Supplemental Material

Mediation of opioid analgesia by a truncated six transmembrane GPCR

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Supplemental Methods

Generation of E1/E11 KO mice

The exons 1 and 11 coding and its adjacent intron regions were replaced with the tdTomato/BGHpolyA (tdT/pA)/PGK-Neo (neo) and ZsGree/SVpolyA (ZsG/pA) cassettes, respectively, in W4 ES cells through homologous recombination with the BAC targeting vector (Figure 1A). The targeting vector was constructed from a 129S7/SvEv mouse BAC clone (bMQ-121P17, spanning ~128 kb from E11 to E4, Source Bioscience) using an established recombineering system (National Cancer Institute Biological Resourced Branch) (1) with a modified Rpsl-kanmycin selection system (2), as previously described (3). We transformed bMQ-121P17 into the SW105 strain by electroporation and used the transformants for all recombineering. A diphtheria toxin (DTA)/amp cassette with 52 bp flanking OPRM1 sequences amplified from pDTA-Amp (a gift from Dr. Yang, The Rockefeller University) was first inserted at 2 kb upstream of exon 11. The loxP site located downstream of sacB marker in the BAC vector, pBACe3.6, was replaced by a PI-SecI site by using Rpsl-kanmycin selection system, which was used for linearlization of the targeting vector in ES cell targeting. Next, the entire E1 coding and a 558 bp adjacent intron region was replaced by a cassette containing ZsGreen (ClonTech), SV40polyA and FRT/PGK-Neo/FRT through homology recombination with a 76 bp OPRM1 flanking sequence. The FRT/PGK-Neo/FRT was then deleted through Flpe expression induced by 0.1% L-arabinose in SW105 cells. Finally, the entire exon 11 coding sequence and a 74 bp adjacent intron region was replaced by a cassette containing tdTomato (ClonTech), BGHpolyA and LoxP/PGK-Neo/LoxP through homology recombination with a 76 bp OPRM1 flanking sequence. The linearlized BAC targeting vector with PI-SecI was transfected into W4 ES cells by electroporation. Genomic DNA isolated from G418 and gancyclovir double-resistant ES clones was digested with EcoRV and screened by Southern blot analysis with a 0.5 kb external 5' probe. Of 288 ES clones screened, we obtained seven positive ES clones. Southern blot analysis was carried out with several restriction enzymes and internal probes, as well as a PGK-Neo probe to further verify single-copy integration of the targeting vector in the correct locus and integrity of the targeting region. Karyotype screening of 6 positive ES clones all showed normal

male karyotypes. Two positive ES clones were injected into C57BL/6J blastocysts to produce chimeras, which then were bred with C57BL/6J. Wildtype (wt) and homozygous mice (E1/E11 KO) generated through heterozygous breeding were used for behavioral studies and lentiviral injection.

Southern blot analysis: Southern blot analysis was performed as described previously (10). Briefly, genomic DNA from ES cells and mouse tails were digested with appropriate restriction enzymes, separated on 0.8% agarose, and transferred onto a PVDF membrane. The membrane was hybridized with the ^{32}P-labeled probes (Figure 1B).

Reverse transcription and polymerase chain reaction (RT-PCR) : Total RNAs were isolated from the spinal cord and brain using RNeasy Kit (Qiagen), treated with DNase I by using Turbo DNA-free reagents (Invitrogen) and reversed transcribed with Superscript III (Invitrogen) and random primers, as described previously (16) . The first-strand cDNAs were used in PCR to amplify a 173 bp fragment of exon 11 (sense primer (SP): 5'-GTC CTT GAG AAT GGA GAG GAT CAG CAA AGC-3' and antisense primer (AP): 5'-GGT AAC TCT TCC CCT CTT GAT TTC CAT C), a 497 bp fragment of exons 1-2 (SP: 5'-GCA GAG GAG AAT ATC GGA CGC TCA G-3' and AP: 5'-GTC TTC ATT TTG GTA TAT CTT ACA ATC ACA TAC ATG-3'), a 315 bp fragment of exons 2-3 (SP: 5'- CAC AAA ATA CAG GCA GGG GTC CA-3' and AP: 5'-GTG GTT TCT GGA ATC GTG ATC AGT GC-3'), a 250 bp fragment of exons 3-4 (SP: 5'-GCA CTG ATC ACG ATT CCA GAA ACC AC-3' and AP: 5'-CCA GAT TTT CTA GCT GGT GGT TAG TTC-3'), and a 242 bp fragment of EGFP (SP: 5'-GGA CGG CAA CAT CCT GGG GC-3' and AP: 5'-CGT TGG GGT CTT TGC TCA GGG C-3'). PCRs for A 450 bp fragment of glyceraldehydes 3-phosphate dehydrogenase (G3PDH) (SP: 5'-ACC ACA GTC CAT GCC ATC AC-3' and AP: 5'-TCC ACC ACC CTG TTG CTG TA-3') were used for an RNA loading control. PCR products were separated on 2% agarose gel containing 66pg/ml ethidium bromide, and imaged using ChemiDoc MP system (Bio-Rad). To quantify expression of EGFP mRNA, we used SYBR green quantitative PCR (qPCR) with the same cDNAs and primers as above, and HotStart-IT SYBR Green qPCR Master Mix (Affymetrix). G3PDH qPCR with the same primers as above was used for normalization. Normalized expression was calculated as $2^{-\Delta C(t)}$ ($\Delta C(t) = C(t)_{EGFP} - C(t)_{G3PDH}$). Fold change was calculated by normalizing with data from week one which is 1.

Generation of the lentivirus constructs

Using lentiviral vectors (gifts from Dr. Didler Trono, Ecole Polytechnique Federale de Lausanne (EPFL), Lausanne, Switzerland), we subcloned a 78 bp polylinker containing XhoI/SpeI/AscI/MluI/NdeI/BamHI/XmaI/XbaI sites into a PmeI site in pWPI. To enhance the translation of the inserted cDNA, a ~500 bp PCR fragment containing the internal ribosome entry site of the Foot and Mouth Disease Virus (FMDV-IRES) was amplified from pMONO-blasti-mcs (Invivogen) with proper primers flanking with AscI and MluI sites, and inserted into AscI/MluI sites of the polylinker as pWPI-IRES (Fig. S2). mMOR-1G was amplified by PCR using a sense primer flanked with MluI (5'-GAT ACG CGT GGC GCG GGA TCT GGG CCG ATG-3') and an antisense primer flanked with XmaI (5'-GAT CCC GGG TCT GGA TGG CGT GGG AC-3') was then

subcloned into the MluI/XmaI sites of the polylinker to construct pWPI-IRES-mMOR-1G (Fig. S2), whose sequence was verified by sequencing. The viral particles were generated by co-transfecting human embryonic kidney (HEK) 293T cells with pWPI-IRES-mMOR-1G or pWPI-IRES, a packaging vector, PAX2, and an envelope vector, pMD2, by using FuGENE HD transfection reagent (Promega) following the manufacture's protocol. After a 48 hr transfection, the supernatants were collected, filtered, and concentrated by ultracentrifugation (100,000 x g, 2 hrs) in Beckman L7-55 ultracentrifuge. The viral titer of the concentrated lentiviral particles was determined by quantifying EGFP-expressing cells in infected HEK293T cells with different dilutions using fluorescent microscope since EGFP is co-expressed in the lentivirus.

Lentivirus injection

The lentivirus was given by intrathecal (i.t.) injections that were performed by lumbar puncture using a Hamilton 10 µl syringe fitted to a 30 gauge needle with V1 tubing under general halothane anesthesia, as described previously (4, 5). Two µl of the lentiviral particles expressing mMOR-1G or vector alone without insertion (1.5×10^9 transducing units/ml) were administrated intrathecally in each mouse on days 1, 3, and 5.

Opioid analgesia

Analgesia was determined using a radiant heat tailflick assay, with a maximal latency of 10 sec to minimize tissue damage, as described previously (4, 5). Baseline latencies typically ranged around 2-3 sec. Results were calculated as percentage of maximum possible effect (%MPE) [(latency after drug – baseline latency)/(10 – baseline latency)*100]. Opioids were administered subcutaneously and analgesia testing carried out 30 min later at peak effect. IBNtxA doses used in cumulative dose-response studies were 0.1, 0.3, 1, 3 or 10 mg/kg. ED_{50} values with 95% confidence intervals were determined using nonlinear regression analysis (GraphPad Prism, Carlsbad, CA).

Opioid receptor binding

Membranes were isolated from mouse spinal cord and prepared as previously described (6, 7). Binding was performed using ^{3}H-DAMGO, or ^{3}H-DPDPE, ^{3}H-U69,593 with 1 ml assay containing 400 µg protein or ^{125}I-IBNtxA in a 0.5 ml assay containing 100 µg protein, as described previously (6, 7). For each replication, tissue was pooled from 2-4 mice for each replication. All binding studies were independently replicated 3 or 4 times. Specific binding was defined as the difference between total binding and nonspecific binding, defined as that remaining in the presence of 10 µM levallorphan. Protein concentrations were determined as described previously by using BSA as the standard (6, 7).

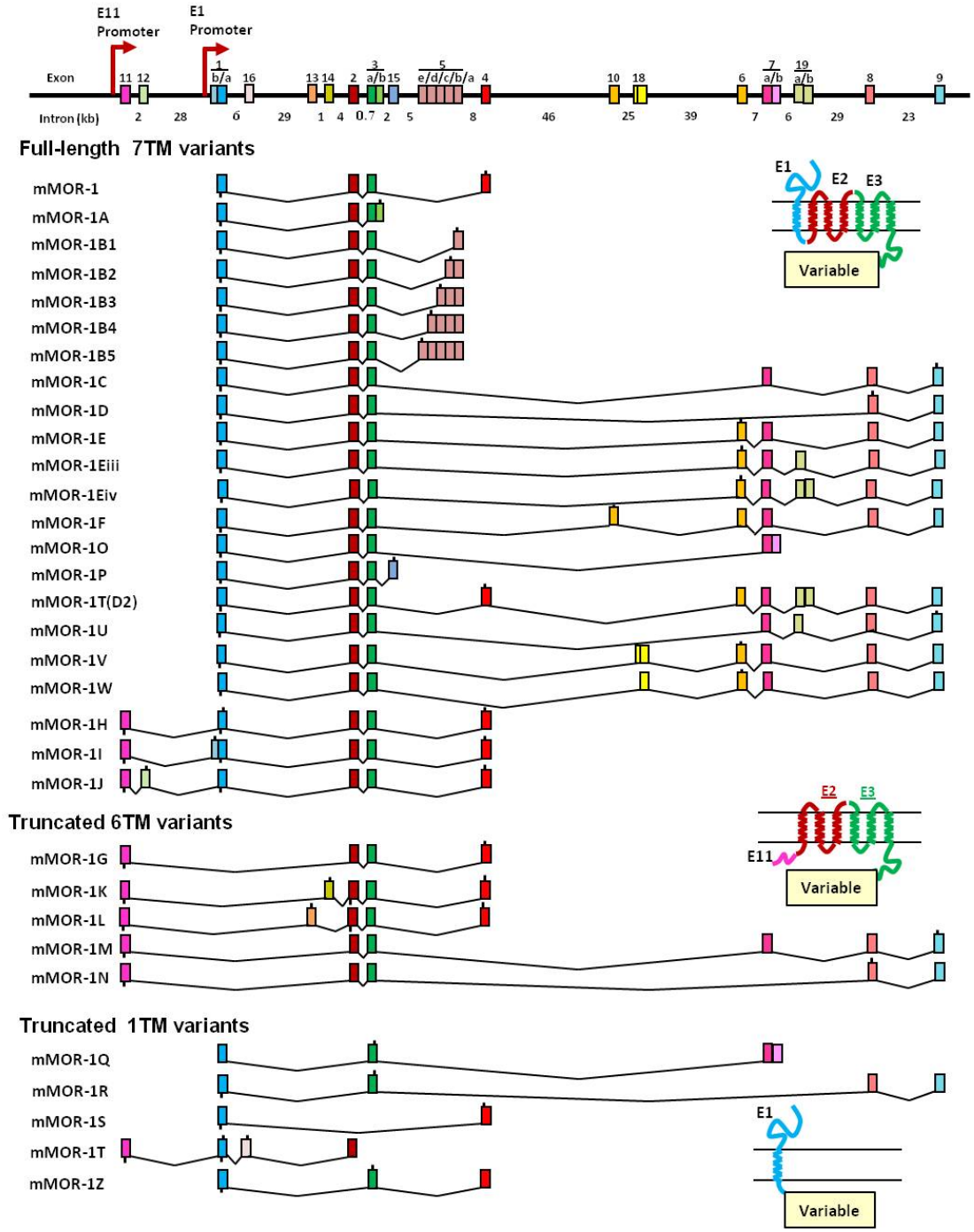
Immunohistochemistry

Mice were anesthetized using ketamine (150 mg/kg, i.p.)/xylazine (15 mg/kg, i.p.) and then received an intracardial perfusion with phosphate-buffered saline (PBS), pH7.4 (30 ml)

followed by 4% paraformaldehyde in PBS, pH7.4 (50 ml). The spinal cords were removed, post-fixed for 2 hrs in the same fixative, cryoprotected overnight in 25% sucrose in PBS, and frozen in Tissue-Tek OCT (Miles, Elkhart, IN). Immunostaining was performed on 15 µm frozen section cut on a Leica Cryomicrotome using a modified tyramide signal amplification (TSA) procedure (8). Briefly, after treatment with 3% H_2O_2 in PBS for 10 min, the section was placed in a blocking solution (0.1M Tris/HCl, pH7.4 and 0.15M NaCl) containing 5% normal goat serum (NGS) for 1 hr at room temperature (RT). For the double EGFP/NeuN staining, the section was then incubated with a chicken anti-GFP antibody (1:100, ab13970, Abcam) and a mouse anti-NeuN antibody (1:500, NAB377, Millipore) in blocking solution containing 3% NGS overnight at 4°C. The section was sequentially incubated with a biotinylated goat-anti-chicken IgG (1:500, 2 hrs at RT, BA-9010, Vector Laboratories) and a strepavidin-horseradish peroxisase (HRP) (1:500, 1 hr at RT, SNN1004, Invitrogen), followed by staining with a TSA fluorescein reagent (PerkinElmer). A goat anti-mouse IgG Alexa Fluor 568 (1:500, 2 hrs at RT, A11029, Invitrogen) was then used for NeuN staining. For the double EGFP and exon 4 epitope staining, the section was incubated with the chicken anti-GFP antibody and a rabbit exon 4 antibody (1:300, RA10104, Neuromics) overnight at 4°C. The section was then stained for EGFP with the same procedure as above followed by staining the exon 4 epitope with the same procedure using a biotinylated goat anti-rabbit IgG (BA1000, Vector Laboratories) and a TSA cyanine 3 reagent (PerkinElmer). All washing before and after each antibody incubation was performed using a wash buffer (0.1M Tris/HCl, pH7.4, 0.15M NaCl and 0.05% Tween 20). All sections were cover-slipped with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (H-1500, Vector Laboratories). The sections were scanned with a Pannoramic 250 FLASH II Scanner (Perkin Elmer).

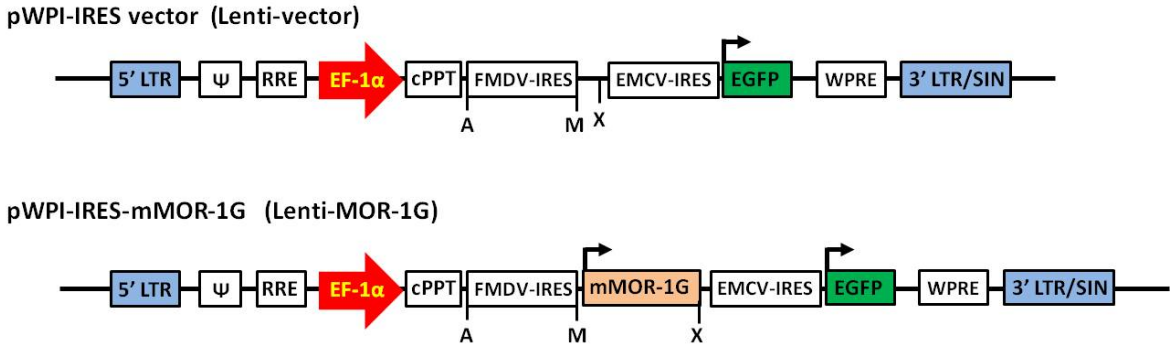
Supplemental Figures

Figure S1: Schematic of the mouse *Oprm1* gene and its alternative splicing



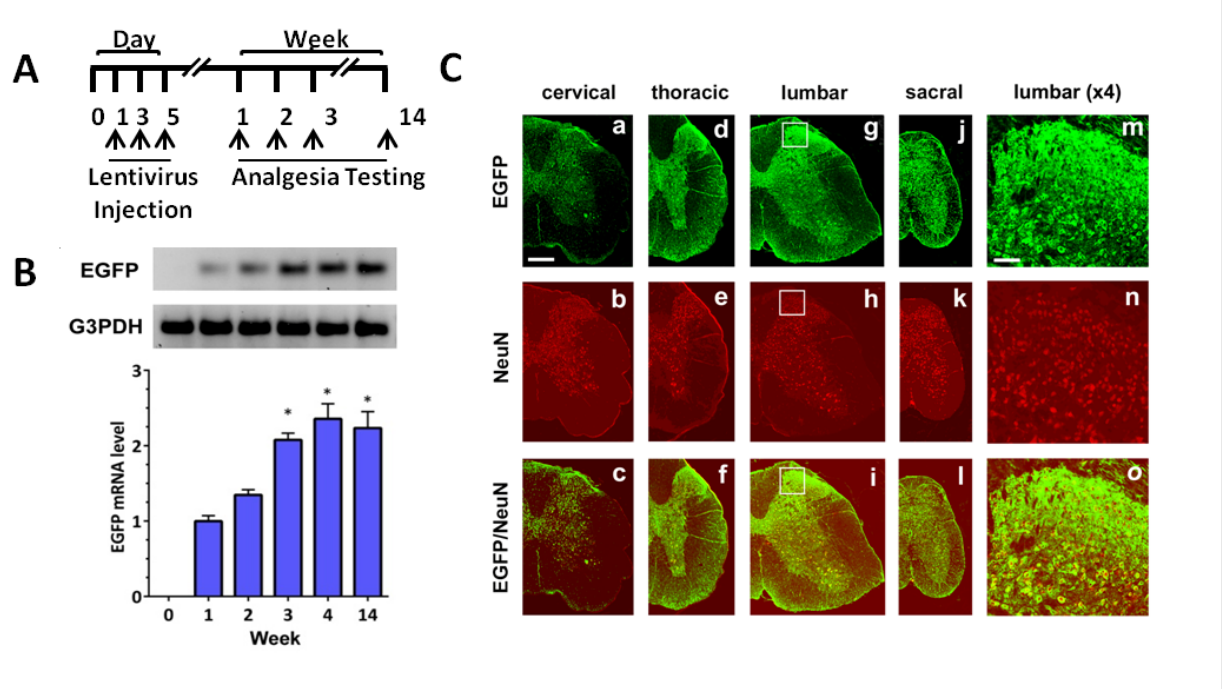
Top: The mouse *Oprm1* gene structure. Exons and introns are indicated by boxes and horizontal lines, respectively. Intron size is indicated below the introns as kilobases (kb). Promoters are showed by arrows. Exons are numbered based upon the published data. **Bottom:** Alternative splicing. Splicing is indicated by tilted lines among exons. Translation start and stop points are shown by bars below and above exon boxes, respectively. Predicted protein structures are shown on right side for each category of the variants. Note: exons were labeled in order of their identification and not by their location within the gene, which is why exon 11 is located approximately 30 kb upstream of exon 1.

Figure S2: Schematic of the lentivector



pWPI-IRES and pWPI-IRES-mMOR-1G constructs were made as described in Supplemental Methods and were used in generating lentivirus expressing mMOR-1G/EGFP (Lenti-mMOR-1G) and EGFP (Lenti-vector), respectively. **Black arrows** indicate translation of EGFP and mMOR-1G enhanced by EMCV-IRES and FMDV-IRES, respectively. **LTR**: long terminal repeat; **Ψ**: a packaging sequence; **EF-1α**: a promoter of human elongation factor-1 alpha; **RRE**: a Rev response element; **cPPT**: a central polypurin track sequence; **FMDV-IRES**: the internal ribosome entry site of the Foot and Mouth Disease Virus; **EMCV-IRES**: the internal ribosome entry site of the encephalomyocarditis virus; **WPRE**: a woodchuck hepatitis virus posttranscriptional regulatory element; **SIN**: a self-inactivation element; **A**: AscI; **M**: MluI; **X**: XmaI.

Figure S3: Lentivirus expression



A) Schematic of the virus injection paradigm. **B)** Time course of spinal EGFP mRNA expression determined by regular PCR (upper panel) and quantified by qPCR (lower panel, n=3-6). *p<0.0001 compared to week 1 (ANOVA followed by Tukey). **C** Distribution of lentiviral EGFP in the spinal cord determined by immunohistochemistry using an anti-GFP antibody. An anti-NeuN antibody was used for staining neurons. m - o are enlarged from j – l, respectively. Scale bars: 200 µm in a-l and 50 µm in m-o. Scale bars: 100 µm in a – i and 10 µm in j – l.

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