1 isoform that is not expressed in GRPR neurons.[31] These findings provide a molecular and neural basis for potentially uncoupling opioid-induced analgesia from opioid-induced itch in the spinal cord. In primates, it has been shown that intrathecal gastrin-releasing peptide induces scratching but does not affect pain sensation.[32,33] Furthermore, aging primates with chronic itch exhibit increased expression of gastrin-releasing peptide in sensory neurons and increased expression of GRPR in the spinal cord.[34] These studies strongly imply that the gastrin-releasing peptide–GRPR itch-specific signaling pathway is evolutionarily conserved between rodents and primates. Nevertheless, it was not clear that this was the mechanism for spinal opioid-induced pruritus in humans.

In this study, we tested the hypothesis that spinal morphine induces pruritus in humans through human *Oprm1* splicing variants that interact with GRPR, in a manner similar to the established interaction between MOR1D and GRPR in mice. We demonstrate the existence of multiple *Oprm1* isoforms in human spinal cord, and our studies suggest that MOR1Y cross-activates GRPR to cause opioid-induced pruritus in humans. These findings have important implications for therapeutic inhibition of pruritus without compromising the efficacy of opioid analgesia.

Materials and Methods

Human Specimens

The study protocol was approved by the Research Advisory Committee of Mid-American Transplant (St. Louis, Missouri). Specimens of human spinal cord were obtained from fresh, cold-stored cadavers. Donors were a 73-yr-old male and a 67-yr-old male. Both had sustained sudden cardiac arrest with resuscitation and subsequent withdrawal of care occurring in early evening, approximately 16 h and 18 h before sample collection, respectively. Both donors had coronary artery disease, hyperlipidemia, type 2 diabetes mellitus, and hypertension. The first donor also had end-stage pulmonary fibrosis, pulmonary hypertension, cardiac failure, renal failure, and chronic steroid medication. A midline posterior incision was made in the thoracolumbar area, carried down to the fascia, and then subperiosteally to expose the lamina, pars, and facet joints. Lamina from T9 to T12 (first specimen) and T8 to T11 (second specimen) were exposed. An oscillating saw was used to cut the lamina laterally at the level of the posterolateral paraspinal gutters. Resection of the lamina was performed using a Kerrison rongeur. On the left side, the pars and facets were resected, exposing the nerve roots to a point beyond their dorsal root ganglion; nerves were cut distal to the dorsal root ganglion. The dura was opened in the midline and the spinal cord was cut proximally and then distally. Sutures were placed to identify the dorsal rostral and dorsal caudal aspects of each specimen. The right sided nerve roots were cut intradurally. A sleeve of dura was left around the nerve roots and the dorsal root ganglion on the left side. The specimens were placed in in RNA stabilizer (RNAlater; QIAGEN, Germany) and kept frozen on dry ice immediately.

Linear-space Similarity Alignment

The linear amino acid sequences alignments of human MOR1 (hMOR1; NP_000905.3), mouse MOR1 (mMOR1;NP_001289722.1), human GRPR (hGRPR; NP_005305.1), and mouse GRPR (mGRPR; NP_032203.1) were performed using William Pearson's lalign program (version 2.1.30).[35]

Identification of Human Oprm1 Isoforms

We designed isoform-specific reverse transcription polymerase chain reaction primers and performed reverse transcriptase polymerase chain reaction using the human spinal cord samples. Sequences of splicing variants specific primers are listed in table 1. Reverse transcriptase polymerase chain reaction was performed as previously described.[31,36] Briefly, total RNA was isolated and genomic DNA was removed in accordance with manufacturer's instructions (RNeasy plus mini kit; QIAGEN). RNA was quantified using Eppendorf BioPhotometer and stored at −80°C. Single-stranded cDNA was synthesized from 1 μg of total RNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) and stored at −20°C until analysis. Thermal cycling was initiated with denaturation at 95°C for 10 min. After this initial step, 35 cycles of polymerase chain reaction (heating at 95°C for 10 s, 56°C for 30 s, and 70°C for 1 min) were performed.

Table 1. Sequences of Primers for hMOR C-Terminal Splicing Variants

Primer Pair	Forward Sequence	Reverse Sequence	Target and Amplicon Size
1	CTGCTGGACTCCCATTCACA	TTAGGGCAACGGAGCAGTTT	hMOR1, NM_000914.4: 325 bp
2	CTGCTGGACTCCCATTCACA	TAATTCTAGAGACTGCGTAC	hMOR1A, NM_001008504.3: 306 bp
3	CTGCTGGACTCCCATTCACA	TTGGTATGCTCACAGTTGAG	hMOR1B1, NM_001145282.2: 336 bp
4	CTGCTGGACTCCCATTCACA	AGTCAGATTTCTGTCTTCTTTC	hMOR1B2, NM_001145283.2: 311 bp hMOR1B3, NM_001145284.3: 579 bp hMOR1Y, AY309009.1: 687 bp
5	CTGCTGGACTCCCATTCACA	AGACGACCCGGCAAGTTGGTC	hMOR1B3, NM_001145284.3: 331 bp hMOR1Y, AY309009.1: 440 bp
6	CTGCTGGACTCCCATTCACA	AGGGTTCATGTCATAGTCAG	hMOR1B4, NM_001145285.2: 308 bp hMOR1B5, NM_001145286.2: 459 bp
7	CTGCTGGACTCCCATTCACA	TGGAGACTGCCCTGCATTGTAG	hMOR1B5, NM_001145286.2: 389 bp
8	CTGCTGGACTCCCATTCACA	TGCCAAGGGTGGCTGATGAT	hMOR1O, NM_001008503.2: 298 bp
9	CTGCTGGACTCCCATTCACA	TCCAGGGTACACAACCAAGC	hMOR1X, NM_001008505.2: 352 bp
10	CTGCTGGACTCCCATTCACA	AACTGCCAAATCGCCACTCC	hMOR1Y, AY309009.1: 361 bp
11	GGGAGACCTGCTCCTCCTAA	GGGGCACAGCTAATGAAGGT	hGRPR, NM_005314.2: 342 bp

hMOR, human μ-opioid receptor.

The plasmids pcDNA3.1/hMOR1, hMOR1B1, hMOR1B3, hMOR1B5, hMOR1O, hMOR1X, and hMOR1Y were provided by Dr. Y. X. Pan, M.D., Ph.D.; and pcDNA/hGRPR was provided by Dr. James Battey, M.D., Ph.D. Splicing variants-specific 3' end sequence of hMOR1A, hMOR1B2, and hMOR1B4 were integrated into reverse primers and synthesized by Integrated DNA Technologies (USA). Full cDNA segments were amplified by polymerase chain reaction from hMOR1 template and sub cloned into pcDNA3.1 between Nhe I and Xba I site. All the plasmids were verified by DNA sequencing.

Cell Culture and Transfections

HEK 293 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2. The cells were first transfected with pcDNA3.1/hGRPR (20 μg/10^7 cells) containing the neomycin resistance by electroporation (GenePulser Xcell; Bio-Rad, USA). Stable transfectants were selected in the presence of 500 μg/ml G418 (Invitrogen, USA). To generate lines coexpressing two receptors, the cells were subjected to a second round of transfection and selected in the presence of 500 μg/ml G418 and 100 μg/ml hygromycin (Roche, Switzerland). Clones expressing hGRPR, hMOR1B1, hMOR1Y, hMOR1B1/hGRPR, and hMOR1Y/hGRPR were generated.

Calcium Imaging

To identify a human MOR1 splicing variant that interacts with GRPR, we performed calcium imaging on HEK 293 cells and examined functional interactions between hGRPR and different human *Oprm1* C terminal splicing variants. HEK 293 cells stably expressing hGRPR were planted on 12-mm round coverslips coated with poly-D-lysine. Plasmids of different human *Oprm1* C terminal splicing variants (1 μg per coverslip) were transfected using Lipofectamine 3000 (ThermoFisher Scientific, USA) in accordance to manufacturer's instructions. Twenty-four hours after transfection, cells were used for calcium imaging as previously described.[37] Fura-2 acetoxymethyl ester (Molecular Probes; ThermoFisher Scientific) was diluted to 2 mM stock in dimethyl sulfoxide/20% pluronic acid. Cells were loaded with fura 2-acetomethoxy ester (Molecular Probes) for 30 min at 37°C. After washing, the coverslips were mounted on a Warner Instruments (USA) recording chamber (RC 26G) perfused with calcium imaging buffer at a rate of ~2 ml/min. An inverted microscope (Eclipse Ti 10X objective; Nikon, Japan) with Roper CoolSNAP HQ2 digital camera was used for fura-2 calcium imaging after 340/380-nm laser excitations (sampling interval, 2 s; exposure time adjusted for each experiment to ~40 ms for 340 nm and to ~30 ms for 380 nm). Responsive cells were identified as regions of interest and F340/F380 ratios were measured using NISElements (version 3.1, Nikon). Calibration of intracellular calcium concentrations ($[Ca^{2+}]_i$) was performed using Fura-2 Calcium Imaging Calibration Kit (Invitrogen) following manufacturer instruction. Each experiment was done at least three times, and at least 30 cells were analyzed each time. Cells were incubated first with morphine (1 μM), and then with gastrin-releasing peptide (1 nM; Bachem, Switzerland), which were dissolved in sterile saline.

For antagonists and transactivator of transcription peptides studies, stable cell lines expressing hMOR1Y/hGRPR were used. Naltrexone (10 μM) and GRPR antagonist (D-Phe-6-Bn(6 to 13)OMe; 1 μM) were preincubated for 15 min before imaging. Transactivator of transcription-hMOR1Y was added into cell cultures 1 h before

fura-2 and present in the incubation buffer for all of the following steps.

Transactivator of Transcription Peptides

C-terminal splicing variants of hMOR1 share identical amino acid sequence from M1 to Q388. Each variant has a unique C terminal tip that may account for their specific functions. To selectively inhibit specific *Oprm1* isoform, we synthesized a transactivator of transcription–fusion peptide containing a transactivator of transcription (YGRKKRRQRRR), a transactivating domain of human immunodeficiency virus protein that can permeate cell membrane,[38] fused to the unique C-terminal tip of the hMOR1Y isoform (IRDPISNLPRVSVF). We only created a transactivator of transcription–fusion peptide for *Oprm1* isoforms that were detected in the spinal cord and that exhibit GRPR specific co-hybridization in the calcium imaging studies. Introduction of transactivator of transcription–fusion peptides permits their competition with corresponding receptors for interaction with hGRPR both *in vitro* and *in vivo*.

Animal Studies

Male C57BL/6J mice of 25 ± 3 g body weight between 7 and 12 weeks old were purchased from the Jackson Laboratory. All mice were housed under a 12 h light/dark cycle with food and water provided *ad libitum*. All experiments were performed in accordance with the guidelines of the National Institutes of Health (Bethesda, Maryland) and the International Association for the Study of Pain (Washington, D.C.) and were approved by the Animal Studies Committee at Washington University (St. Louis, Missouri).

Nineteen mice were randomly assigned to three groups (0, 0.1, and 0.4 nmol of transactivator of transcription-hMOR1Y) for morphine-induced scratching behaviors and analgesia using simple randomization. Twenty-one mice were randomly assigned to three groups (0, 0.1, and 0.4 nmol of transactivator of transcription-hMOR1Y) for gastrin-releasing peptide-induced scratching behaviors. Behavioral experiments were performed between 9:00 and 11:00 AM. The injection area was shaved 2 days before experiments. To examine the effect of transactivator of transcription–fusion peptide at the spinal cord level, saline or different doses of transactivator of transcription–hMOR1Y (0.1 nmol and 0.4 nmol) were administered *via* intrathecal injections. Animals were treated in sequential order. After 30 min, each mouse was placed in a plastic arena (10 × 11 × 15 cm) for another 30 min to acclimatize. Mice were then briefly removed from the chamber for intrathecal injections of morphine or gastrin-releasing peptide. Morphine was injected at the dose of 0.3 nmol per mouse that induces robust scratching behaviors and thermal analgesic effect.[31]

Scratching behaviors were videotaped (HDR-CX190; Sony, Japan) for 30 min. The videos were played back on a computer and the quantification of mice behaviors were performed by investigators who were blinded to the treatments. One scratching episode is defined as a lifting of the hind limb toward the body and then a replacing of the limb back to the floor or the mouth, regardless of how many scratching strokes take place between those two movements.

Warm water tail immersion assay was conducted as described previously.[39] Thirty minutes after morphine injection, mice tails were dipped beneath the 50°C water in a temperature-controlled water bath (IITC Inc., USA). The latency to withdrawal was measured with a 15-s cutoff. For opioid analgesia, tail-flick results were expressed as percentage of maximum possible effect [%MPE = (post drug latency − pre drug latency) × 100/ (cutoff time − pre drug latency)].

Statistical Analysis

One-way ANOVA with one between-subject factor of drug treatment followed by *post hoc* analysis (Dunnett) was used for multiple group comparisons. Two-tailed independent *t* test was used for two group comparisons. Normality and equal variance assumptions were tested with the Kolmogorov–Smirnov and the Brown-Forsythe median test, respectively. All statistical analyses were carried out with Prism 8 (v8.0.1; GraphPad, USA). The data are presented as mean ± SD, and $P < 0.05$ was considered statistically significant. No *a priori* statistical power calculation was conducted to guide sample size; sample size was based on our previous studies. No data points were omitted as outliers.

Results

Identification of *Oprm1* Splicing Variants from Human Spinal Cord

To identify *Oprm1* splicing variants from human spinal cord, we first isolated cDNA from the dorsal horn and ventral horn of human spinal cords. Using reverse transcription polymerase chain reaction, we were able to detect distinct bands corresponding to hMOR1, hMOR1A, hMOR1B5, and hMOR1Y (fig. 1A). In the ventral horn, reverse transcription polymerase chain reaction bands of hMOR1 and hMOR1A were present, suggesting that these two variants are broadly expressed (fig. 1B). A band corresponding to h*Grpr* was also present in the dorsal horn of human spinal cord (fig. 1C). Reverse transcription polymerase chain reaction results demonstrate that various hMOR splicing variants are expressed in human spinal cord (fig. 1A).

Human MOR1Y Can Cross-activate GRPR Signaling

We previously showed that morphine induces itch signaling by activating MOR1D–GRPR cross-talk[31] in mice. Of note, the amino acid sequences of MOR and GRPR are highly conserved in human and mice (fig. 1, D and E). As such, a key criterion for identifying a human *Oprm1*

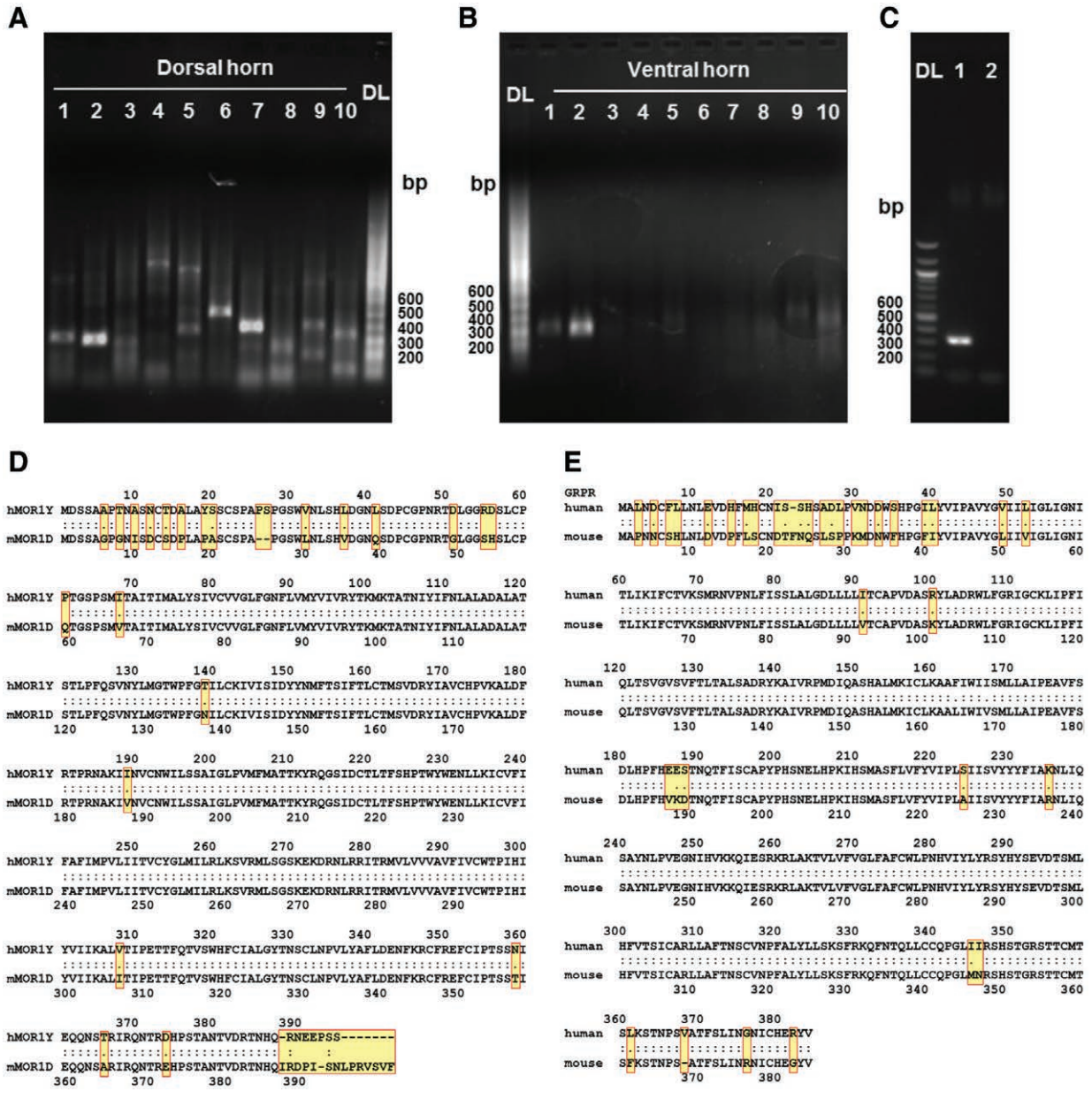

Fig. 1. Detection of hMOR1Y from human spinal cord. (*A*) Gel image of RT-PCR using C-terminal splicing variants specific primers showed that bands corresponding to hMOR1Y were detected in the dorsal horn of the spinal cord by primer pair 4 (687 bp) and primer pair 10 (361 bp), respectively. RT-PCR bands corresponding to hMOR1, hMOR1A, hMOR1B5, and hMOR1O were also detected by primer pair 1 (325 bp), primer pair 2 (306 bp), primer pair 6 (459 bp), primer pair 7 (389 bp), and primer pair 8 (298 bp), respectively. (*B*) In the ventral horn, hMOR1 and hMOR1A transcripts were detectable by RT-PCR. A faint band of hMOR1Y was also detected by primer pair 10. (*C*) *hGrpr* transcript was detected in the dorsal horn (lane 1), but not in the ventral horn (lane 2) by RT-PCR. (*D*) Alignment of amino acid sequences of hMOR1Y and mMOR1D shows that MOR is highly conserved with 93.8% identity and 97.2% similar in 388 aa amino acids overlap (1 to 388:1 to 386). (*E*) Alignment of amino acid sequences of human GRPR (M1-V384) and mouse GRPR (M1-V384) shows that GRPR is highly conserved with 89.9% identity. *Rectangles* in parts *D* and *E* highlight differences between two sequences. DL, DNA ladder; GRPR, human gastrin-releasing peptide receptor human; hMOR, human μ-opioid receptor; MOR, μ-opioid receptor; mMOR, mouse μ-opioid receptor; RT-PCR, reverse transcription polymerase chain reaction

splicing variant that interacts with GRPR in a similar manner to the behavior of MOR1D in mice, is that the isoform should mediate calcium transients in HEK 293 cells expressing GRPR when morphine is added.[31] Of five splicing variants that were detected in human dorsal horn, only hMOR1Y cross-activated calcium signaling of hGRPR after morphine incubation (fig. 2A). Morphine failed to evoke calcium responses when other splicing variants were coexpressed with hGRPR (fig. 2B and Supplemental Digital Content 1, http://links.lww.com/ALN/B956). In contrast, morphine did not induce calcium responses in HEK 293 cells that were singly transfected with hMOR1Y or hGRPR (fig. 2C). When cells were preincubated with naltrexone, a MOR antagonist that inhibits morphine-induced pruritus in primates,[33] morphine-induced calcium spikes were completely blocked in hGRPR/hMOR1Y cells (mean difference, 56 ± 3 nM) (fig. 3, A and B, *red*). Morphine-induced calcium spike was also blocked by the GRPR antagonist (mean difference, 54 ± 3 nM) (fig. 3, A and B, *blue*). These data suggest that hMOR1Y is capable of mediating the effect of morphine on cross activation of hGRPR.

C-terminal of hMOR1Y Is Required for Cross-activation of hGRPR Signaling

Our previous studies using a transactivator of transcription–peptide approach showed that the seven amino acid sequence in the C terminal of MOR1D in mice is critical for cross-talk between MOR1D and GRPR because Tat-peptide could attenuate MOR1D–GRPR cross-signaling.[31] To test whether hMOR1Y and hMOR1B1 may cross-activate hGRPR through their C-terminal, we synthesized transactivator of transcription–fusion peptides containing a transactivator of transcription (YGRKKRRQRRR), fused to the C terminal of hMOR1Y (IRDPISNLPRVSVF) that we demonstrated to interact with hGRPR, and tested whether the transactivator of transcription–peptide could attenuate the cross-talk with hGRPR. We first incubated transactivator of transcription–hMOR1Y with HEK 293 cells expressing hGRPR/hMOR1Y. Remarkably, preincubation of transactivator of transcription–hMOR1Y for 2 h dose-dependently blocked morphine-induced calcium spikes in hGRPR/hMOR1Y-expressing cells (mean difference, 28 ± 2 nM, vehicle *vs.* 50 μM; 66 ± 2 nM, vehicle *vs.* 200 μM) (fig. 3, C and D). High concentration of Tat-hMOR1Y (200 μM) also showed inhibiting effect on gastrin-releasing peptide-induced calcium responses (mean difference, 12 ± 2 nM) (fig. 3, C and D). These results suggest that the C-terminus of hMOR1Y may direct interact with hGRPR.

C-terminal of hMOR1Y Recapitulates the Effect of mMOR1D on Itch and Analgesia in Mice

The finding that the transactivator of transcription–hMOR1Y could block hMOR1Y and hGRPR cross-talk supports two important notions. First, GRPR between human and mouse is conserved (fig. 1E). Second, the C-terminal of hMOR1Y and mMOR1D may share important biochemical characteristics enabling their cross-activation of GRPR, irrespective of species (table 2). To further examine this, we tested the effect of transactivator of transcription–hMOR1Y on mouse scratching behaviors induced by intrathecal injections of morphine or gastrin-releasing peptide. Remarkably, transactivator of transcription–hMOR1Y dose-dependently attenuated morphine-induced scratching behaviors (mean difference, 24 ± 16, 0 nmol *vs.* 0.1 nmol; 54 ± 17, 0 nmol *vs.* 0.4 nmol) (n = 6 to 7 mice per group; $P = 0.011$, one-way ANOVA) (fig. 4A) with no effect on morphine-induced antinociception (fig. 4B). Interestingly, transactivator of

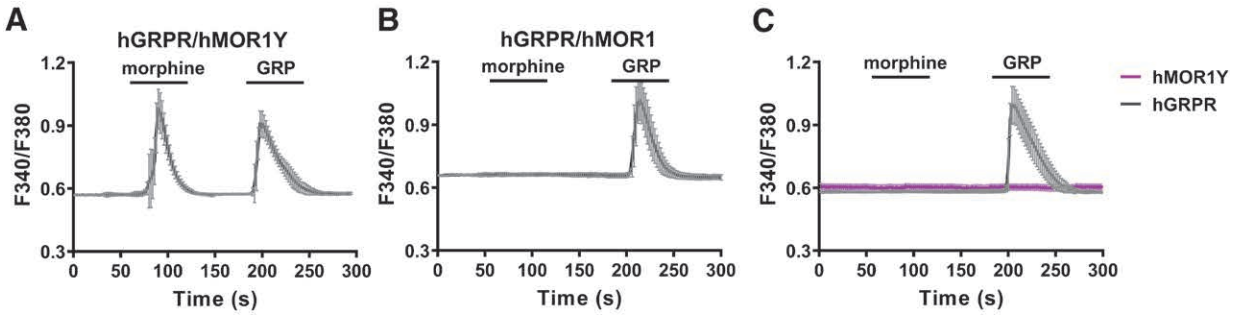

Fig. 2. Morphine cross activates hGRPR/Ca^{2+} signaling pathway when hGRPR is co-expressed with hMOR1Y. (*A*) Both morphine (1 μM) and GRP (1 nM) induced calcium responses in hGRPR-expressing HEK 293 cells transfected with and hMOR1Y. (*B*) Morphine (1 μM) did not induce calcium response in hGRPR-expressing HEK293 cells transfected with hMOR1. (*C*) Morphine (1 μM) did not induce calcium response in HEK 293 cells singly expressing hMOR1Y (*purple*) or hGRPR (*grey*). Data are presented as mean ± SD. Each experiment was done three times and at least 30 cells were analyzed each time. GRP, gastrin-releasing peptide; hGRPR, human gastrin-releasing peptide receptor; hMOR, human μ-opioid receptor.

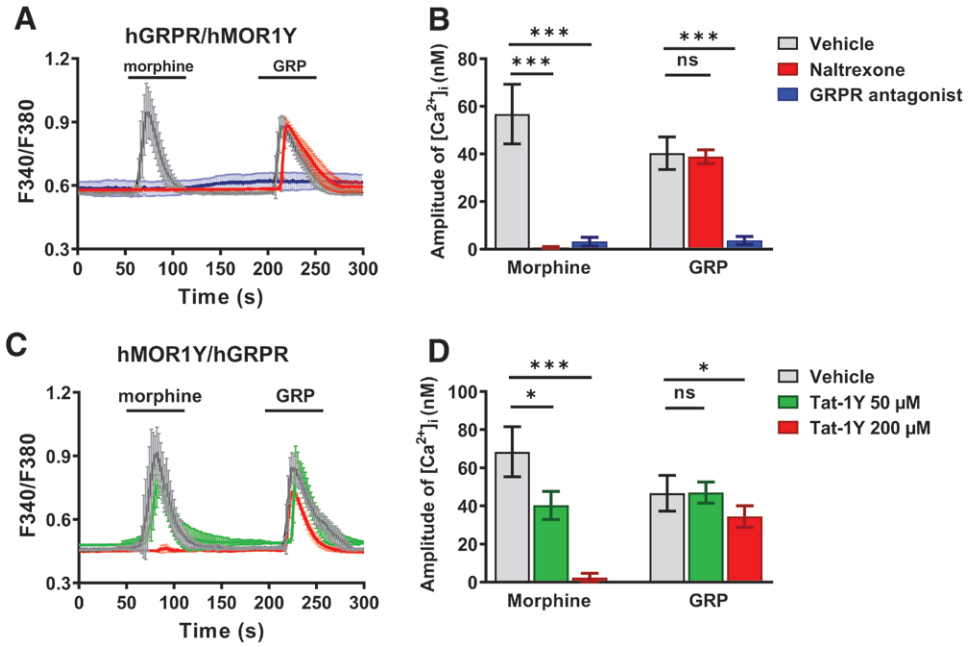

Fig. 3. Morphine induced hGRPR calcium responses through hMOR1Y. (*A*) Naltrexone (10 μM, *red*) blocked morphine-induced calcium responses in HEK 293 cells co-expressing hGRPR and hMOR1Y. The GRPR antagonist (1 μM) (*blue trace*) completely blocked morphine and GRP-induced Ca^{2+} increase. (*B*) Quantified data of (*A*) show that naltrexone (*red*) significantly inhibited the peak concentrations of intracellular calcium ($[Ca^{2+}]_i$) induced by morphine. ***$P < 0.001$ *versus* vehicle, F (2, 87) = 604.3 for morphine, F (2, 87) = 717.6 for GRP, one-way ANOVA followed by Dunnett *post hoc* test. (*C* and *D*) Incubation of Tat-1Y with hMOR1Y/hGRPR coexpressing cells significantly blocked morphine-induced calcium spikes. High concentration of Tat-1Y (200 μM, *red*), but not low concentration of Tat-1Y (50 μM, *green*), significantly inhibited GRP-induced calcium responses. Data are presented as mean ± SD. *$P < 0.05$, ***$P < 0.001$ *versus* vehicle, F (2, 87) = 426.9 for morphine, F (2, 87) = 30.8, one-way ANOVA followed by Dunnett *post hoc* test. Each experiment was done three times and at least 30 cells were analyzed each time. GRP, gastrin-releasing peptide; hGRPR, human gastrin-releasing peptide receptor; hMOR, human μ-opioid receptor; ns, not significant; Tat, transactivator of transcription.

transcription–hMOR1Y also blocked gastrin-releasing peptide-induced scratching behaviors (mean difference, 20 ± 18, 0 nmol *vs.* 0.1 nmol; 6 ± 18, 0 nmol *vs.* 0.4 nmol) (n = 7 mice per group; $P = 0.013$, one-way ANOVA) (fig. 4C). These results suggest that transactivator of transcription–hMOR1Y may directly block mouse GRPR function. To test this, we performed calcium imaging on HEK 293 cells coexpressing mMOR1D/mGRPR. Preincubation of transactivator of transcription–hMOR1Y for 2 h significantly blocked morphine and gastrin-releasing peptide-induced calcium spikes (mean difference, 101 ± 6 nM for morphine; 103 ± 4 nM for gastrin-releasing peptide) (fig. 4, D and E). Thus, transactivator of transcription–hMOR1Y could function as a GRPR antagonist in MOR1D/GRPR neurons. Taken together, the expression of hMOR1Y in the dorsal horn of the spinal cord and its interactions with hGRPR suggests that hMOR1Y may play important roles in morphine-induced pruritus.

Table 2. Amino Acid Sequences of C-Terminal Tips of Mouse MOR1D and Human MOR C-terminal Splicing Variants

MOR C-terminal Splicing Variants	Sequences
mMOR1D	NHQ-RNEEPSS
hMOR1	NHQ-LENLEAETAPLP
hMOR1A	NHQ-VRSL
hMOR1B5	NHQ-VELNLDCHCENAKPWPLSYNAG
hMOR1Y	NHQ-IRDPISNLPRVSVF

hMOR, human μ-opioid receptor; mMOR, mouse μ-opioid receptor.

Discussion

This work presents several major findings that are directly relevant to our understanding of itch transmission in humans. First, we demonstrate the existence of hGRPR in human spinal cord. GRPR was discovered to be an itch receptor in mice in 2007.[28] The present work represents the evidence suggesting that GRPR also functions as an itch receptor that mediates opioid-induced pruritus in human spinal cord. Second, we demonstrate that the *Oprm1* gene undergoes extensive alternative splicing in human spinal cord, with marked regional differences between the ventral and dorsal horns. Strikingly, the regional variation of

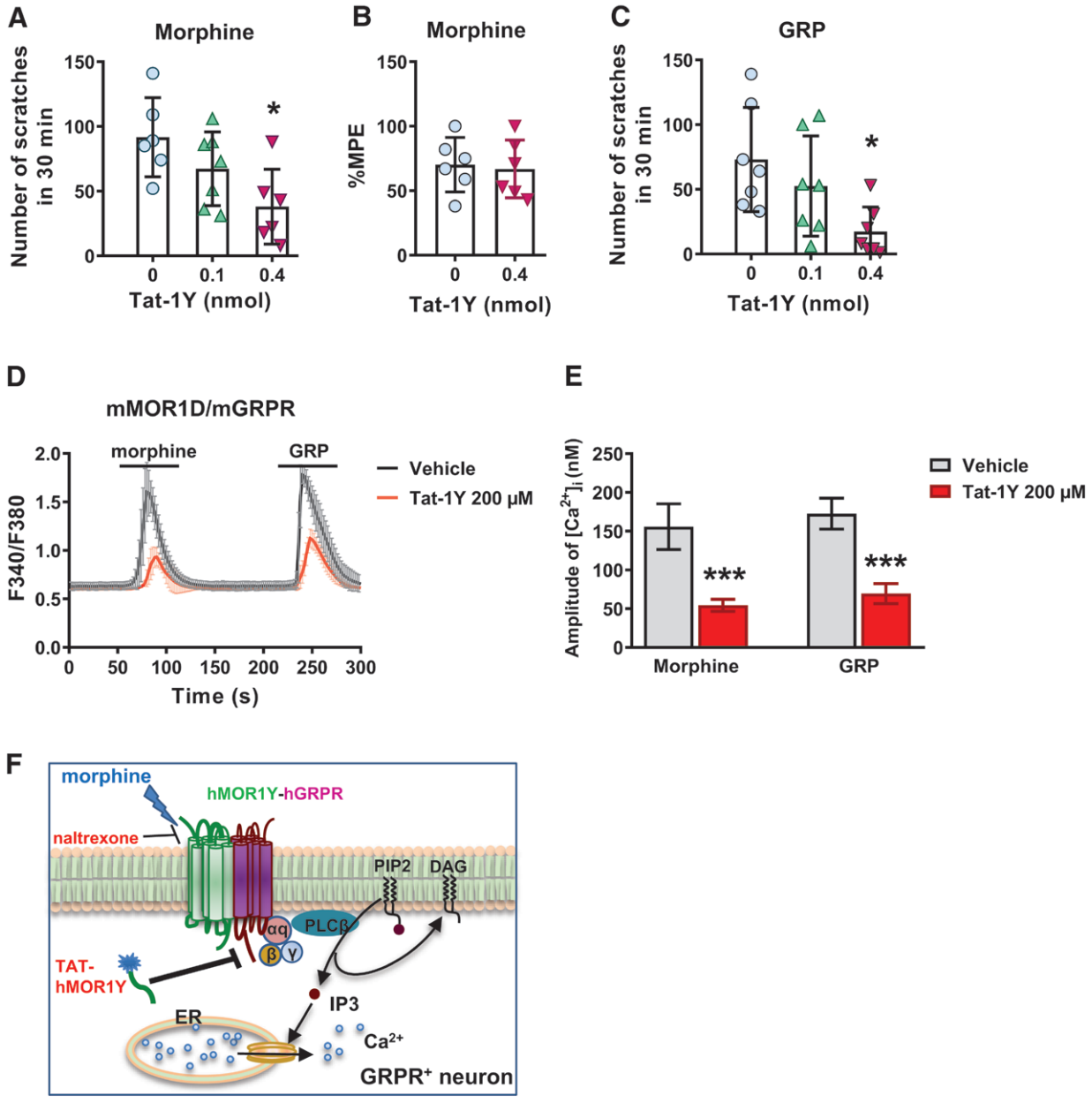

Fig. 4. C-terminal end of hMOR1Y is responsible for the interactions between hGRPR and hMOR1Y. (*A*) Preinjection of Tat-1Y (0.4 nmol, i.t.) for 1 h significantly reduced scratching behaviors induced by i.t. injection of morphine (0.3 nmol). n = 6 to 7 mice per group. *$P < 0.05$ *versus* Tat-1Y 0 nmol, F (2, 16) = 5.04, one-way ANOVA followed by Dunnett's *post hoc* test. (*B*) Morphine-induced analgesia was not affected by Tat-1Y (0.4 nmol, i.t.) as tested by warm water tail-immersion assay. n = 6 mice per group. $P = 0.796$, t = 0.27, degrees of freedom = 10, two-tailed unpaired *t* test. (*C*) Tat-1Y dose-dependently inhibited scratching behaviors induced by i.t. injection of GRP (0.05 nmol). n = 7 mice per group. *$P < 0.05$ *versus* Tat-1Y 0 nmol, F (2, 18) = 4.78, one-way ANOVA followed by Dunnett *post hoc* test. (*D* and *E*) Incubation of Tat-1Y (200 μM, *red*) for 2 h significantly inhibited morphine and GRP-induced calcium responses in mMOR1D/mGRPR coexpressing cells. *** $P < 0.001$ *versus* vehicle, two-tailed unpaired *t* test, t = 18.19, degrees of freedom = 58 for morphine, t = 23.68, degrees of freedom = 58 for GRP. (*F*) A diagram shows that morphine activates hMOR1Y, which cross-activates hGRPR-mediated the PLC-Ca^{2+} signaling pathway for itch that can be blocked by either naltrexone or TAT-hMOR1Y. Data are presented as mean ± SD. GRP, gastrin-releasing peptide; hGRPR, human gastrin-releasing peptide receptor; hMOR, human μ-opioid receptor; i.t., intrathecal; mMOR, mouse μ-opioid receptor; Tat, transactivator of transcription.

expression patterns of splicing variants of *Oprm1* in the human spinal cord mirrors that in mouse spinal cord,[40–42] further supporting the notion that the *Oprm1* splicing variants are highly conserved across animal species.[22] Third, our data suggest that hMOR1Y and hGRPR can cross-talk, and the C-terminal of hMOR1Y is required for cross-activation of GRPR-mediating itch transmission. This finding is reminiscent of mouse MOR1D–GRPR cross-talk in the spinal cord,[31] strongly indicating a functional conservation of opioid-induced itch pathways between mice and humans. Lastly, we show that transactivator of transcription–hMOR1Y can function as a GRPR antagonist and exert its anti-GRPR signaling in the absence of morphine but the presence of hMOR1Y. These findings imply that the mere presence of hMOR1Y may alter the conformation and functionality of hGRPR, and thereby enable transactivator of transcription–hMOR1Y to interfere the binding of gastrin-releasing peptide to GRPR.

We previously demonstrated uncoupling between the desired spinal opioid-induced antinociceptive effects mediated *via* the MOR1 isoform and the unwanted spinal opioid-induced scratching behavior mediated *via* the MOR1D isoform in mice.[31] This double disassociation of function occurs at different cell types in mouse spinal cord because MOR1D is expressed in GRPR neurons which are dedicated to itch but not pain transmission, while MOR1 is expressed in non-GRPR neurons, most likely nociceptive neurons.[29] Interfering with the seven amino acids at the C-terminal of MOR1D using a transactivator of transcription–peptide approach blocks MOR1D–GRPR crosstalk and thereby attenuates the scratching behavior following spinal opioids.[31] Therefore, opioids induce itch and analgesia through completely distinct neural pathways in the mouse spinal cord.

In this report, we have identified the sequence for each of the *Oprm1* isoforms of the human spinal cord. Assessment of the free cytosolic calcium concentration in transfected HEK cells showed that the human MOR1Y isoform can be activated by both morphine and gastrin-releasing peptide, and this activation can be blocked by a transactivator of transcription–peptide we designed to bind specifically to the unique C-terminal of the hMOR1Y isoform and by the MOR antagonist naltrexone (fig. 4F). Furthermore, we showed that transactivator of transcription–hMOR1Y dose-dependently attenuated scratching behaviors induced both by morphine and by gastrin-releasing peptide in mice, with no effect on morphine-induced analgesia. To our surprise, we could not find similarity between the C-terminals of hMOR1Y and mMOR1D (12 *vs.* 7 amino acids in length). This suggests that the precise mechanisms by which the C-terminals interact with GRPR may differ between mice and humans. Alternatively, it is possible that the cross-talk may occur following conformational change of one or two receptors rather than a direct physical heteromeric interaction. The C-terminal could exert the effect through interfering in downstream signaling events rather than direct heteromeric interaction in humans. Because specific antibody against hMOR1Y is not available, we are unable to assess this possibility in this study. Future function-structural characterization is required to identify minimal amino acids of the C-terminals required for crosstalk and signaling components affected.

Owing to technical difficulties in human studies, particularly the lack of a specific hMOR1Y antibody, we were unable to determine whether hMOR1Y and hGRPR are expressed in distinct subpopulations of neurons in the spinal cord, nor to functionally verify the role of hMOR1Y by siRNA knockdown as we did in mice. However, the observations that hMOR1Y and GRPR engage in cross-talk and that perturbation of the C-terminal of hMOR1Y impairs GRPR signaling and scratching behavior in mice strongly argues that structurally and functionally the cross-talk between splicing *Oprm1* isoform and GRPR is evolutionarily conserved between human and mouse.

There are two important clinical applications of our findings. First, the study provides a potential pharmacologic target to treat or prevent spinal opioid-induced pruritus in human, resulting in a drug which could be coadministered together with spinal opioids. It is conceivable that two novel pharmacologic strategies could be employed to tackle opioid-induced pruritus. First, one may target GRPR by the coadministration of morphine with a GRPR antagonist. In addition, opioid-induced pruritus may be specifically alleviated by the coapplication of morphine and a transactivator of transcription–peptide targeting the hMOR1Y C-terminal.[43] These approaches would be preferable to the current practice where opioid antagonists[44,45] or histamine antagonists[46,47] have been used to attempt to tackle severe opioid-induced pruritus, since these drugs are—at best—only partially effective, and since opioid antagonists compromise the desired analgesic effects of spinal opioids. Second, the discovery of multiple splicing variants in the human spinal cord raises an important question as to their specific function in humans, which has been largely ignored and remain unknown to date. A major challenge for overcoming the limitation of opioid drugs in pain management is to diminish their unwanted side effects without affecting their analgesic action.[48,49] Our findings should significantly strengthen the notion that activation of distinct *Oprm1* isoforms may account for some side effects associated with opioid drugs in pain management. As such, these findings may herald future efforts to uncouple desired opioid analgesia from other adverse side effects, such as addiction and respiratory depression.

Acknowledgments

The authors gratefully appreciate the assistance of Mid-American Transplant, St Louis, Missouri, who facilitated our harvesting of human spinal cord samples.

Research Support

Supported by the National Institutes of Health (Bethesda, Maryland) grant Nos. AR056318-06, NS094344, and RO1DA037261-01A1 (to Dr. Chen).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Ginosar: Department of Anesthesiology, Hadassah Hebrew University Medical Center, Ein Karem, P.O. Box 12000 Jerusalem, Israel 91120. ginosar@wustl.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Pert CB, Kuhar MJ, Snyder SH: Opiate receptor: Autoradiographic localization in rat brain. Proc Natl Acad Sci U S A 1976; 73:3729–33
2. Behar M, Magora F, Olshwang D, Davidson JT: Epidural morphine in treatment of pain. Lancet 1979; 1:527–9
3. Eltzschig HK, Lieberman ES, Camann WR: Regional anesthesia and analgesia for labor and delivery. N Engl J Med 2003; 348:319–32
4. Carvalho B, Wang P, Cohen SE: A survey of labor patient-controlled epidural anesthesia practice in California hospitals. Int J Obstet Anesth 2006; 15:217–22
5. Practice Guidelines for Obstetric Anesthesia: An Updated Report by the American Society of Anesthesiologists Task Force on Obstetric Anesthesia and the Society for Obstetric Anesthesia and Perinatology. ANESTHESIOLOGY 2016; 124: 270–300
6. Block BM, Liu SS, Rowlingson AJ, Cowan AR, Cowan JA Jr, Wu CL: Efficacy of postoperative epidural analgesia: A meta-analysis. JAMA 2003; 290:2455–63
7. Mhuircheartaigh RJ, Moore RA, McQuay HJ: Analysis of individual patient data from clinical trials: Epidural morphine for postoperative pain. Br J Anaesth 2009; 103:874–81
8. American Society of Anesthesiologists Task Force on Acute Pain M: Practice guidelines for acute pain management in the perioperative setting: An updated report by the American Society of Anesthesiologists Task Force on Acute Pain Management. ANESTHESIOLOGY 2012; 116: 248–73
9. Bujedo BM, Santos SG, Azpiazu AU: A review of epidural and intrathecal opioids used in the management of postoperative pain. J Opioid Manag 2012; 8:177–92
10. Pöpping DM, Elia N, Marret E, Wenk M, Tramèr MR: Opioids added to local anesthetics for single-shot intrathecal anesthesia in patients undergoing minor surgery: A meta-analysis of randomized trials. Pain 2012; 153:784–93
11. Pöpping DM, Elia N, Wenk M, Tramèr MR: Combination of a reduced dose of an intrathecal local anesthetic with a small dose of an opioid: A meta-analysis of randomized trials. Pain 2013; 154:1383–90
12. Kumar K, Kelly M, Pirlot T: Continuous intrathecal morphine treatment for chronic pain of nonmalignant etiology: Long-term benefits and efficacy. Surg Neurol 2001; 55:79–86; discussion 86–8
13. Mercadante S, Intravaia G, Villari P, Ferrera P, Riina S, David F, Mangione S: Intrathecal treatment in cancer patients unresponsive to multiple trials of systemic opioids. Clin J Pain 2007; 23:793–8
14. Lyons G, Columb M, Hawthorne L, Dresner M: Extradural pain relief in labour: Bupivacaine sparing by extradural fentanyl is dose dependent. Br J Anaesth 1997; 78:493–7
15. Polley LS, Columb MO, Wagner DS, Naughton NN: Dose-dependent reduction of the minimum local analgesic concentration of bupivacaine by sufentanil for epidural analgesia in labor. ANESTHESIOLOGY 1998; 89:626–32
16. Lim Y, Jha S, Sia AT, Rawal N: Morphine for post-caesarean section analgesia: Intrathecal, epidural or intravenous? Singapore Med J 2005; 46:392–6
17. Ballantyne JC, Loach AB, Carr DB: Itching after epidural and spinal opiates. Pain 1988; 33:149–60
18. Szarvas S, Harmon D, Murphy D: Neuraxial opioid-induced pruritus: A review. J Clin Anesth 2003; 15:234–9
19. Waxler B, Dadabhoy ZP, Stojiljkovic L, Rabito SF: Primer of postoperative pruritus for anesthesiologists. ANESTHESIOLOGY 2005; 103:168–78
20. Pan YX: Diversity and complexity of the mu opioid receptor gene: Alternative pre-mRNA splicing and promoters. DNA Cell Biol 2005; 24:736–50
21. Pasternak GW: Molecular insights into mu opioid pharmacology: From the clinic to the bench. Clin J Pain 2010; 26 Suppl 10:S3–9
22. Pasternak GW, Pan YX: Mu opioids and their receptors: Evolution of a concept. Pharmacol Rev 2013; 65:1257–317
23. Uhl GR, Sora I, Wang ZJ: The mu opiate receptor as a candidate gene for pain: Polymorphisms, variations in expression, nociception, and opiate responses. Proceedings of the National Academy of Sciences of the United States of America 1999; 96: 7752–55
24. Shabalina SA, Zaykin DV, Gris P, Ogurtsov AY, Gauthier J, Shibata K, Tchivileva IE, Belfer I, Mishra B, Kiselycznyk C, Wallace MR, Staud R, Spiridonov NA, Max MB, Goldman D, Fillingim RB, Maixner W, Diatchenko L: Expansion of the human mu-opioid

receptor gene architecture: Novel functional variants. Hum Mol Genet 2009; 18:1037–51
25. Barry DM, Li H, Liu XY, Shen KF, Liu XT, Wu ZY, Munanairi A, Chen XJ, Yin J, Sun YG, Li YQ, Chen ZF: Critical evaluation of the expression of gastrin-releasing peptide in dorsal root ganglia and spinal cord. Mol Pain 2016; 12
26. Barry DM, Liu X-T, Yang Q, Liu X-Y, Zeng X, Liu B-L, Chen Z-F: Exploration of sensory and spinal neurons expressing GRP in itch and pain. bioRxiv 2018
27. Barry DM, Munanairi A, Chen ZF: Spinal mechanisms of itch transmission. Neurosci Bull 2018; 34:156–64
28. Sun YG, Chen ZF: A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. Nature 2007; 448:700–3
29. Sun YG, Zhao ZQ, Meng XL, Yin J, Liu XY, Chen ZF: Cellular basis of itch sensation. Science 2009; 325:1531–4
30. Zhao ZQ, Huo FQ, Jeffry J, Hampton L, Demehri S, Kim S, Liu XY, Barry DM, Wan L, Liu ZC, Li H, Turkoz A, Ma K, Cornelius LA, Kopan R, Battey JF Jr, Zhong J, Chen ZF: Chronic itch development in sensory neurons requires BRAF signaling pathways. J Clin Invest 2013; 123:4769–80
31. Liu XY, Liu ZC, Sun YG, Ross M, Kim S, Tsai FF, Li QF, Jeffry J, Kim JY, Loh HH, Chen ZF: Unidirectional cross-activation of GRPR by MOR1D uncouples itch and analgesia induced by opioids. Cell 2011; 147:447–58
32. Ko MC, Naughton NN: An experimental itch model in monkeys: Characterization of intrathecal morphine-induced scratching and antinociception. Anesthesiology 2000; 92:795–805
33. Lee H, Ko MC: Distinct functions of opioid-related peptides and gastrin-releasing peptide in regulating itch and pain in the spinal cord of primates. Sci Rep 2015; 5:11676
34. Nattkemper LA, Zhao ZQ, Nichols AJ, Papoiu ADP, Shively CA, Chen ZF, Yosipovitch G: Overexpression of the gastrin-releasing peptide in cutaneous nerve fibers and its receptor in the spinal cord in primates with chronic itch. J Invest Dermatol 2013; 133:2489–92
35. Huang XQ, Miller W: A time-efficient, linear-space local similarity algorithm. Advances in Applied Mathematics 1991; 12: 337–57
36. Liu XY, Wan L, Huo FQ, Barry DM, Li H, Zhao ZQ, Chen ZF: B-type natriuretic peptide is neither itch-specific nor functions upstream of the GRP-GRPR signaling pathway. Mol Pain 2014; 10:4
37. Zhao ZQ, Liu XY, Jeffry J, Karunarathne WK, Li JL, Munanairi A, Zhou XY, Li H, Sun YG, Wan L, Wu ZY, Kim S, Huo FQ, Mo P, Barry DM, Zhang CK, Kim JY, Gautam N, Renner KJ, Li YQ, Chen ZF: Descending control of itch transmission by the serotonergic system via 5-HT1A-facilitated GRP-GRPR signaling. Neuron 2014; 84:821–34
38. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF: *In vivo* protein transduction: Delivery of a biologically active protein into the mouse. Science 1999; 285:1569–72
39. Chen ZF, Rebelo S, White F, Malmberg AB, Baba H, Lima D, Woolf CJ, Basbaum AI, Anderson DJ: The paired homeodomain protein DRG11 is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. Neuron 2001; 31:59–73
40. Pan YX, Xu J, Bolan E, Abbadie C, Chang A, Zuckerman A, Rossi G, Pasternak GW: Identification and characterization of three new alternatively spliced mu-opioid receptor isoforms. Mol Pharmacol 1999; 56:396–403
41. Pan YX, Xu J, Bolan E, Chang A, Mahurter L, Rossi G, Pasternak GW: Isolation and expression of a novel alternatively spliced mu opioid receptor isoform, MOR-1F. FEBS Lett 2000; 466:337–40
42. Pan YX, Xu J, Mahurter L, Bolan E, Xu M, Pasternak GW: Generation of the mu opioid receptor (MOR-1) protein by three new splice variants of the Oprm gene. Proc Natl Acad Sci U S A 2001; 98:14084–9
43. Kang T, Gao X, Chen J: Harnessing the capacity of cell-penetrating peptides for drug delivery to the central nervous system. Curr Pharm Biotechnol 2014; 15:220–30
44. Paech M, Sng B, Ng L, Nathan E, Sia A, Carvalho B: Methylnaltrexone to prevent intrathecal morphine-induced pruritus after Caesarean delivery: A multicentre, randomized clinical trial. Br J Anaesth 2015; 114:469–76
45. Wu Z, Kong M, Wang N, Finlayson RJ, Tran QH: Intravenous butorphanol administration reduces intrathecal morphine-induced pruritus after cesarean delivery: A randomized, double-blind, placebo-controlled study. J Anesth 2012; 26:752–7
46. Kung AT, Yang X, Li Y, Vasudevan A, Pratt S, Hess P: Prevention *versus* treatment of intrathecal morphine-induced pruritus with ondansetron. Int J Obstet Anesth 2014; 23:222–6
47. Prin M, Guglielminotti J, Moitra V, Li G: Prophylactic ondansetron for the prevention of intrathecal fentanyl- or sufentanil-mediated pruritus: A meta-analysis of randomized trials. Anesth Analg 2016; 122:402–9
48. Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, Glaser SE, Vallejo R: Opioid complications and side effects. Pain Physician 2008; 11(2 Suppl):S105–20
49. Porreca F, Ossipov MH: Nausea and vomiting side effects with opioid analgesics during treatment of chronic pain: Mechanisms, implications, and management options. Pain Med 2009; 10:654–62