Supplementary Materials for

Genetic behavioral screen identifies an orphan anti-opioid system

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This PDF file includes:

Materials and Methods
Figs. S1 to S14
Tables S1

Other Supplementary Materials for this manuscript includes the following:

Movies S1 to S10

Materials and Methods

C. elegans and mouse strains

All *C. elegans* strains were maintained using standard methods. The following worm strains were used: N2 strain (wild isolate, Bristol, UK), CB4856 polymorphic Hawaiian strain (wild isolate, Hawaii, USA), tgMOR (*bggIs10*), *frpr-13 (bgg9), egl-19 (bgg8), rsbp-1 (vs163), pha-1 (e2123), ttTi5605mos1, unc-119 (ed3), oxEx1578*.

The *Gpr139*$^{-/-}$ mouse strain (Gpr139$^{tm1.1(KOMP)Vlcg}$) was created from ES cell clone 10338B-A5, generated by Regeneron Pharmaceuticals, Inc. and made into mice by the KOMP Repository at the University of California Davis. Sperm obtained from KOMP was used for *in vitro* fertilization of recipient C57Bl6 females at the Mouse Genetics Core of the Scripps Research Institute. All mice evaluated in this study were littermates obtained by crossing heterozygous parents. Mice were housed in groups (unless otherwise stated) on a 12-hour light-dark cycle with food and water available *ad libitum*. All studies involving mice were carried out in accordance with the National Institute of Health guidelines and every procedure was reviewed and approved by the IACUC committee at the Scripps Research Institute.

Reagents

ActOne Membrane Potential Dye was purchased from Codex BioSolutions, Inc (Gaithersburg, MD). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Dulbecco Modified Eagle Medium (DMEM), MEM non-essential amino acids (NEAA), and sodium pyruvate were from Life Technologies (Grand Island, NY). NanoGlo reagent was purchased from Promega (Madison, WI). Morphine sulfate salt, anti-FLAG M2 antibody,

anti-MOR antibody, anti-NeuN antibody, anti-GAD67 antibody, anti-TH antibody, and fetal bovine serum (FBS) were purchased from Millipore-Sigma (Burlington, MA). Anti-rabbit secondary antibody was acquired from Kindle Biosciences, LLC (Greenwich, CT). DAMGO was purchased from Tocris Bioscience (Minneapolis, MN). Matrigel was purchased form Corning Life Sciences (Corning, NY) and cOmplete EDTA-free protease inhibitor tablets were from Roche (Boston, MA). Protein G Sepharose Fast Flow was from GE Healthcare (Chicago, IL). GPR139 antibody was produced in rabbit by Pocono Rabbit Farm & Laboratory (Canadensis, PA) and purified by peptide affinity. The antibody epitope corresponds to the carboxy-terminal 19 amino acids of mouse GPR139 (CIKMLVYQYDKHGKPIKVSP). pHO4d-Cas9 was a gift from Michael Nonet (Addgene plasmid # 67881).

Molecular biology and transgenics in *C. elegans*

A pan-neuronal expression plasmid P_{rgef-1}::FLAG::MOR (pBG-GY400) was generated using the mouse MOR cDNA sequence (NM_001302793.1). MOR cDNA was amplified by PCR, cloned into pCR8 Topo GY (Invitrogen) and recombined into P*rgef*-1 FLAG destination vector (pBG-GY134). For FRPR-13 native promoter rescue ($P_{frpr-13}$FRPR-13), we generated a MosSCI plasmid (pBG-316) that contained 2000bp 5' UTR, *frpr-13* ORF and 2000bp 3'UTR. For pan-neuronal rescue, we generated a MosSCI plasmid (P_{rab-3}FRPR-13, pBG-GY808). For rescue with human GPR139, we built a MosSCI plasmid ($P_{frpr-13}$ GPR139, pBG-GY818) containing human GPR139 cDNA (NM_001002911.3). All plasmids were confirmed by sequencing.

The tgMOR strain, *bggIs10* [P_{rgef-1}FLAG::MOR; P_{ttx-3}RFP], was generated by UV/TMP integration of *bggEx125* [P_{rgef-1}FLAG::MOR; P_{ttx-3}RFP]. To generate *bggEx125* P_{rgef-1}::FLAG::MOR (10ng/µL), P_{ttx-3}::RFP (50 ng/µL) and pBluescript (50 ng/µL) were injected into N2 animals.

All transgenic rescues were done using Mos1-mediated single copy insertion (MosSCI). To generate tgMOR; *ttTi5605mos1* animals, *ttTi5605mos1*; *unc-119(ed3)*; *oxEx1578* animals were crossed into tgMOR animals, and *unc-119(ed3)* and *oxEx1578* were removed. TgMOR; *ttTi5605mos1* was crossed with tgMOR; *frpr-13(bgg8)* to generate the tgMOR; *frpr-13(bgg8); ttTi5605mos1* strain. For FRPR-13 rescue experiments, tgMOR; *frpr-13(bgg8); ttTi5605mos1* animals were injected with a DNA mixture containing P_{eft-3}Transposase (50ng/µL), P_{hsp}PEEL-1 (10 ng/µL), P_{rab-3}mCherry (10 ng/µL), P_{myo-2}mCherry (2.5 ng/µL) P_{myo-3}mCherry (5 ng/µL) and $P_{frpr-13}$FRPR-13, P_{rab-3}FRPR-13 or $P_{frpr-13}$hGPR139 (50ng/µL). Injected animals were selected using G418 (0.4 mg/mL) and integrated MosSCI transgenes and genotypes were confirmed PCR genotyping or sequencing as needed.

C. elegans behavior

Thrashing assays. Young adults were placed in 50 µL assay buffer (M9 + 0.01% Tween-20) or assay buffer containing indicated concentration of drug in 96 well plates. Individual wells contained a single animal. Thrashing behavior was scored for 1 minute by counting body bends at different time intervals following drug application. Animals were grown at 20$_{o}$C and experiments performed at room temperature. For each genotype, drug condition and time point, data was collected from 30 or more total animals obtained across 3

independent experiments. Statistical analysis was done using two-way ANOVA. P values reported in figures represent analysis of differences between genotypes or drug doses and time. While not reported in our figures, significant differences between genotypes and drug doses were further evaluated at each individual time point using Fisher's LSD *post-hoc* test with Bonferroni correction. All data are shown as mean ± S.E.M.

Multi-Worm Tracker recording. To record animal movement in liquid over time (Fig. 1C), we adapted Multi-Worm Tracker (MWT) . For tracking, animals were plated in 96 well plate lid with 30 µl assay buffer with or without 10 µM fentanyl. Images were captured for 1 min after animals were initially added to liquid (time 0) or after 90 minutes in buffer or fentanyl. Supplemental Movies 1 to 10 were generated using ImageJ.

Genetic screen and mutagenesis

Synchronized, L4 tgMOR animals were treated with 50mM ethyl methanesulfonate (EMS) for 4h at 20$_{o}$C with constant rotation. These P0 animals were washed with M9 and healthy animals were plated for 16 h to obtain F1s. F1 animals were removed by washing plates and remaining F2 eggs were grown to adulthood. Adult F2 animals were collected and washed with M9 buffer. Animals were transferred to a fresh glass tube and treated with morphine (300 µM). After 15 min treatment, animals were transferred to the start zone of 10 cm agar plate (Fig 2A). Animals were allowed to move for 15 min, and agar corresponding to start zones was then removed leaving only harvest zones (Fig 2A). Animals were harvested, moved to fresh plates and recovered for 24h. We screened mutants a second time using similar procedures, but treated animals for 75 min with

fentanyl (10 μM).

Genetic Mapping

Independently isolated tgMOR; mutants generated on the N2 background were crossed with CB4856 males and F1 cross-progeny were isolated. At least, 20 F2 progeny (independent recombinants) were isolated and phenotypically verified for each mutant. For a given mutant, all recombinants were starved, pooled and genomic DNA was isolated. For Whole Genome Sequencing (WGS), DNA libraries was prepared and WGS was done using MiSeq DNA Sequencing System (Illumina) with pair-end 250 nucleotide reads. WGS data was analyzed using CloudMap on the Galaxy platform (42).

CRISPR/Cas9 gene editing

To test mutations in the mapped regions for a given lesion, we engineered single point mutations of interest into the starting parental tgMOR strain. We directly injected asS.E.Mbled Cas9-crRNA-tracrRNA complexes with in vitro synthesized oligonucleotide templates to perform homology-directed repair (43). *Pha-1 (e2123*, ts allele) was simultaneously repaired by co-CRISPR to increase efficiency of isolating gene edited animals. 42 nt-length crRNA (20nt gene-specific sequence followed by 22 nt universal sequence), 74 nt-length tracrRNA, and repair template (containing ~35nt homology arms) was synthesized (Dharmacon and IDT). Recombinant 6xHis-Cas9 protein was purified from *E. coli* BL21 transformed with the plasmid pHO4d-Cas9. tgMOR; *pha-1* animals were injected with a mixture of recombinant Cas9 (10μg) and crRNA-tracrRNA-repair complexes (desired gene and *pha-1*) that were incubated for 15 min at 37 °C prior to

injection. Gene edited animals were isolated by growing animals at non-permissive temperature for *pha-1* (23 °C). Desired gene edits were confirmed by PCR genotyping and sequencing.

C. elegans immunoprecipitation and Western blotting

To detect FLAG::MOR, we performed immunoprecipitation. Wild-type or tgMOR worms were grown in liquid culture and harvested by centrifugation. After flash freezing in liquid N_2, worms were ground by Cryomill and extracted with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% Glycerol and 1% Triton X-100). 20mg of total protein was immunoprecipitated with protein G agarose beads and 2µL mouse monoclonal anti-FLAG antibody (M2, Sigma). Precipitates were incubated with 50 µL urea-SDS sample buffer (Urea: 8M) 37 °C for 30 min and run on 4-20% gradient gels (Bio-rad), and transferred to PVDF membranes. Rabbit polyclonal anti-FLAG antibody (Cell Signaling) was used for immunoblotting and blots were visualized with HRP conjugated secondary antibodies, ECL (Pierce, Pico West) and x-ray film.

Cell culture and transfection

HEK239T/17 cells were maintained in culture medium (DMEM supplemented with 10% v/v fetal bovine serum, MEM non-essential amino acids, and 1 mM sodium pyruvate). On the day of transfection, cells were split to 3 x 10^6 cells on 60 mm plates in culture medium supplemented with 1 µg/mL Matrigel and allowed to adhere for four hours. After four hours, cells were transfected using Lipofectamine 2000 (Invitrogen). All transfected DNA's were balanced to 10 µg using pcDNA3.1 and added dropwise per manufacturer's instructions.

Membrane Potential Dye Assay

HEK293T/17 cells were transfected with 1.26 µg MOR-FLAG pcDNA3.1; 0 µg (0x), 0.42 µg (1x), or 5.04 µg (12x) of GPR139 pcDNA3.1; 1.26 µg of GIRK1 pcDNA3.1; 1.26 µg of GIRK2a pcDNA3.1; 0.21 µg of Gαo pcMV5; balanced to 10 µg using pcDNA3.1 and added dropwise to cells per manufacturer's instructions. The next day, cells were re-seeded into a clear-bottom, black-walled 96-well plate at 1.5 x 10^5 cells per well and incubated for 3-4 hours at 37°C and 5% CO_2. Cells were then loaded with an equal volume of 1x membrane potential dye and incubated at room temperature in the dark for 2 hours. Changes in membrane potential were measured on the BMG POLARstar Omega plate reader after in-line injection of 0.1 µM morphine per manufacturer's instructions. Data were fit to a one-phase decay equation and the maximum inward current was calculated. Quantitative analysis was performed on 3 independent experiments with 4 technical replicates in each. One-way ANOVA with Dunnett's *post-hoc* analysis was applied to evaluate statistical significance.

Coimmunoprecipitation

HEK293T/17 cells were transfected with 0.84 ug of MOR-FLAG pcDNA3.1 and 0.84 ug of myc-GPR139 for 24 hours. The next day, cells were harvested, washed, and lysed in lysis buffer (1x phosphate-buffered saline (PBS), 150 mM NaCl, and Roche Complete EDTA-free protease inhibitors). Lysis was performed by sonication for 10 seconds at 30% for three cycles on ice. Homogenates were centrifuged at 100k x g for 30 minutes and cell pellets were extracted with detergent buffer (Lysis buffer + 1% Triton X-100) for 30 minutes at 4°C with gentle rocking. Solubilized membranes were centrifuged at 14,000 g

for 10 minutes. The extract (input) was incubated overnight at 4°C with 2 µg anti-FLAG antibody (Sigma) coupled to Protein G beads. Beads were subsequently washed 3 times with detergent buffer and eluted in 2x SDS-sample buffer (100mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 2% 2-mercaptoethanol, 0.00016% bromophenol blue, 20% (v/v) glycerol). Samples were western blotted using anti-MOR or anti-GPR139 antibody and visualized with chemiluminescent anti-rabbit HRP secondary antibodies using the KwikQuant Imager (Kindle Biosciences, LLC).

Cell Surface Abundance

Cell surface content of MOR was determined using Promega's NanoGlo® HiBiT Extracellular Detection System. HEK293T/17 cells were transfected with 0.84 µg HiBiT-tagged MOR pcDNA3.1 and 0 µg (0x), 0.42 µg (1x), or 5.04 µg (12x) of GPR139 pcDNA3.1 and incubated overnight. The next day, cells were re-seeded at 1.5 x 10^4 cells per well in culture medium without phenol red in a white-walled 96-well plate. Cells were further incubated for 3-4 hours at 37°C and 5% CO_2. Substrate buffer containing NanoGlo® reagent and LargeBiT protein was prepared per manufacturer's instructions. 50 µl of overlaying media was removed from the cells and 50 µl of substrate buffer was added to each well. Luminescence was immediately measured every 2.5 minutes for 20 minutes using the Perkin Elmer Envision plate reader at 0.1 seconds per well to ensure all wells reached a maximum, steady-state signal. Data are the mean of the normalized data from the final luminescence read from three independent experiments.

NanoBiT β-arrestin2 Assay

Agonist-induced β-arrestin2 recruitment to MOR was measured using Promega's NanoBiT® protein:protein interaction assay system. MOR tagged with a carboxy-terminal Small BiT (SmBiT) pcDNA3.1 (0.84 µg) was co-transfected with 0.42 µg β -arrestin2-LargeBiT pcDNA3.1 and 0 µg (0x), 0.42 µg (1x), or 5.04 µg (12x) of HA-GPR139 pcDNA3.1. The next day, cells were re-seeded into a white-walled 96-well plate at 1.5 x 10_4 cells per well in culture media without phenol red and incubated at 37°C and 5% CO_2 for an additional 3-4 hours. NanoGlo® substrate buffer was prepared by diluting NanoGlo® substrate 1:100 with BRET buffer (1x PBS, 0.5 mM $MgCl_2$, 0.1% (w/v) D-glucose). 25 µl of NanoGlo® reagent was dispensed into 25 µl of cell media and incubated for 60 seconds. Five second baseline recording was acquired measuring luminescence at 0.1 seconds per well in the PheraStar FSX plate reader. After five seconds, 10 µM DAMGO was injected and β-arrestin2 recruitment was measured as an increase in luminescence. Data are the average of three independent experiments.

***In situ* hybridization**

mRNA coexpression with single-cell resolution of *Gpr139* and *Oprm1* (MOR) was assessed with ViewRNA™ 2-plex *In Situ* Hybridization Assay (Panomics, Santa Clara, CA). Probes for *Gpr139* (NM_001024138.1; Cat# VB6-3197064-VT) and *Oprm1* (NM_001039652; Cat# VB1-13575) were used as previously described. Antibody staining for markers of cell subpopulations were performed at the end of the *in situ* hybridization procedure if Nissl staining was not used. Briefly, sections were blocked in 10% Donkey Serum, 0.1% Triton X-100, in PBS (PBST) for 30 minutes, primary antibodies were

incubated in 2% Donkey Serum/PBST for 1 hour, washed and fluorescent-dye (Alexa488) conjugated secondary antibodies (Invitrogen; Cat#A21206 and A21202) were applied in 2% Donkey Serum/PBST for 1 hour. Sections were then mounted using Fluoromont-DAPI (SouthernBiotech, Cat#0100-20). Confocal images were acquired at the Light Microscopy Facility, Max Planck Florida Institute, using a confocal microscope Zeiss LSM 880 Airy Scan (Carl Zeiss; Plan-Apochromat 20x/0.8 M27) setting the fluorescence intensity to non-saturating conditions. Quantification of coexpression was done using Fiji software (NIH).

G protein BRET Assay

HEK293T/17 cells were transfected with MOR-FLAG pcDNA3.1; 0 µg (0x), 0.42 µg (1x), or 5.04 µg (12x) of GPR139 pcDNA3.1; 0.84 µg Gαo pcMV5; 0.42 µg Venus(1-155)-Gγ2 pcDNA3.1; 0.42 µg Venus(156-299)-Gβ1 pcDNA3.1; 0.42 µg mas-GRK3-NanoLuc pcDNA3.1, and DNAs were balanced to 10 µg using pcDNA3.1 as described (44). Cells were incubated overnight, washed the next day in two mL of BRET buffer (0.1 % (w/v) glucose, 1x phosphate-buffered saline, and 0.5 mM $MgCl_2$), then gently lifted from the plate using 2 mL of BRET buffer. Cells were centrifuged at 500 g for 5 minutes and gently resuspended in 2 mL of BRET buffer. Twenty-five µl of 2x NanoGlo reagent was injected into 25 µl of cell suspension in a white-walled 96-well plate and a 5 second baseline recording was taken. One µM morphine was injected into each well and activation of MOR was measured kinetically on a PhereaStar FSX plate reader. Data are expressed as the change in BRET ratio (535 nm / 475 nm). Quantitative analysis was performed on 3 independent experiments with 4 technical replicates in each. One-way ANOVA with Dunnett's *post-hoc* analysis was applied to evaluate statistical significance.

Electrophysiology

Medial Habenula

Electrophysiological recordings of medial Habenula (MHb) neurons were performed with mice of either sex aged between 2-4 months. For preparation of acute brain slices used in loose-seal, cell-attached recording, mice were anesthetized with isoflurane and decapitated. The brain was quickly removed and rested for 30 seconds in ice-cold oxygenated NMDG cutting solution containing (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2$·, 10 $MgCl_2$, (adjusted to 7.2–7.4 pH with HCl). Coronal slices (280 μm thick) containing the habenula were cut on a vibratome (VT1200S, Leica) mounted on a porous membrane and incubated for 30 minutes at 34° C in oxygenated ACSF containing the following (in mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 18 $NaHCO_3$, 1.2 NaH_2PO_4, 10 glucose, then allowed to recover for at least 1 hour at room temperature before recording. For recordings, slices were transferred to a submerged recording chamber where they were continuously perfused at 2 ml/min with oxygenated ACSF containing picrotoxin (100 μM) and Kynurenic acid (3 mM). Loose-seal, cell-attached recordings of MHb neurons were obtained using glass electrodes (5–7 MΩ) filled with ACSF from neurons located in the basolateral MHb, at the border of the stria medullaris where we detected the highest levels of colocalization between *Oprm1* and *Gpr139*. Spontaneous firing was recorded under basal condition for at least 10 minutes followed by bath application of the selective MOR agonist (10 min), DAMGO {[D-Ala2-MePhe4-Gly(ol)5] enkephalin} (Tocris Bioscience, USA) and drug wash out (10 min). Changes in firing frequency are quantified as the mean value of the last

3 minutes of recording in the presence of drug, and normalized with respect to the last 3 minutes of baseline preceding bath application of drug.

To calculate the net inhibition of neuronal firing by DAMGO we used a built-in function from Graph Prism 7 to compute the area under the curve (AUC) during the different phases. Net inhibition expressed as fold changes was calculated as AUC during DAMGO application and wash-out divided by AUC of baseline. To calculate the recovery kinetics of neuronal firing, the average firing rate during the wash-out period was normalized to the first minute of wash-out and computed for the best fit of a nonlinear regression. The time of 50% recovery was plotted for comparison.

Current clamp signals were high pass filtered at 300 Hz. Acquisition was done using Clampex 10.5, MultiClamp 700B amplifier and Digidata 1440A (Molecular Devices, CA). Data sets were analyzed using unpaired two-tailed Student's *t*-test.

Locus Coeruleus

Mice of both sexes aged between 2-4 months were used. Horizontal slices containing Locus Coeruleus (LC) neurons were prepared as previously described (45). Briefly, mice were anesthetized and decapitated. The brains were quickly removed, blocked, and mounted in a chamber of vibrating tissue slicer (VT1200, Leica). Horizontal slices (260 µm) were cut in warm (28-32˚C) aCSF (in mM: 75 NaCl, 50 sucrose, 2.5 KCl, 6 $MgCl_2$, 0.1 $CaCl_2$, 1.2 NaH_2PO_4, 2.5 D-glucose, 25 $NaHCO_3$, 0.01 MK801) equilibrated with 95% O_2/5% CO_2. Slices divided into two hemispheres along the midline and each hemisphere was placed into an individual well of a custom slice incubation chamber where they remained in oxygenated aCSF (in mM: 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 1.2 NaH_2PO_3, 11 D-

glucose, and 21.4 $NaHCO_3$) in 35°C water bath until use. During recording, slices were transferred to a submerged recording chamber where they were continuously perfused at 1 – 2 ml/min with oxygenated aCSF with picrotoxin (100 µM) and Kynurenic acid (3 mM), and maintained at 32 – 36°C. Whole-cell recordings were obtained from LC neurons with borosilicate glass pipettes (2–5 MΩ) filled with the following solution (in mM): 115 potassium methanesulfonate, 20 KCl, 1.5 $MgCl_2$, 5 HEPES (potassium salt), 10 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, 2 Mg-ATP, and 0.2 Na-GTP, with a pH of 7.4 and osmolarity of 280 mOsmol. Series resistance was monitored without compensation and remained < 15 MΩ for inclusion. All data were collected with a HEKA EPC10 amplifier system (HEKA Instruments, Holliston, MA), and transferred to a PC computer using an ITC-16 digital-to-analog converter (HEKA Instruments). The signals were filtered at 2.9 kHz and digitized at 10 kHz using Patchmaster software (HEKA Instruments). Voltages were not corrected for the liquid-liquid junction potential. Data were analyzed offline using Patchmaster and Igor Pro (Version 6.35, WaveMetrics). To analyze the effect of morphine on spontaneous firing frequency of LC neurons, neurons were recorded under current clamp mode at resting membrane potential. After 3 – 5 min stabilization, 0.1 µM morphine were bath applied for 5 min. Firing frequency was calculated with a 10s bin and normalized to the average frequency before morphine application. Data sets was analyzed using paired two-tailed Student's *t*-test.

Hotplate

Animals (n = 14 -17 per group; approximately 3 months old, males and females) were tested for analgesic effects of morphine using hotplate set to 52°C. Mice were injected (0,

10, and 20 mg/kg morphine, s.c.) 30 minutes prior to start, then individually placed onto hotplate, observed for any physical signs of sensitivity to heat (i.e. licking paw), and quickly removed. Any mice that showed no signs of discomfort after 50 seconds were removed. Animals were tested every 30 minutes for 2 hours. Time (seconds) spent on hotplate was graphed as %MPE (test latency – baseline latency/ cut-off time – baseline latency x 100). Data were analyzed by two-way ANOVA with Dunnett's *post-hoc* test.

Tai immersion

Animals (n = 11 per group; approximately 3 months old, males and females) were tested for nociceptive reaction and as an evaluation of morphine's analgesic effects by tail immersion. Individual mice were transferred to experimentation room and restrained using a tube with airholes. Once animal was immobilized, 2/3 of entire tail was dipped into a water bath heated to 53°C. Latency to remove tail from water bath was recorded. Data were analyzed by two-way ANOVA with Dunnett's *post-hoc* test.

Von Frey

Animals (n = 11 per group; approximately 3 months old, males and females) were subjected to manual von Frey microfilaments test to look at mechanical allodynia and hyperalgesia. Animals were habituated to experimental room and chamber (30 cm x 30cm x 30 cm black plexiglass with 0.5cm plastic mesh floor) for 30 minutes one day prior and immediately before experiment to prevent additional stress. During experimentation, subjects were timed for withdrawal responses to monofilament applied perpendicularly to plantar surface of hind paw until filament buckles (approximately 0.2 to 13.7mN) using the up down

method for filament size (all animals began with 4.08mN microfilament) Once animals showed brisk paw withdrawal, lick or shaking/flicking of paw, stimulus was removed. Time from when monofilament is applied to hind paw to the point of animal's withdrawal response were recorded. All animals were tested 5 times in 10 second intervals. Data were analyzed by two-way ANOVA with Dunnett's *post-hoc* test.

Conditioned Place Preference

Mice (n = 8 per group; approximately 3-5 months old, females and males) were initially placed into a three-chamber box (black and white designated chambers with connecting tunnel, built in house) and tracked (ANY-Maze, Stoelting Co., Wood Dale, IL USA) for 30 minutes. All animals had scored less than 60% of total time in all chambers and deemed unbiased. Animals were then drug paired, using unbiased method, to either white or black chamber for morphine stimulus (7.5, 10, 20mg/kg, s.c.) and other chamber paired to saline control. Animals were trained for 6 consecutive days, alternating in paired chambers. Once training was completed, mice were placed into tunnel and given 30 minutes to explore all chambers. Place preference score was graphed as time spent in morphine-paired chamber minus time spent in saline paired chamber. Data were analyzed by two-way ANOVA with Dunnett's post *hoc-test.*

Withdrawal

Naïve animals (n = 8 - 10 per group; 3 – 4 months old, males and females) were individually housed a few days prior to start. Identities of all subjects were concealed and the investigators were blinded at the beginning of study. Animals were injected once daily in

increasing dose of 20, 40, 60, and 80 mg/kg morphine (s.c.). On the day 5, animals were injected 100mg/kg morphine (s.c.). Withdrawal was precipitated 3 hours later by injecting 1m/kg naloxone (s.c.). Withdrawal was scored immediately for 30 minutes by number of jumps, dog shakes, paw tremor, back-walking, and tremor. Ptosis was also scored for every 5-minute interval over the 30 minutes. Scores are presented normalized to 100 for *Gpr139*$^{+/+}$ in comparison with *Gpr139*$^{-/-}$. Data were analyzed by two-way ANOVA Dunnett's post hoc test. Global withdrawal score was calculated for each animal after subtracting signs observed during baseline. The behavior of *Gpr139*$^{+/+}$ mice was used as reference and was given a global score of 100 points, with each sign receiving equal weight. Global withdrawal score was then analyzed by unpaired two-tailed Student's *t*-test. Animals were weighed daily for weight loss in comparison to pre-injected weight. Weight loss is shown as percentage of weight loss (final weight / pre-injected weight x 100). Data set was analyzed using unpaired two-tailed Student's *t*-test.

Food/Morphine Self-Administration

Naïve mice (n = 6 per group; 3 – 4 months old, males) were placed into operant conditioning chambers (MedAssociates Inc., Maine USA) and trained to differentiate between two levers: one paired with food reward and the other with no consequences (FR5TO20). When all subjects had met criteria (FR5TO20; at least 20 rewards obtained in an hour session; 85% accuracy between levers) mice were feed *ad libitum* for 2 days prior to surgical implantation of catheter into jugular vein. Mice were anesthetized with 1-3% isoflurane in oxygen and implanted with indwelling intravenous catheters inserted into the right external jugular vein. Catheters consisted of 6 cm length of plastic tubing fitted to a

guide cannula (Plastics One, Wallingford, CT), bent to a curved right angle and encased in dental acrylic. Catheters were threaded subcutaneously from the right jugular vein to the animals' back, exiting the body via a guide cannula. All subjects were given recovery periods for at least 48 h after the surgery. After recovery, animals were placed into food self-administration sessions for 2 days and transitioned onto morphine (0.3 mg/kg/infusion) under identical condition (FR5TO20; 2-hour sessions). When subjects met standard (at least 15 infusions per session; 85% accuracy between the active and inactive levers; less than 20% variation of the running mean without a trend of an increase of a decrease for three consecutive sessions), animals were subjected to various doses of either vehicle and/or JNJ-63533054 (GPR139 agonist) compound. Vehicle/compound was administered via cannula infusion immediately before each session. Data were analyzed by two-way ANOVA with Dunnett's *post-hoc* test.

Open Field

Animals (n = 8 - 10 per group; approximately 3 months old, males and females) were placed into center of open field arena (140cm x 140cm x 140cm) and movement was monitored (ANY-Maze, Stoelting Co., Wood Dale, IL USA) for 1 hour. Graphs were plotted as total distance travelled and distance at 10-minute intervals. Data were analyzed using unpaired two-tailed Student's *t*-test and two-way ANOVA with Dunnett's *post-hoc* test.

RotaRod

Mice (n = 8 - 10 per group; approximately 3 months old, males and females) were placed into RotaRod apparatus (RotaRod, IITC Inc., Woodland Hills, CA USA) for 6 sessions per day for 3 consecutive days. Animals were tested on motor and spatial capabilities at gradual ascending speed. Cut off time was 120 seconds. RotaRod results were graphed with RPM achieved during sessions over days. Data were analyzed by two-way ANOVA with Dunnett's *post-hoc* test.

Metabolic measurements

Weight and body composition were taken from littermates (n = 12 -13 per group; approximately 3 months old, males and females). Mice were weighed prior to body composition analysis done using NMR minispec system (Bruker Energy & Supercon Technologies Inc.; Billerica MA, USA). Percent body composition were graphed as: grams of fat and muscle over total weight multiplied by 100. Data sets was analyzed using unpaired two-tailed Student's *t*-test.

Compound testing

Subjects used in pain paradigms tested with either vehicle and/or JNJ-63533054 (GPR139 ligand) compound (o.g.) at various doses prior to start of final sessions. Data were analyzed by one-way ANOVA with Dunnett's *post-hoc* test.

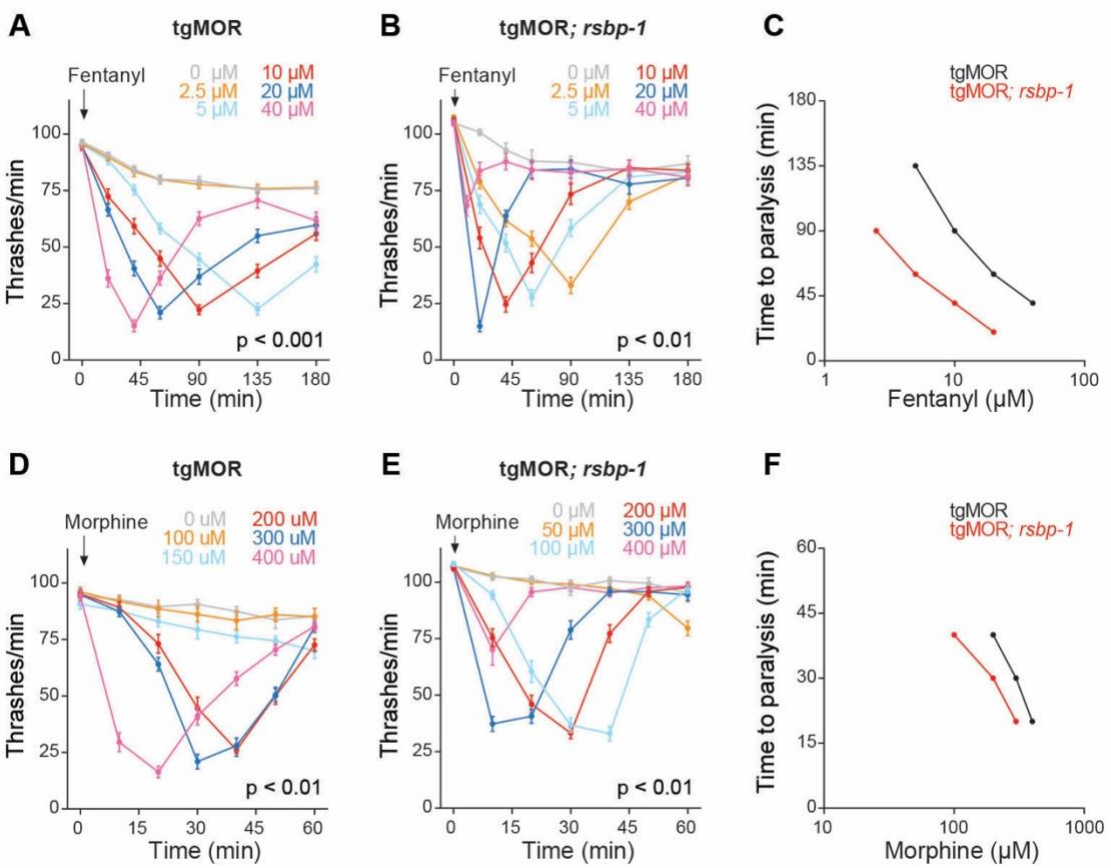

Fig. S1. Expanded dose effects showing tgMOR; *rsbp-1* animals are hypersensitive to fentanyl and morphine.

Time course shows fentanyl concentrations inducing paralysis on (**A**) tgMOR or (**B**) tgMOR; *rsbp-1* mutants. (**C**) Dose response showing tgMOR; *rsbp-1* mutants are hypersensitive to fentanyl. Time course shows morphine concentrations inducing paralysis in (**D**) tgMOR and (**E**) tgMOR; *rsbp-1* mutants. (**F**) Dose response showing tgMOR; *rsbp-1* mutants are hypersensitive to morphine. For all genotypes and drug conditions, means are shown from 30 or more animals obtained from three independent experiments. Significance tested using two-way ANOVA. All results presented as mean ± S.E.M.

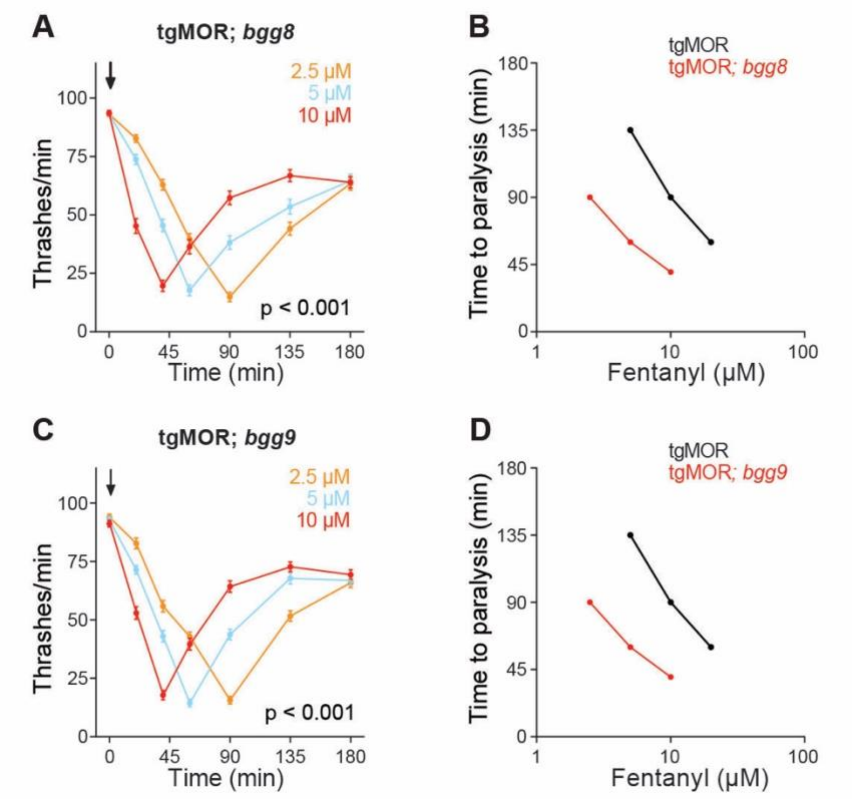

Fig. S2. Dose response showing tgMOR; *bgg8* and tgMOR; *bgg9* mutants are hypersensitive to fentanyl.
(**A**) Time course of fentanyl concentrations inducing paralysis on tgMOR; *bgg8* animals. (**B**) Dose response showing tgMOR; *bgg8* mutants are hypersensitive to fentanyl. (**C**) Time course of fentanyl concentrations inducing paralysis on tgMOR; *bgg9* animals. (**D**) Dose response showing tgMOR; *bgg9* mutants are hypersensitive to fentanyl. For all genotypes and drug conditions, means are shown from 30 or more animals obtained from three independent experiments. Significance tested using two-way ANOVA. All results presented as mean ± S.E.M.

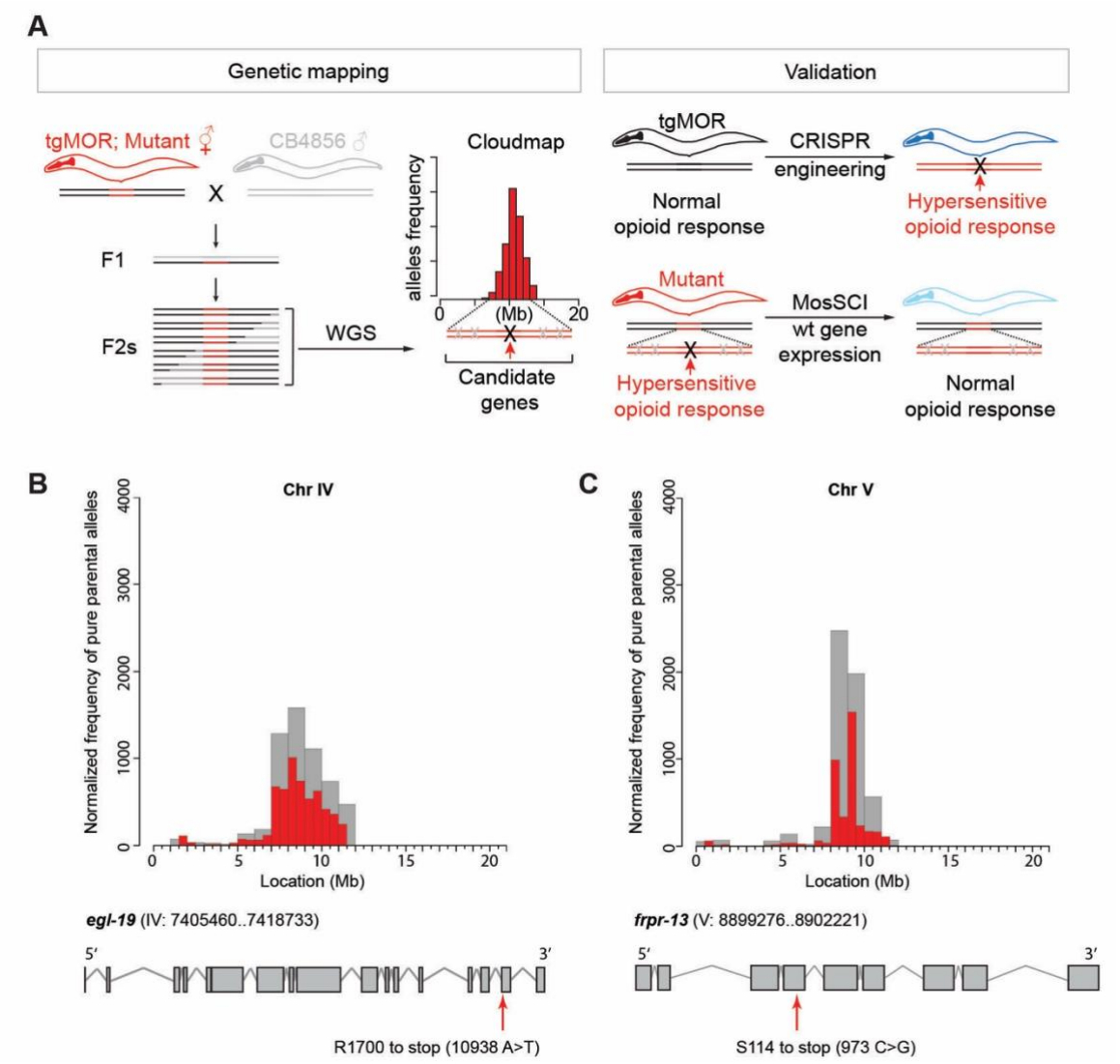

Fig. S3. Strategy and identification of mutations in tgMOR; *bgg8* and tgMOR; *bgg9*. (**A**) Strategy for evaluating tgMOR mutants isolated from forward genetic screen. Whole-genome sequencing and mapping identifies mutations. Individual mutations are then validated for effects on opioid sensitivity using CRISPR/Cas9 gene editing and MosSCI transgene expression. (**B**) Plot showing mapped region of chromosome IV (red and grey bars) containing *bgg8*. Gene diagram showing mutation in *egl-19* contained in mapped region. (**C**) Mapping plot for *bgg9* (red and grey) on chromosome V. Gene diagram showing mutation in *frpr-13* contained in mapped region.

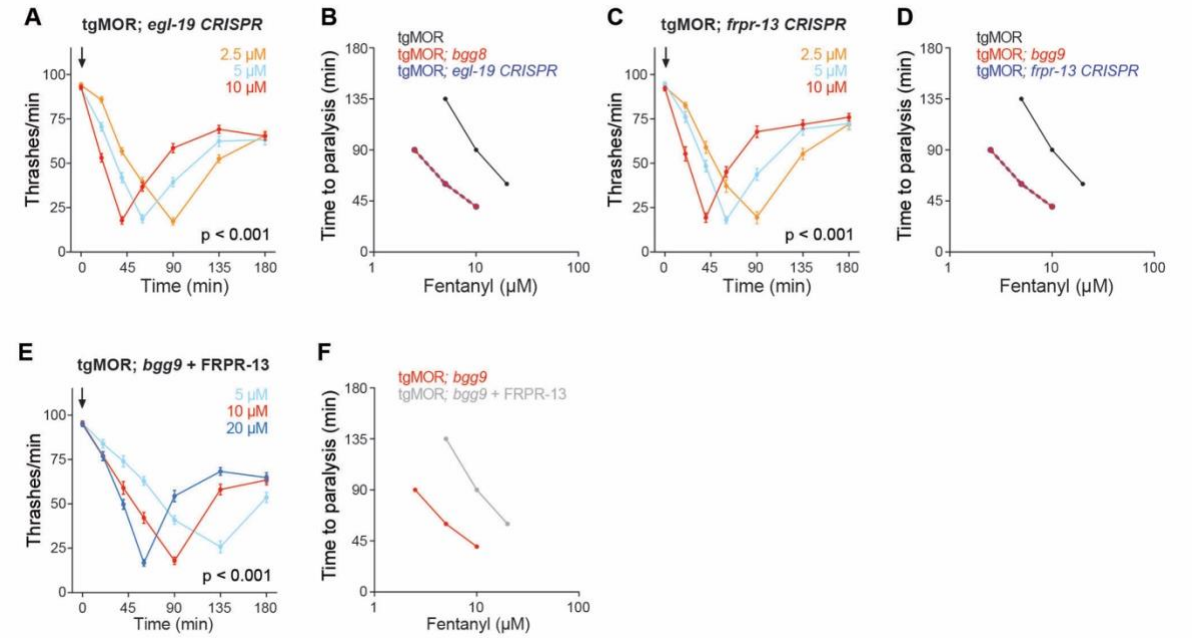

Fig. S4. CRISPR editing and MosSCI transgene expression show mutations in *egl-19* and *frpr-13* cause hypersensitivity to fentanyl.

(**A**) Time course of fentanyl concentrations inducing paralysis on tgMOR; *egl-19 CRISPR* animals. (**B**) Fentanyl dose response showing tgMOR; *egl-19 CRISPR* and tgMOR; *bgg8* both cause hypersensitivity to fentanyl (leftward shift) compared to tgMOR animals. (**C**) Time course of fentanyl concentrations inducing paralysis on tgMOR; *frpr-13 CRISPR* animals. (**D**) Fentanyl dose response showing tgMOR; *frpr-13 CRISPR* and tgMOR; *bgg9* both cause hypersensitivity to fentanyl. (**E**) Time course of fentanyl concentrations inducing paralysis on tgMOR; *bgg9* animals carrying a single copy of FRPR-13 driven by its native promoter. (**F**) Fentanyl dose response showing hypersensitivity (leftward shift) of tgMOR; *bgg9* animals are reversed by transgenic FRPR-13. Arrows denote fentanyl (10 µM) application. For all genotypes and drug conditions, means are shown from 30 or more animals obtained from three independent experiments. Significance tested using two-way ANOVA. All results presented as mean ± S.E.M.

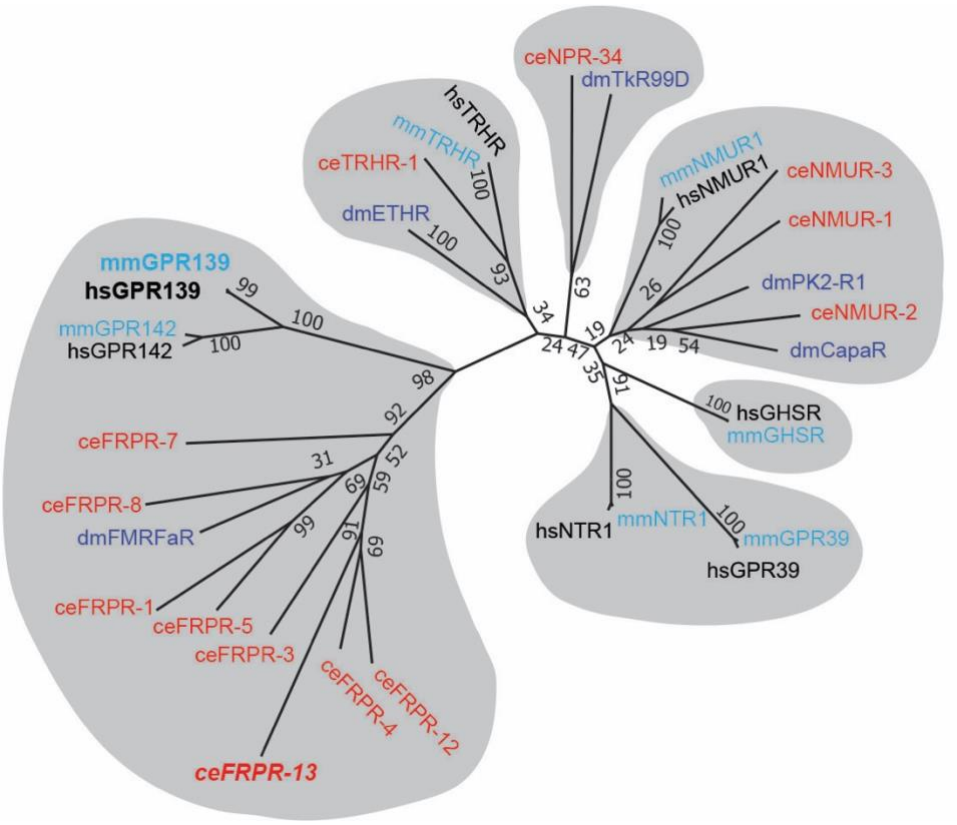

Fig. S5. FRPR-13 is one of several *C. elegans* receptors with homology to mouse and human GPR139.

Phylogeny of different receptors in the Neurotensin/GPR139 cluster of GPCRs from human (black), mouse (green), fly (blue) and *C. elegans* (red). *C. elegans* FRPR-13 is part of an expanded group of receptors in worms with closest homology to human and mouse GPR139 and GPR142. Phylogenetic analysis done using protein sequences and MEGA 5 software.

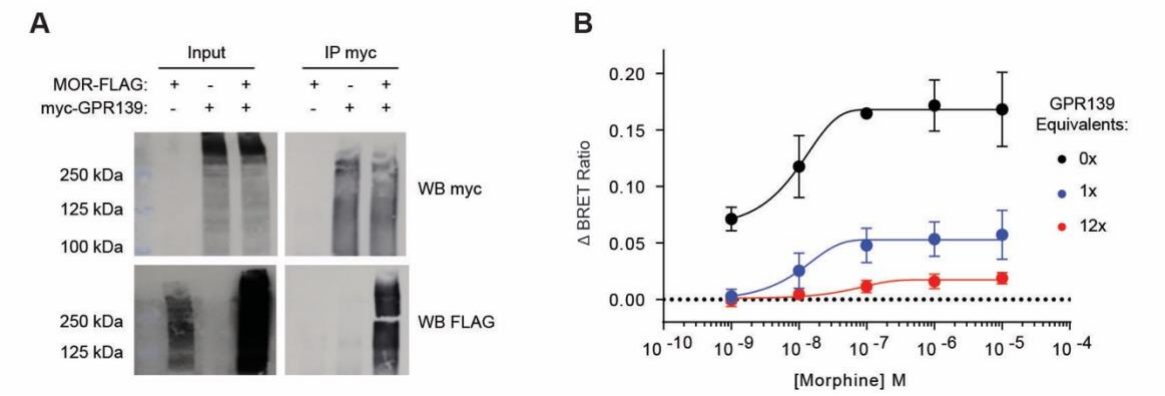

Fig. S6. Interaction of GPR139 and MOR in HEK293T cells and its functional impact. (**A**) Coimmunoprecipitation of myc-GPR139 with MOR-FLAG from HEK293T cells. Western blot shown is representative from three independent experiments. Note orientation of coIP is reversed from experiment shown in Figure 3D. (**B**) GPR139 coexpression reduces morphine-induced activation of Gαo by MOR across a range of morphine doses. Shown is mean from three independent experiments ± S.E.M. Two-way ANOVA indicates significance of the differences between conditions, Tukey *post hoc* test $p<0.001$ for 1X and 12X as compared to 0X.

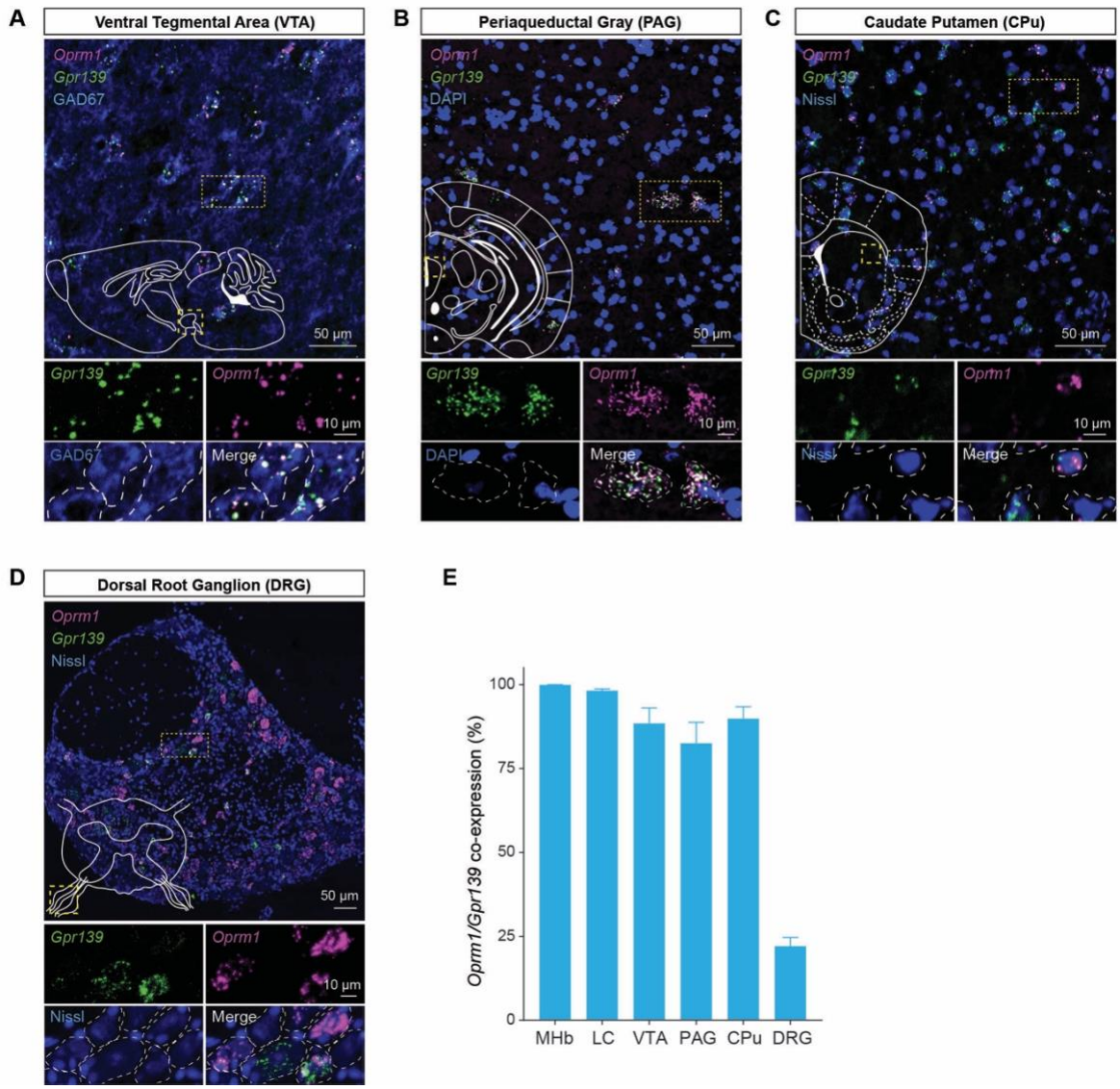

Fig. S7. *Gpr139* is coexpressed with *Oprm1* in multiple brain regions.
Representative images of double *in situ* hybridization using probes against *Oprm1* (red) and *Gpr139* (green) in (**A**) Ventral Tegmental Area (VTA), (**B**) Periaqueductal Gray (PAG), (**C**) Caudate Putamen (CPu), and (**D**) Dorsal Root Ganglion (DRG). Soma of each cell (blue) is identified by (**A**) GAD67 antibody staining, (**B**) DAPI staining, or (**C and D**) Nissl staining. Soma boundaries are designated by dashed lines. (**E**) Quantification of *Gpr139* and *Oprm1* coexpression in each brain region. An average of 1,200 neurons per region from 2-3 mice were quantified. All results were reported as mean ± S.E.M. For **A-D**, Red dashed boxes highlight regions shown below at higher magnification.

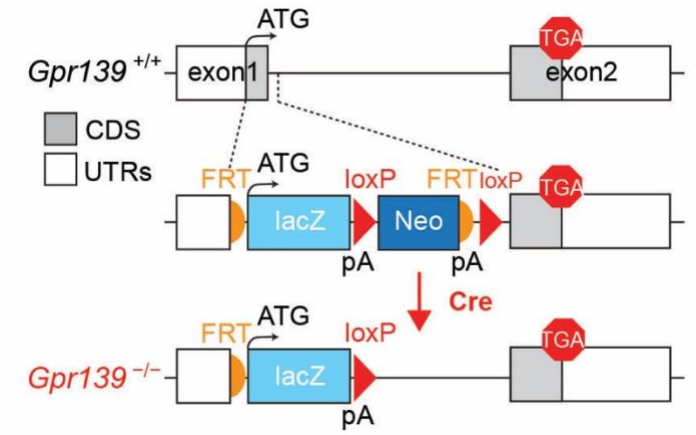

Fig. S8. *Gpr139* knockout strategy.
Schematic showing strategy for deleting *Gpr139*. In *Gpr139*$^{-/-}$ mice, the coding sequence (CDS) of exon 1 is replaced by lacZ insertion creating a *Gpr139* null allele.

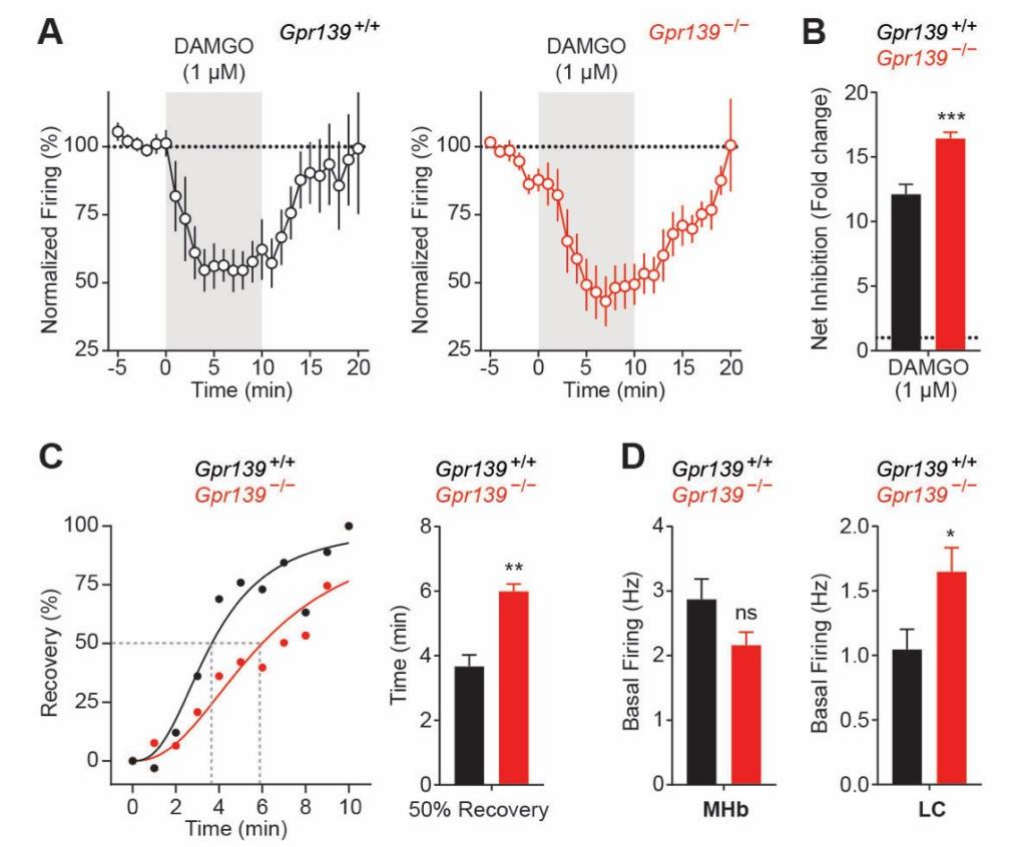

Fig. S9. Electrophysiological properties of MHb and LC neurons from *Gpr139*$^{-/-}$ mice.

(**A**) Effect of saturating DAMGO concentration on firing of MHb neurons. (**B**) Quantification of net inhibition of neuronal firing produced by DAMGO (1 μM, 10 minute application) shows significantly larger effect in *Gpr139*$^{-/-}$ neurons. N=9 cells from 4-5 mice per genotype. (**C**) Recovery kinetics of MHb neurons following washout of DAMGO (1 μM) is significantly slower in *Gpr139*$^{-/-}$ neurons. (**D**) Lack of GPR139 does not affect spontaneous firing of MHb neurons but increases firing rate of LC neurons (n = 19-20 cells from 8-11 mice per genotype). Significance tested using unpaired two-tailed Student's *t*-test. All results presented as mean ± S.E.M. * $p < 0.05$; ** $p<0.01$; ns, no significant difference.

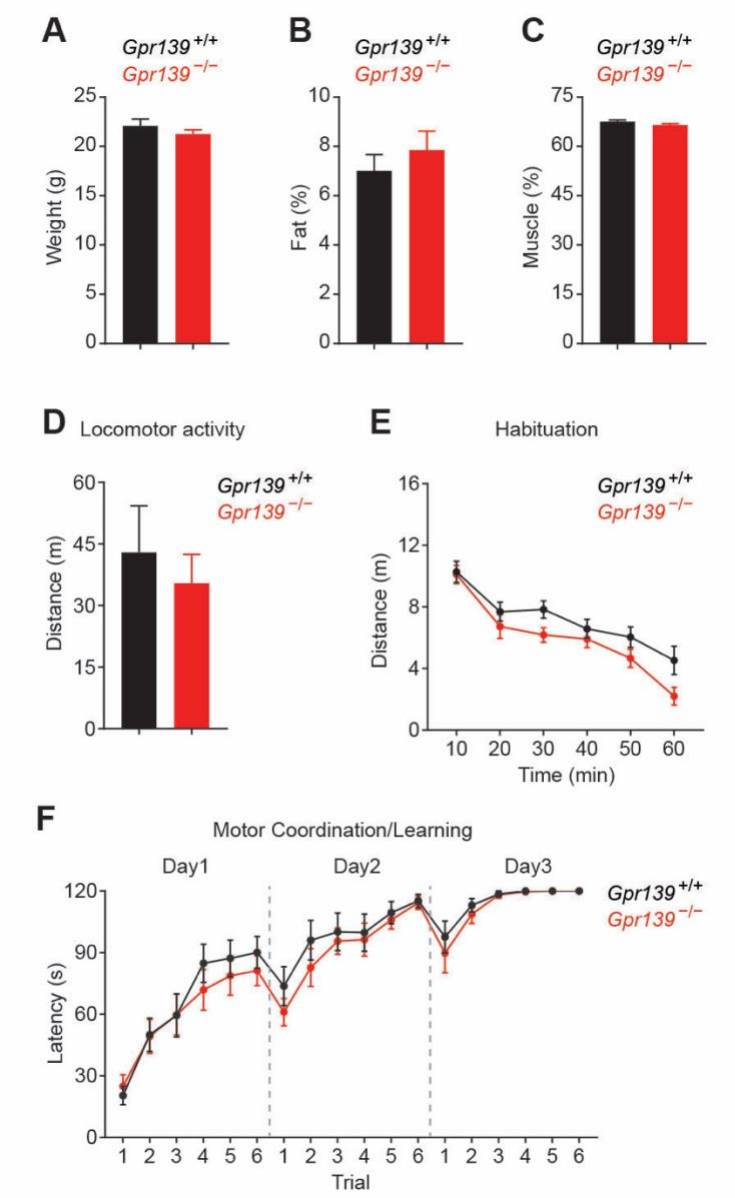

Fig. S10. *Gpr139* knockout mice have normal weight, metabolism, locomotion and motor learning.

(**A**) Body weight of *Gpr139* $^{+/+}$ and *Gpr139*$^{-/-}$ mice at approximately 4 months of age (n = 12 - 13 per group; males and females). (**B**) Percent body fat of *Gpr139* $^{+/+}$ and *Gpr139*$^{-/-}$ mice at approximately 4 months old (n = 12 - 13 per group; males and females). (**C**) Percent body muscle of *Gpr139* $^{+/+}$ and *Gpr139*$^{-/-}$ mice at approximately 4 months old (n = 12 - 13 per group; males and females). (**D**), Open Field (total distance, n = 8 - 10 per group; males and females). (**E**) Open Field distance over time (n = 8 - 10 per group; males and females). Two-way ANOVA. (**F**) RotaRod (n = 8 - 10 per group; males and females). For **A-D**, significance tested using unpaired two-tailed Student's *t*-test. For **E and F**, significance tested using two-way ANOVA. Note no significant differences ($p>0.05$) found between *Gpr139*$^{+/+}$ and *Gpr139*$^{-/-}$ mice for all results, which are presented as mean ± S.E.M.

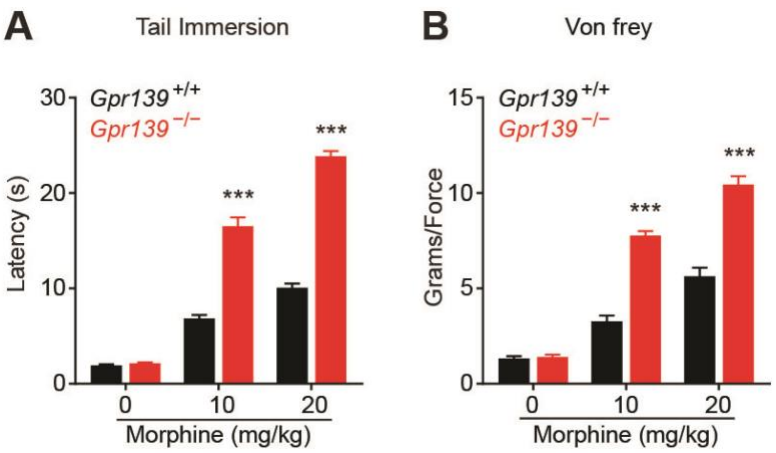

Fig. S11. Gpr139 knockout mice show increased dose-dependent analgesic effect to morphine.
(**A**) *Gpr139*$^{-/-}$ mice have increased tail immersion threshold (latency) in response to morphine (n = 11 per dose; males and females). **(B)** *Gpr139*$^{-/-}$ mice have increased Von Frey threshold (grams/force) in response to morphine (n = 11 per dose; males and females). Significance tested using two-way ANOVA. All results were presented as mean ± S.E.M. *** $p<0.001$ for genotype comparisons

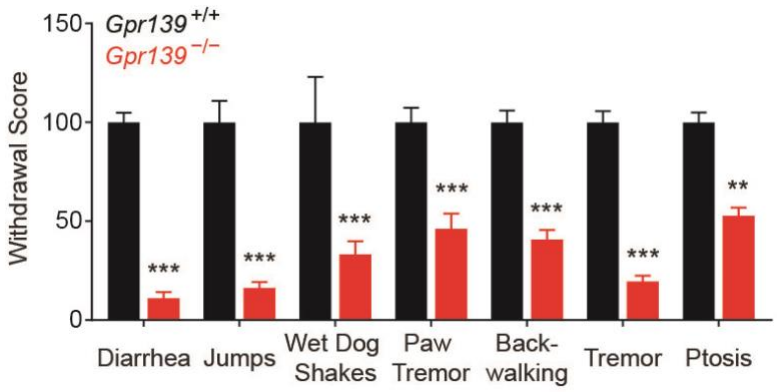

Fig. S12. *Gpr139* knockout mice show decreased effects in a range of behaviors with naloxone-precipitated somatic withdrawal following chronic morphine exposure.
Effects of morphine withdrawal are reduced in *Gpr139*$^{-/-}$ mice compared to *Gpr139*$^{+/+}$ littermates (n = 8 ~ 10 per genotype; males and females). Behaviors (jumps, wet dog shakes, paw tremor, back-walking, tremor, and ptosis) were scored and normalized to values observed in *Gpr139*$^{+/+}$. Significance tested using two-way ANOVA. All results presented as mean ± S.E.M. ** $p<0.001$, *** $p<0.001$.

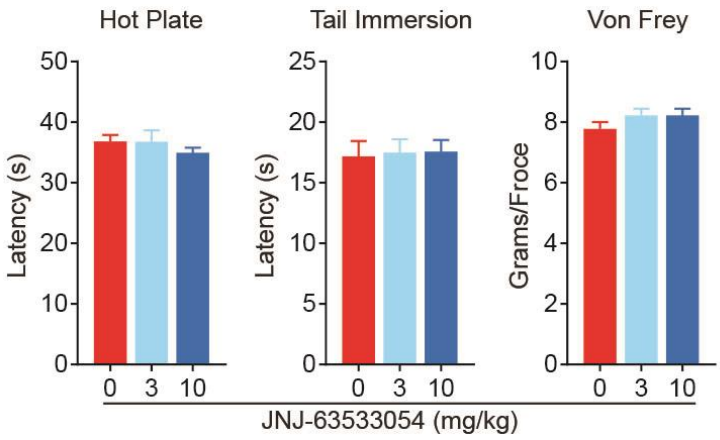

Fig. S13. Lack of JNJ-63533054 effect on morphine analgesia in *Gpr139* knockout mice.

Multiple doses of JNJ-63533054 do not affect morphine-induced (10mg/kg) analgesia in *Gpr139*$^{-/-}$ mice. Hot plate assay (n = 14 per group; males and females), tail immersion assay (n = 6 per dose; males and females), von Frey assay (n = 9 per dose; males and females). Significance tested using one-way ANOVA. Note no significant differences ($p>0.05$) found between JNJ treated *Gpr139*$^{-/-}$ mice and 0mg/kg controls. All results presented as mean ± S.E.M.

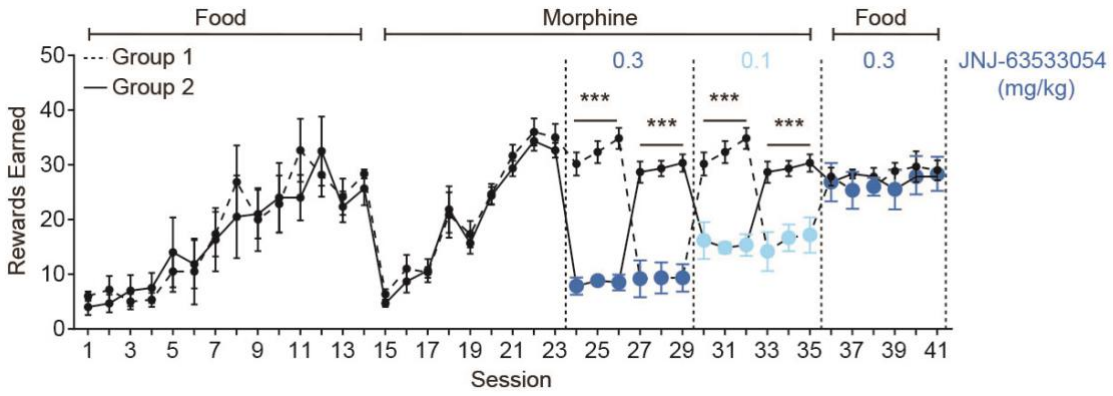

Fig. S14. Activation of GPR139 by JNJ-63533054 reverses morphine self-administration.

Naïve *Gpr139*$^{+/+}$ mice (n = 6 per group; 3 – 4 months old, males) were trained with food-administration and switched to morphine administration (0.3 mg/kg/infusion). GPR139 agonist JNJ-63533054 significantly reduced morphine intake but not food intake. Significance tested using two-way ANOVA. All results presented as mean ± S.E.M.
*** $p < 0.001$.

MHb	% *Oprm1*$^+$ expressing *Gpr139*	% of NeuN$^+$ expressing only GPR139	% of NeuN$^+$ expressing only MOR
	99.94 ± 0.06	0.06 ± 0.06	0.03 ± 0.03
LC	% *Oprm1*$^+$ expressing *Gpr139*	% of TH$^+$ expressing only GPR139	% of TH$^+$ expressing only MOR
	98.23 ± 0.48	0.00 ± 0.00	1.70 ± 0.51
VTA	% *Oprm1*$^+$ expressing *Gpr139*	% of GAD67$^+$ expressing only GPR139	% of GAD67$^+$ expressing only MOR
	88.46 ± 4.58	2.18 ± 1.20	8.87 ± 3.70
PAG	% *Oprm1*$^+$ expressing *Gpr139*		
	82.51 ± 6.25		
CPu	% *Oprm1*$^+$ expressing *Gpr139*		
	89.84 ± 3.57		
DRG	% *Oprm1*$^+$ expressing *Gpr139*	% of Nissl$^+$ expressing only GPR139	% of Nissl$^+$ expressing only MOR
	22.07 ± 2.59	15.82 ± 3.13	47.20 ± 3.15

Table S1. Quantification of Oprm1 and Gpr139 expression in neuronal populations

Supplemental Movie Legends

Movie S1. Movie simultaneously showing wt animals and tgMOR animals treated with buffer or 10 μM fentanyl at time 0. Movie is 51 seconds shown at 2x speed.

Movie S2. Movie showing wt animals treated with buffer at time 0. Movie is 51 seconds shown at 2x speed.

Movie S3. Movie showing tgMOR animals treated with buffer at time 0. Movie is 51 seconds shown at 2x speed.

Movie S4. Movie showing wt animals treated with 10 μM fentanyl at time 0. Movie is 51 seconds shown at 2x speed.

Movie S5. Movie showing tgMOR animals treated with 10 μM fentanyl at time 0. Movie is 51 seconds shown at 2x speed.

Movie S6. Movie simultaneously showing wt animals and tgMOR animals 90 minutes after initial treatment with buffer or 10 μM fentanyl. Movie is 54 seconds shown at 2x speed.

Movie S7. Movie showing wt animals 90 minutes following initial treatment with buffer. Movie is 54 seconds shown at 2x speed.

Movie S8. Movie showing tgMOR animals 90 minutes following initial treatment with buffer. Movie is 54 seconds shown at 2x speed.

Movie S9. Movie showing wt animals 90 minutes following initial treatment with 10 μM fentanyl. Movie is 54 seconds shown at 2x speed.

Movie S10. Movie showing tgMOR animals 90 minutes following initial treatment with 10 μM fentanyl. Movie is 54 seconds shown at 2x speed.