Supplementary Data

Supplementary Materials and Methods

Expression of recombinant proteins

The KRX/pFN2A-NR1 (segments C0-C1-C2; NM_008169.3), KRX/pFN2A-HINT1 (NM_00824) and KRX/pFN2A-RGSZ2 (NM_001161822) strains were obtained as previously described (12, 13). Murine full-length σ1R (AF004927) and the short variant (AB721301) were cloned after PCR amplification from PAG cDNA into the pFN2A (GST) Flexi vector (Promega, Madrid, Spain). Specific primers containing upstream *Sgf*I and downstream *Pme*I restriction sites were used. The PCR products were cloned downstream of the GST coding sequence and the TEV protease site. The sequenced proteins were identical to the GenBank™ sequences. The vector was introduced into *Escherichia coli* BL21 (KRX #L3002; Promega), and clones were selected on solid medium containing ampicillin. After overnight induction at room temperature (1 m*M* IPTG and 0.1% Rhamnose), the cells were collected by centrifugation, and the pellets were maintained at −80°C. The purification of GST fusion proteins was improved using SUMO1-agarose (#UL-740; Boston Biochem, Cambridge, MA), which binds the SIM domain of σ1R; this was followed by purification under native conditions on GStrap FF columns (17-5130-01; GE Healthcare, Barcelona, Spain) and when necessary, the fusion proteins that were retained were cleaved on the column with ProTEV protease (#V605A; Promega); further purification was achieved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroelution of the corresponding gel band (GE 200; Hoefer Scientific Instruments, San Francisco, CA) and immobilized through agarose-coupled affinity-purified IgGs directed to the target protein. Site-directed mutagenesis was performed with Accuprime Pfx DNA Polymerase (Invitrogen, Madrid, Spain) using NR1 segments C0-C1-C2 as the template. Threonine 879 was mutated to Alanine (T879A), and the amplified fragment was cloned into pFN2A (GST) Flexi® Vector (Promega). The sequences were confirmed through automated capillary sequencing. The protein Calmodulin (#208694) was obtained from Calbiochem (Merck-Millipore, Barcelona, Spain); PKCγ was from Millipore (14-483) and Abnova (P4756; Taipei City, Taiwan); and Gαi2 subunits were from Calbiochem (#371796).

Drugs and intracerebroventricular injection

NMDA (#0114), MK801 (#0924), BD1063 (#0883), BD1047 (#0956), PRE084 (#0589), NE100 (#3133), clonidine (#0690), and WIN55,212-2 (#1038) were obtained from Tocris Bioscience (Bristol, United Kingdom). We also used BD1063 (EST0013430.A; 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydro-chloride) and the newly synthesized σ1R antagonist S1RA (EST-52862.A; 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine) (3) obtained from Laboratorios Esteve (Barcelona, Spain). Progesterone (P7556), Pregnenolone (P9129), Pregnenolone acetate (P49902), and Pregnenolone sulfate (P162) were obtained from Sigma (Barcelona, Spain), and Gö7874 (Calbiochem #365252) was obtained from Merck-Millipore. Morphine sulfate was purchased from Merck (Darmstadt, Germany). L-NG nitroarginine and NG-nitro-L-arginine methyl ester were from Tocris Bioscience (L-NNA, 0664; L-NAME, 0665). The substances were each injected into the lateral ventricle of mice at 4 μl as previously described (8). Compounds were dissolved in saline except σ1R ligands that were prepared in ethanol:cremophor EL:saline (1:1:18). Doses of the compounds were taken from previous studies (5, 13, 14).

Immunohistochemistry

The mice were deeply anesthetized with chloral hydrate 28% weight/volume (0.1 ml/100 g body weight), before intracardially perfusing them with a physiological solution and then with 4% paraformaldehyde in 0.1 *M* phosphate buffer (PB, pH 7.4) at 4°C. The mice's brains were removed, cut into small blocks, and postfixed for 4 h at room temperature in the same fixative. The blocks were cryoprotected at 4°C in a 30% sucrose solution in PB saline (PBS), snap frozen on dry ice, and stored at −80°C. Coronal cryosections (10 μm, 2800 Frigocut; Reichert-Jung, Depew, NY) were mounted on gelatin-coated slides and permeabilized in PBS containing 0.3% Triton X-100 (PBT) for 30 min at room temperature. The sections were incubated with PBT containing 1% bovine serum albumin for 1 h at room temperature, and a rabbit IgG labeling kit (Zenon Tricolor Rabbit IgG labeling Kit #1; Molecular Probes, Eugene, OR) was then used to detect the rabbit polyclonal IgGs used that fulfilled the recommended criteria for use in immunohistochemistry (15): anti-MOR (C-terminal region, Abcam, Cambridge, United Kingdom, ab134054; Alexa Fluor 647) (2), anti-NMDAR NR1 subunit (C-terminus, Merck-Millipore, Chemicon AB9864; Alexa Fluor 555) (11), and anti-σ1R (internal region139-157, Invitrogen 42-3300; Alexa Fluor 488) (1). The labeling of the used antibodies was performed according to the Zenon Complex Formation protocol and diluted in PBT to the desired working concentration. The sections were incubated with the labeled IgGs in a humidified chamber overnight at 4°C, and then the sections were washed with PBS, mounted, and examined with confocal microscopy (Leica TCS SP-5/LAS AF Lite Software; Microsystems, GmbH, Hohenstein-Ernstthal, Germany). DAPI (4′-6-diamidino-2-phenylindole) nuclear counterstaining was performed to localize the cells. For each channel, the first four microphotographs at a distance of 1 μm apart were Z-merged and the background was subtracted to enhance the specificity and visibility of the signals (ImageJ; NIH, Bethesda, MD) (16). Controls for immunohistochemistry were performed following standard protocols (for further details see Supplementary Fig. S3).

Immunoprecipitation and Western blotting

After intracerebroventricular (icv) morphine, groups of eight mice were killed at various intervals postopioid; periaqueductal grey matter (PAG) were obtained and

processed to obtain the synaptosomal pellet as previously described (13), and used for mu-opioid receptor (MOR) and NR1 immunoprecipitation and co-precipitation of HINT1, NR1, and MOR. This procedure has been described elsewhere (4, 7). Briefly, the PAGs were collected and homogenized in 10 volumes of 25 m*M* Tris-HCl pH 7.4, and 0.32 *M* sucrose supplemented with a phosphatase inhibitor mixture (P2850; Sigma), H89 (B1427; Sigma), and a protease inhibitor cocktail (P8340; Sigma). The homogenate was centrifuged at 1000 *g* for 10 min to remove the nuclear fraction. The supernatant (S1) was centrifuged twice at 20,000 *g* for 20 min to obtain the crude synaptosomal pellet (P2). The final pellet was diluted in Tris buffer supplemented with a mixture of protease inhibitors (0.2 m*M* phenylmethylsulphonyl fluoride, 2 μg/ml leupeptin, and 0.5 μg/ml aprotinin), followed by division into aliquots and freezing at -80°C.

For immunoprecipitation studies, the PAG from eight mice were typically pooled. The assays were repeated at least twice on samples receiving an identical treatment and collected at the same interval postadministration. The affinity purified IgGs against the extracellular domains of the MOR 2EL (205-216: MATTKYRQGSID; GenScript Co., Piscataway, NJ) and the NMDAR NR1 subunit (483-496: KFGTQERVNNSNKK; GenScript Co.) were labeled with biotin (Pierce #21217 and 21339). Pilot assays were performed to optimize the amount of IgG and sample protein needed to precipitate the desired protein in a single run. Target proteins were subsequently immunoprecipitated from solubilized membranes and resolved through SDS/PAGE as previously described (13). The Nonidet P-40 solubilized proteins were incubated overnight at 4°C with biotin-conjugated primary antibodies raised against the target protein, for example, murine MOR or NR1 subunits. The immunocomplexes were recovered and resolved by SDS-PAGE. HINT1 was resolved in 4%–12% Bis–Tris gels (NuPAGE NP0341; Invitrogen) with NuPAGE MES SDS running buffer (NP0002; Invitrogen) and SeeBlue Plus2 prestained Standards (3–188 kDa, LC5925; Invitrogen). Sufficient protein was obtained by immunoprecipitation to load about four gel lanes.

The separated proteins were then transferred onto 0.2 μm polyvinylidene difluoride (PVDF) membranes (162-0176; Bio-Rad, Madrid, Spain) and probed overnight at 6°C with the selected primary antibodies diluted in Tris-buffered saline pH 7.7 (TBS) + 0.05% Tween 20 (TTBS) in DecaProbe chambers (PR 150; Hoefer-GE, Barcelona, Spain). Those were detected using secondary antibodies conjugated to horseradish peroxidase. Antibody binding was visualized by chemiluminescence (#170-5061; Bio-Rad) and recorded using a ChemiImager IS-5500 (Alpha Innotech, San Leandro, CA). Densitometry was performed using Quantity One Software (Bio-Rad), and expressed as the mean ± SEM of the integrated volume (average optical density of the pixels within the object area/mm^2). Immunosignals are shown relative to these of control animals that were attributed an arbitrary value of 1. Equal loading among the postopioid intervals was verified after determining the target protein in parallel blots of the same immunoprecipitated samples used to study co-precipitation. Each bar is the mean ± SEM of three assays performed on PAG samples obtained from independent groups of mice.

Effect of zinc ions and NO generators on the recruitment of PKCγ to PAG MORs in synaptosomal membranes

The effect of zinc ions, the NO generator (5)-nitroso-*N*-acetylpenicillamine (SNAP [#0598; Tocris Bioscience], and the metal ion chelator *N*′,*N*′,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine [TPEN; Fluka WA16827]) on the association of PKCγ with MORs was studied in PAG synaptosomal membranes. Membranes were incubated with zinc chloride (Puratronic, 231-592-0; Alfa Aesar, Ward Hill, MA) for 4 h at 4°C before the MORs were immunoprecipitated. Subsequently, free zinc ions were removed by centrifugation and extensive washing. The synaptosomal membranes were then solubilized in Nonidet P-40 buffer as described (13). The solubilized membranes were incubated overnight at 4°C with $\sim$3 μg of a biotin (Pierce #21217 and 21339) conjugated primary antibody (affinity-purified IgGs) raised against extracellular sequences in MOR. The MOR-associated proteins were then separated by SDS-PAGE and analyzed by Western blotting.

Antibodies

The primary antibodies included a newly synthesized anti-σ1R against the murine peptide sequence SRLIVELRRLHPGH (amino acids 59–72; GenScript Co.) exhibiting <10% homology to other known protein sequences (EMBL, GenBank and SwissProt databases). Anti-σ1R IgGs were purified by affinity chromatography using the antigenic peptide coupled to NHS-activated Sepharose 4 Fast Flow (#17-0906-01; GE Healthcare). Other antibodies included anti-σ1R (AB2 #42-3300; Invitrogen); anti-MOR CT (13); anti-NMDAR1 (#MAB1586; Merck-Millipore); anti-NMDAR1 phospho-S890 (#3381; Cell Signaling, Danvers, MA); anti-NMDAR1 phospho-S896 (#ABN88; Merck-Millipore); anti-NMDAR1 phospho-S897 (#ABN99; Merck-Millipore); anti-NMDAR2A phospho-Y1325 (#ab16646; Abcam); anti-NMDAR2B phospho-Y1472 (#ab59205; Abcam); anti-CAMKIIpan (#3362; Cell Signaling); anti-CaMKIIα phospho-T286 (#3361; Cell Signaling); anti-calmodulin (#4830; Cell Signaling); and anti-PKCI/HINT1 (#H00003094-A01; Abnova). Anti-RGSZ2 was raised against aa 192-215, GenScript Co. (6); Anti-PKCγ (#ab4145; Abcam); anti-Actin (#CSA-400; Stressgen); Phosphoserine detection kit (clones 1C8, 4A9 and 16B4; #525282; Calbiochem); and Phosphothreonine detection kit (#525288; Calbiochem).

References

1. Bangaru ML, Weihrauch D, Tang QB, Zoga V, Hogan Q, and Wu HE. Sigma-1 receptor expression in sensory neurons and the effect of painful peripheral nerve injury. *Mol Pain* 9: 47, 2013.
2. Cui Y, Ostlund SB, James AS, Park CS, Ge W, Roberts KW, Mittal N, Murphy NP, Cepeda C, Kieffer BL, Levine MS, Jentsch JD, Walwyn WM, Sun YE, Evans CJ, Maidment NT, and Yang XW. Targeted expression of mu-opioid receptors in a subset of striatal direct-pathway neurons restores opiate reward. *Nat Neurosci* 17: 254–261, 2014.
3. Diaz JL, Cuberes R, Berrocal J, Contijoch M, Christmann U, Fernandez A, Port A, Holenz J, Buschmann H, Laggner C, Serafini MT, Burgueno J, Zamanillo D, Merlos M, Vela JM, and Almansa C. Synthesis and biological evaluation of the 1-arylpyrazole class of sigma (1) receptor antagonists:

Identification of 4-{2-[5-methyl-1-(naphthalen-2-yl)-1H-pyrazol-3-yloxy]ethyl}morpholine (S1RA, E-52862). *J Med Chem* 55: 8211–8224, 2012.
4. Garzón J, Rodríguez-Muñoz M, López-Fando A, and Sánchez-Blázquez P. Activation of μ-opioid receptors transfers control of Gα subunits to the regulator of G-protein signaling RGS9-2: role in receptor desensitization. *J Biol Chem* 280: 8951–8960, 2005.
5. Garzón J, Rodríguez-Muñoz M, Vicente-Sánchez A, Bailón C, Martínez-Murillo R, and Sánchez-Blázquez P. RGSZ2 binds to the neural nitric oxide synthase PDZ domain to regulate mu-opioid receptor-mediated potentiation of the N-methyl-D-aspartate receptor-calmodulin-dependent protein kinase II pathway. *Antioxid Redox Signal* 15: 873–887, 2011.
6. Garzón J, Rodríguez-Muñoz M, Vicente-Sánchez A, García-López MA, Martínez-Murillo R, Fischer T, and Sánchez-Blázquez P. SUMO-SIM Interactions Regulate the Activity of RGSZ2 Proteins. *PLoS One* 6: e28557, 2011.
7. Garzón J, Torre-Madrid E, Rodríguez-Muñoz M, Vicente-Sánchez A, and Sánchez-Blázquez P. Gz mediates the long-lasting desensitization of brain CB1 receptors and is essential for cross-tolerance with morphine. *Mol Pain* 5: 11, 2009.
8. Haley TJ and McCormick WG. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br J Pharmacol Chemother* 12: 12–15, 1957.
9. Hecker CM, Rabiller M, Haglund K, Bayer P, and Dikic I. Specification of SUMO1- and SUMO2-interacting motifs. *J Biol Chem* 281: 16117–16127, 2006.
10. Kerscher O. SUMO junction-what's your function? New insights through SUMO-interacting motifs. *EMBO Rep* 8: 550–555, 2007.
11. Lee JH, Pradhan J, Maskey D, Park KS, Hong SH, Suh MW, Kim MJ, and Ahn SC. Glutamate co-transmission from developing medial nucleus of the trapezoid body—lateral superior olive synapses is cochlear dependent in kanamycin-treated rats. *Biochem Biophys Res Commun* 405: 162–167, 2011.
12. Rodríguez-Muñoz M, Sánchez-Blázquez P, Vicente-Sánchez A, Bailón C, Martín-Aznar B, and Garzón J. The histidine triad nucleotide-binding protein 1 supports mu-opioid receptor-glutamate NMDA receptor cross-regulation. *Cell Mol Life Sci* 68: 2933–2949, 2011.
13. Rodríguez-Muñoz M, Sánchez-Blázquez P, Vicente-Sánchez A, Berrocoso E, and Garzón J. The mu-opioid receptor and the NMDA receptor associate in PAG neurons: implications in pain control. *Neuropsychopharmacology* 37: 338–349, 2012.
14. Rodríguez-Muñoz M, Torre-Madrid E, Sánchez-Blázquez P, and Garzón J. NO-released zinc supports the simultaneous binding of Raf-1 and PKCgamma cysteine-rich domains to HINT1 protein at the mu-opioid receptor. *Antioxid Redox Signal* 14: 2413–2425, 2011.
15. Saper CB and Sawchenko PE. Magic peptides, magic antibodies: guidelines for appropriate controls for immunohistochemistry. *J Comp Neurol* 465: 161–163, 2003.
16. Schneider CA, Rasband WS, and Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9: 671–675, 2012.