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Supplementary Materials for

The prolactin receptor long isoform regulates nociceptor sensitization and opioid-induced hyperalgesia selectively in females

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/12/529/eaay7550/DC1)

Data file S1 (Microsoft Excel format). Raw data.

Materials and Methods

Animals

All procedures were approved by the University of Arizona Institutional Animal Care and Use Committee in accordance with the guidelines of the committee for Research and Ethical Issues of the International Association for the Study of Pain. Female and male 8-12-week old mice were used in this study. Mice were housed 2-5 per cage and were maintained in a climate-controlled room on a 12-hour light/dark cycle with access to food and water ad libitum.

C57BL/6J mice were purchased from Jackson Laboratories . PRLR-L^{Cre} mice were made by Dr. Ulrich Boehm (University of Saarland School of Medicine, Homberg, Germany) and kindly provided by Dr. Armen Akopian (University of Texas Health Science Center at San Antonio, San Antonio, Texas). In these mice, an internal ribosome entry site (IRES) followed by Cre recombinase cDNA is inserted immediately after exon 10 in the *prlr* gene *(41)*. MOR-mCherry$^{+/+}$ mice (B6;129S2-Oprm1tm4Kff/J; Stock No: 029013) and Ai6 green fluorescent protein (GFP$^{flx/flx}$) reporter mice (B6.Cg-Gt(ROSA)26Sortm6(CAG-ZsGreen1)Hze/J; Stock No: 007906) were purchased from Jackson Laboratories. To make PRLR-L$^{Cre/-}$/GFP$^{flx/-}$/MOR-mCherry$^{+/-}$ mice, we first bred MOR-mCherry$^{+/-}$/GFP$^{flx/-}$ mice by crossing MOR-mCherry$^{+/+}$ with GFP$^{flx/-}$ mice. We then crossed MOR-mCherry$^{+/-}$/ GFP$^{flx/-}$ with PRLR-L$^{Cre/-}$ mice to avoid the off-target Cre expression in the germline.

Drugs and routes of administration

Drugs. Morphine pellets (75 mg) and morphine sulfate (10, 30, 100 mg/kg s.c. in saline) were obtained from NIDA Drug Supply Program (NDSP). BIBN 4096 (10 μg i.th. in 10%

DMSO, 10% Tween 80, 80% saline) was purchased from Tocris Bioscience (Batch# 5A/211400). (+)-MK-801 maleate (3.5 nM i.t. in saline) was purchased from EMD Millipore Corp (Lot# 3103998). Cabergoline (1.2 mg/kg i.p. in 10% DMSO, 10% Tween 80 and 80% saline) was from Tocris Bioscience (Batch# 2A/206222). Naloxone (3 mg/kg s.c. in saline) was purchased from Tocris. Rat CGRP (1 ug, i.th. in PBS) was purchased from Millipore Sigma).

Subcutaneous injection. In lightly restrained and unanesthetized mice, a 27G needle placed on a 1 ml syringe was inserted into the loose skin over the neck followed by injection of 10 ml/kg solution.

Intrathecal injection. In lightly anesthetized (2% isoflurane) mice, a 30G needle attached to a 25-µl Hamilton syringe were inserted between the L5 and L6 vertebral bones, puncturing through the dura indicated by a reflexive flick of the tail or formation of an "S" shape by the tail, then followed by injection of 5 µl of the indicated solution.

Intraperitoneal injection. In lightly restrained and unanesthetized mice, a 27G needle attached to a 1 ml syringe was inserted to the lower right quadrant of the abdomen, followed by injection of 10 ml/kg solution.

Dural injection. In lightly anesthetized mice (2% isoflurane), 5 µl of solution was injected on the dura by using a modified internal-cannula (In-vivo1, part #8IC313ISPCXC, Internal Cannula, standard, 28 gauge, fit to 0.7 mm). The injection site was at the intersection of the lambdoidal and sagittal sutures. The length of the projection was modified using calipers to be from 0.6 to 0.7 mm in order not to puncture the dura.

gRNA design and cloning for CRISPR targeting

Our strategy to delete the PRLR-L focused on targeting the first exon specific to this alternatively spliced variant (ENSMUST00000124470.7) using a guide RNA (gRNA) as described previously *(65)*. We identified the exon 10 (ENSMUSE00000770736) of the mRNA coding the PRLR-L to be amenable for CRISPR targeting. Targeting this exon will not remove the ligand binding fragment of the PRLR-L but will delete the intracellular fragment responsible for signaling transduction (gRNA PRLR-L: GTATCTGTGTTTCAATAGAA, quality score 55). To delete total PRLR (PRLR-S and PRLR-L), the gRNA was designed on the first exon common to all mRNA arising from the *Prlr* gene (ENSMUST00000128921.7, gRNA total PRLR: GTGTCAGGGGAACGACATTTG, quality score 97). To delete the μ-opioid receptor the gRNA (gRNA oprm1: CATGCGGTCCTAACCGCACG, quality score 55) was designed on the first coding exon (ENSMUSE00001280325) common to all mRNA arising from the oprm1 gene (ENSMUST00000056385.13). Using CRISPR, we expect minimal to none off-target activity of the Cas9 enzyme as we and others verified before. The indicated gRNA sequence was inserted into the *Esp3I* restriction site of the pL-CRISPR.EFS.tRFP lentiplasmid (Cat#57819, Addgene) *(66)* as described before *(67)*. All plasmids were verified by Sanger sequencing (Eurofins).

Construction of the PRLR-L-GFP expression plasmid

The coding sequence corresponding to murine PRLR-L (ENSMUST00000124470.7) was synthesized and cloned between the XbaI and BamHI restriction sites in the pLenti-CMV-GFP-puro backbone (Addgene #17448) by Genscript.

In vivo transfection of CRISPR plasmids

For *in vivo* transfection, the indicated plasmids were diluted to 0.4 µg/µl in 5% sterile glucose solution. Then, Turbofect *in vivo* transfection reagent (Cat#R0541, Thermo Fisher Scientific) was added following manufacturer's instructions. Finally, 5 µl of the plasmid complexes were delivered by lumbar puncture in mice.

Culturing primary dorsal root ganglia (DRG) neurons and micro-electrode array (MEA) analysis

DRG neurons were isolated from the indicated mice following established procedures *(68)*. Dissociated DRG neurons were maintained in media containing Neurobasal (Cat# 21103049, Thermofisher), 2% B-27 (Cat# 17504044, Thermofisher), 1% penicillin/streptomycin sulfate from 10,000 µg/ml stock, 30 ng/ml nerve growth factor, and 10% fetal bovine serum (Hyclone). Collected cells were re-suspended in DRG media and seeded as a 10-µl drop on the poly-D-lysine coated electrodes of the micro-electrode array (MEA) (24-well plate, Cat# MED-Q2430L, MED64). The cells were allowed to adhere for 30 min and then flooded with DRG media. The next day, cells were analyzed on a MED64 presto where 24 wells containing each 16 electrodes could be recorded simultaneously. After a first recording of 5 min (MEA symphony software), 50 nM of mouse recombinant PRL (kindly provided by Dr. A.F. Parlow, Director, Pituitary Hormones and Antisera Center, NIDDK), was added to the indicated wells by pipetting a 2x concentrated conditioned DRG media. The cells were allowed to incubate for 5 min before another recording was done. The data was analyzed on MEA symphony and Mobius offline toolkit to extract the firing rate of the active electrodes before and after prolactin treatment. Firing rate is shown as Hz (event per second) for the electrodes that showed spontaneous activity

before the addition of prolactin. No electrodes with spontaneous activity were detected in any culture from naïve animals.

Immunoblot preparation and analysis

Tissue lysates were prepared and analyzed with western blot as described before *(67)* with the liquid transfer step done using TG (25mM Tris pH=8.5, 192mM glycine), 20% (vol/vol) methanol as transfer buffer. The following primary antibodies were used (anti-PRLR: Cat# ab2772 and ab170935, Abcam; anti-GFP: Cat# AB3080, Millipore; anti-tRFP: Cat# AB233, Evrogen and anti-βIII-tubulin: Cat# G7121, Promega). Horseradish peroxidase-conjugated light chain and Fc-specific secondary antibodies were Jackson immunoresearch. For western blots of PRLR-L, we used the antibody ab2772 (Clone U5) which has a better affinity for PRLR-L and yields a low signal for PRLR-S (as shown in figure 1L). To detect PRLR-S, we used the antibody ab170935 has a better affinity for PRLR-S and gives a low signal for PRLR-L (as shown in figure 1N). For this reason, the absolute expression ratio of PRLR-L/PRLR-S in our samples cannot be calculated. Samples from different groups were always run on the same gels using the same antibodies for each western experiment allowing relative comparisons and reliable results. For all experiments, protein expression was always normalized to βIII-tubulin in the same sample.

Fluorescence microscopy

Naïve PRLR-$L^{Cre/-}$/$GFP^{flx/-}$/MOR-$mCherry^{+/-}$ mice were anesthetized and transcardially perfused with 4% paraformaldehyde. Lumbar L3, 4 and 5 DRGs (left and right) were collected

and postfixed for 2 h in the fixative. 10-µm thick sections were cut on a Microm HM 525 cryostat microtome and mounted on Surgipath X-tra microscope slides (Leica Biosystems). Sections were permeabilized with 0.2% TritonX100 in phosphate buffered saline (PBS), blocked with 1% BSA and 5% normal goat serum and incubated overnight with rabbit polyclonal anti-mCherry antibody (1:1,000; GeneTex GTX128508) followed by anti-rabbit Alexa568 secondary antibody (1:1,000, Thermo Fisher A-11036). The sections were examined under an Olympus BX51 microscope equipped with a Hamamatsu C8484 digital camera using HC Image Live Imaging Software (Hamamatsu Corporation, Version 4.1.6.0). Micrographs of 2 sections per each DRG (100 µm apart) were taken using 10X objective and processed using NIH ImageJ software. The number of PRLR-$L^{Cre/-}$ positive (green) cells and PRLR-$L^{Cre/-}$/MOR-$mCherry^{+/-}$ co-expressing (green and red) cells were manually counted by a blinded observer. The number of cells in the left and right L3-5 DRGs from 5 female and 3 male mice were averaged. Confocal micrographs were acquired on a Leica SP5-II confocal microscope, using oil 40x/1.25NA PL Apo objective.

Calcitonin gene-related peptide (CGRP) release from spinal lumbar tissue

After decapitation under deep anesthesia with 5% isoflurane, cervical and lumbar vertebral incisions were made to expose the spinal cord. A 24 G needle on a 10-ml saline-filled syringe was inserted into the lumbar vertebral foramen and then steady pressure was applied until the spinal cord was extruded. The lumbar region of the spinal cord was dissected and placed in the Tyrode solution-filled beaker on the ice to wash out the blood and the sample collection protocol was started. The individual lumbar regions were then placed in the individual wells of 24-well plate filled with Tyrode solution and were incubated at 37°C. After a 10-min incubation, Tyrode

solution was collected from individual wells as samples of baseline step. The spinal cord lumbar regions were then incubated with 100 nM capsaicin (Fisher Scientific, Hampton, NH, CAS#404-86-4) in Tyrode solution to evoke CGRP release and the solution was collected after 10 mins as the sample of the treatment step. The new Tyrode solution was added to the wells and 10 mins later, the solution was collected as the sample of washout step. 50 nM water-soluble 17beta-estradiol (500 μM stock solution in 100% ethanol, Sigma, Lot# SLBW5325) was added in Tyrode solution used in every step. The concentration of CGRP released into the samples was measured by enzyme-linked immunosorbent assay (Cayman Chemical) and was normalized by the weight of the spinal lumbar tissues.

Surgery

Subcutaneous pellet implantation. In lightly anesthetized (2% isoflurane) mice, a horizontal incision was made at the shaved base of the neck, followed by inserting control or morphine pellets into the underlying subcutaneous space; incisions were closed by surgical wound clips (Stoelting, 9 mm Stainless Steel). To prevent the mice from immediate overdose, the pellets were wrapped with 2x2 cm square, two-layer nylon mesh as reported previously *(69–71)* (Greenbrier International, INC) and tied with 3-0 non-absorbable surgical suture (Henry Schein INC).

Spared nerve injury (SNI). In lightly anesthetized (2% isoflurane) mice, the area from slightly below the left knee to the hip was shaved and a 1-cm incision was made in a longitudinal direction proximal to the left knee. The muscle layers were separated by blunt dissection using a pair of sterile tweezers to visualize the sciatic nerve. A tight surgical knot was made by suture (6-0 suture) was applied around the tibial and common peroneal nerve branches from the sciatic nerve without touching the sural branch. The nerves below the suture were grabbed by a pair of

small tweezers and the nerves were cut above and below the tweezers. The suture ends were cut off and the muscle layers were gently closed. The animals were allowed to recover for 7 days before behavioral testing.

Subcutaneous minipump implantation. In lightly anesthetized (2% isoflurane) mice, a horizontal incision was made at the shaved base of the neck. Osmotic minipumps (Model 2991, Alzet Cupertino) (1μl/hr) delivering vehicle (saline), (-)-, or (+)-oxymorphone or morphine (both at 20 mg/kg/day) for 7 days were inserted into the underlying subcutaneous space; (-) or (+)-oxymorphone were synthesized by Dr. Kenner Rice, NIH. The oxymorphone dose was determined according to a previous study *(72)*. The animals were allowed to recover for 5 days before following experiments.

Periorbital and hindpaw tactile allodynia assessment

As described previously *(73)*, the mice were acclimated for 2 hours individually in clear Plexiglas chambers (3 X 3 X 7 inch) wrapped with black poster board on elevated wire-mesh platforms to allow access to the forehead and ventral surface of hindpaws. For response frequency measurement, successive applications (10 consecutive times at ~30 s-intervals) of von Frey filaments were applied to the forehead (force 0.4 g) and to the hindpaws (force 0.6 g for females and 1.0 g for males). Attack, face washing, and scratching by hindpaws were considered as positive responses to facial stimulation. Hindpaw withdrawal and licking were positive responses to hindpaw stimulation. Only mice with positive response frequencies < 50% to the 10 applications were selected for the subsequent experiments. For hindpaw withdrawal threshold measurement, the calibrated von Frey filaments (Stoelting Co) were applied perpendicularly to the plantar hindpaw with sufficient force to bend the filament for 1 s. Positive responses were

hindpaw withdrawal and licking. The hindpaw withdrawal threshold was determined by the up-down statistical algorithm *(74)*.

Mouse prolactin ELISA

Mice were anesthetized with isoflurane (2%) and whole blood was collected by cardiac puncture and coagulated at room temperature for 1 hour before isolating serum by centrifugation at 6,000 rcf, 10 mins, 4 °C. Serum samples were collected and stored at -80 °C until use. Serum prolactin was quantified by a mouse prolactin ELISA kit according to manufacturer instructions (Abcam, ab100736).

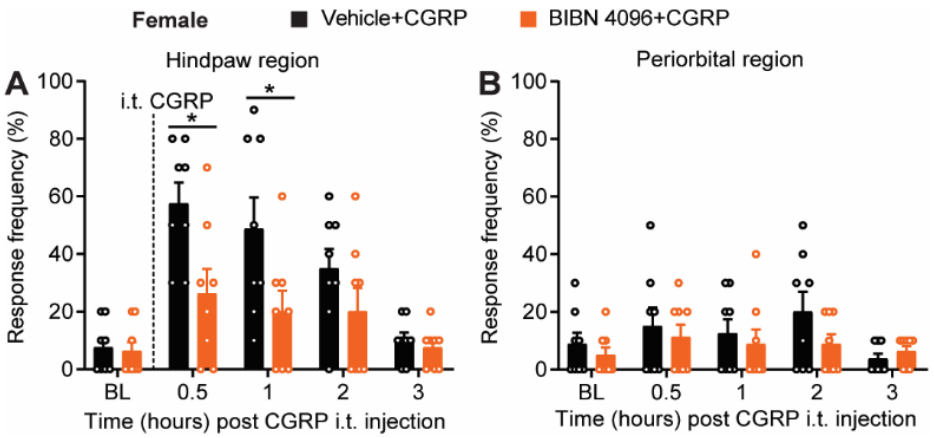

Fig. S1. Intrathecal pretreatment of BIBN 4096 prevents hindpaw allodynia induced by intrathecal injection of CGRP in naïve female mice. Hindpaw (**A**) periorbital (**B**) allodynia in naïve female mice with intrathecal administration of BIBN 4096 prior to intrathecal injection of CGRP (*n*=8/group.Hindpaw *P*=0.0122, Periorbital *P*=0.3782). Two-way repeated measures ANOVA with Sidak's multiple comparisons. **P*<0.05. All data are expressed as mean ± s.e.m. Each data point represents an individual animal.

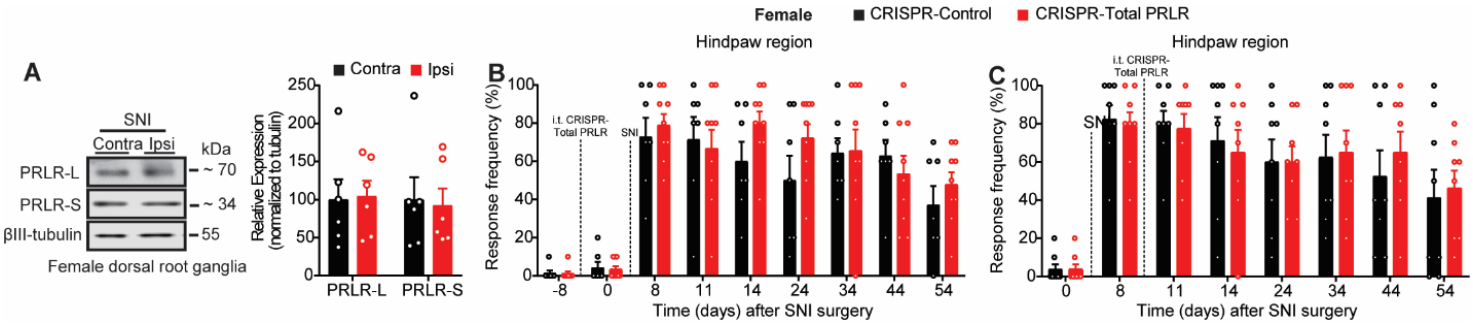

Fig. S2. The PRL/PRLR system is not critical for the development of trauma-induced neuropathic pain in female mice. (**A**) Representative western blot images (left panel) and quantification (right panel) of PRLR isoform expression in ipsilateral and contralateral DRGs from SNI female mice (*n*=6/group. PRLR-L: *P*=0.9372, PRLR-S: *P*>0.9999). (**B** and **C**), Hindpaw allodynia in female mice with intrathecal CRISPR-Total-PRLR editing prior to (**B**) or following (**C**) SNI surgery (B. *n*=7 sham, n=9 SNI; *P*=0.3614. C. *n*=8/group, *P*=0.9165). Two-tailed Mann-Whitney test (**A**). Two-way repeated measures ANOVA with Sidak's multiple comparisons test (**B** and **C**). *P<0.05. All data are expressed as mean ± s.e.m. Each data point represents an individual animal.

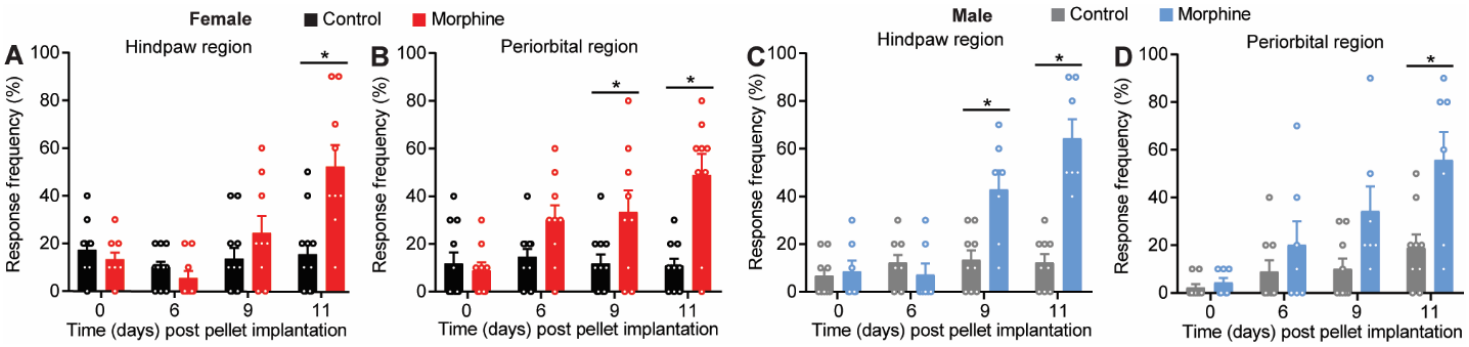

Fig. S3. Subcutaneous implantation of a single morphine pellet induces hindpaw and periorbital allodynia in female and male mice. Time course of hindpaw and periorbital tactile allodynia in female (**A** and **B**) and male mice (**C** and **D**) implanted with a single control or morphine pellet (Female: *n*=9 control, *n*=11 morphine; Hindpaw $P<0.0001$, Periorbital $P=0.0003$. Male: *n*=9/group; Hindpaw $P<0.0001$, Periorbital $P=0.0057$). Two-way repeated measures ANOVA with Sidak's multiple comparisons test. *P<0.05. All data are expressed as mean ± s.e.m. Each data point represents an individual animal.

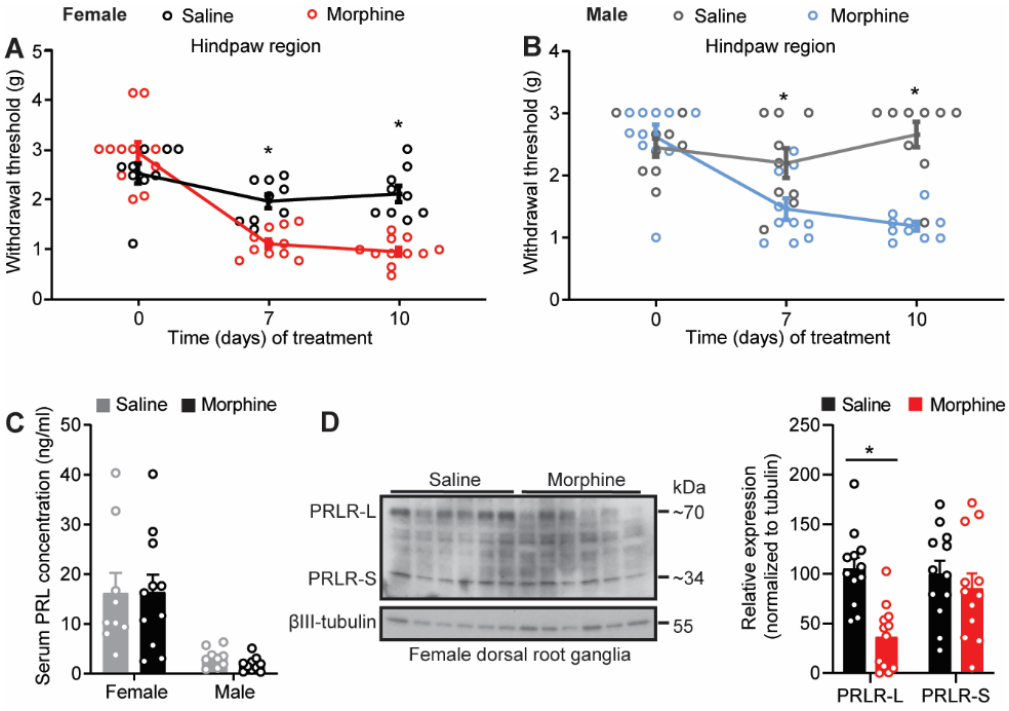

Fig. S4. Alteration in the PRL/PRLR system in female and male mice with daily injections of morphine. (**A** and **B**) Timecourse of hindpaw allodynia in female (A) and male (B) mice with daily injection of escalating doses of morphine (Female: *n*=9 saline, *n*=11 morphine; *P*<0.0001. Male: *n*=9 saline, *n*=10 morphine; *P*=0.0001). (**C**) Serum PRL concentration in female and male mice at day 10 of morphine treatment (Female: *n*=9 saline, *n*=11 morphine; *P*=8238. Male: *n*=8 saline, *n*=7 morphine; *P*=0.7789). (**D**) Representative western blot images (left panel) and quantification (right panel) of PRLR-L expression in DRGs from saline or morphine treated female mice (*n*=12/group. PRLR-L: *P*=0.0002, PRLR-S: *P*=0.5137). Two-tailed Mann-Whitney test (C and D). Two-way repeated measures ANOVA with Sidak's multiple comparisons test (**A** and **B**). *P<0.05. All data are expressed as mean ± s.e.m. Each data point represents an individual animal.

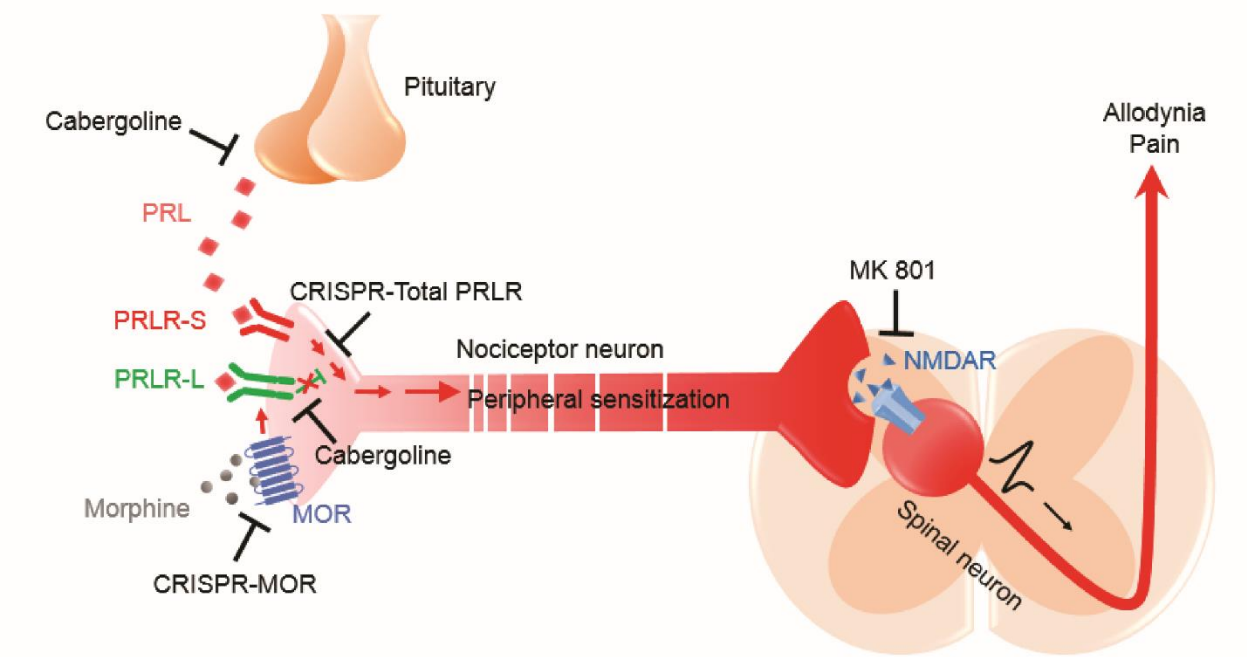

Fig. S5. Schematic of circulating PRL/PRLR system mediating nociceptor sensitization and OIH in females. MOR activation results in down-regulation of protective PRLR-L isoform in DRGs promoting nociceptor sensitization by circulating PRL from the pituitary, and allodynia through spinal glutamatergic mechanisms. Disruption of total PRLR or MOR expression, or suppression of circulating PRL by cabergoline inhibits nociceptor sensitization and attenuates OIH selectively in females. The figure is adapted from *(14)*.

Table S1. Binding affinity of (–)- and (+)-oxymorphone.

Compound	[^{3}H]DAMGO, Ki (nM)
(-)-oxymorpphone	3.67
(+)-oxymorphone	n.c.
morphine	4.65

Kd ([^{3}H]DAMGO) = 3.0 nM

n.c.: no competition (less than 10% inhibition at 10 μM)

Data file S1. Raw data. Provided as an Excel file.