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FK506 binding protein 12 modulates μ opioid receptor phosphorylation and PKCε-dependent signaling by its direct interaction with the receptor

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Running title: FKBP12 and morphine-induced PKCε activation

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Abbreviations: OPRM1, μ-opioid receptor; PKC, protein kinase C; FKBP12, FK506 binding protein 12; OPRM1P353A, OPRM1 with P353A mutation; DAMGO, [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin; GRK, G protein-coupled receptor kinase; PPIase, peptidyl prolyl cis-trans isomerase; RyR, ryanodine receptor; IP_3R, $Ins(1,4,5)P_3$ receptor; TGFβ, transforming growth factor β; CT, carboxyl tail; OPRD1, δ-opioid receptor C-tail; OPRK1, κ-opioid receptor; mKG, monomeric Kusabira-Green; mKGN, N-terminal fragment of mKG; mKGC, C-terminal

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fragment of mKG; MN, N-terminus of the mKGN or mKGC; MC, C-terminus of the mKGN or mKGC PDB, protein data bank; MOE, Molecular Operating Environment; MD, molecular dynamics; siRNA, short interfering RNA; ERK1/2, extracellular signal-regulated kinases

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Abstract

Protein kinase C (PKC) activation plays an important role in morphine-induced μ-opioid receptor (OPRM1) desensitization and tolerance development. It was recently shown that receptor phosphorylation by G protein-coupled receptor kinase regulates agonist-dependent selective signaling and that inefficient phosphorylation of OPRM1 leads to PKCε activation and subsequent responses. Here, we demonstrate that such receptor phosphorylation and PKCε activation can be modulated by FK506 binding protein 12 (FKBP12). Using a yeast two-hybrid screen, FKBP12 was identified to specifically interact with OPRM1 at the Pro^{353} residue. In HEK293 cells expressing OPRM1, the association of FKBP12 with OPRM1 decreased the agonist-induced receptor phosphorylation at Ser^{375}. The morphine-induced PKCε activation and the recruitment of PKCε to the OPRM1 signaling complex were attenuated both by FKBP12 siRNA treatment and in cells expressing OPRM1 with a P353A mutation (OPRM1P353A), which leads to diminished activation of PKC-dependent extracellular signal-regulated kinases. Meanwhile, the over-expression of FKBP12 enabled etorphine to activate PKCε. Further analysis of the receptor complex demonstrated that morphine treatment enhanced the association of FKBP12 and calcineurin with the receptor. The blockade of the FKBP12 association with the receptor by the siRNA-mediated knockdown of endogenous FKBP12 or the mutation of Pro^{353} to Ala resulted in a reduction in PKCε and calcineurin recruitment to the receptor signaling complex. The receptor-associated calcineurin modulates OPRM1 phosphorylation, as demonstrated by the ability of the calcineurin autoinhibitory peptide to increase the receptor phosphorylation. Thus, the association of FKBP12 with OPRM1 attenuates the phosphorylation

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of the receptor and triggers the recruitment and activation of PKCε.

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Introduction

Morphine is widely prescribed in the treatment of moderate to severe pain. However, its use is limited because of its high propensity to induce antinociceptive tolerance, physical dependence, and addiction (Rezvani et al., 1983; Whistler et al., 1999). Similarly to other opioids, morphine exerts its function through the activation of opioid receptors, which can be classified into three classes: μ-, δ-, and κ-opioid receptors. Of these, the μ-opioid receptor (OPRM1) has been identified to mediate the opioid-induced analgesic effects and the development of tolerance and physical dependence (Kieffer, 1999; Matthes et al., 1996).

OPRM1 belongs to the rhodopsin subfamily of G protein-coupled receptors. Upon activation by most opioids, such as etorphine and [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO), this receptor is phosphorylated by G protein-coupled receptor kinase (GRK) and recruits β-arrestins, which promotes the uncoupling of the receptor to G proteins and subsequent internalization. However, morphine induces receptor phosphorylation inefficiently and activates PKC via the G protein-dependent pathway (Bohn et al., 2002; Zheng et al., 2008a; Zheng et al., 2008b). The participation of activated PKC in receptor signal desensitization and tolerance development has been extensively reported (Bailey et al., 2009; Bohn et al., 2002; Chu et al., 2010; Smith et al., 2002; Zheng et al., 2008b).

Recently, the extent of receptor phosphorylation was shown to regulate the agonist-specific activation of PKC (Zheng et al., 2011). The over-expression of GRK2 to increase morphine-induced receptor phosphorylation impairs its ability to activate PKC, whereas the

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blockade of receptor phosphorylation switches etorphine and DAMGO signaling from β-arrestin- to PKC-dependent.

All of the studies described above establish a link between agonist-induced receptor phosphorylation, PKC activation, and chronic drug responses. However, the exact relationship between all of these cellular signaling events remains unresolved. In the current study, by identifying a novel interaction between FK506 binding protein 12 (FKBP12) and OPRM1, we found that FKBP12 regulates the morphine-induced PKC activation via its modulation of receptor phosphorylation.

FKBP12, a 12-kDa FK506 binding protein that was first discovered as a receptor for the immunosuppressant drug FK506, is a peptidyl prolyl cis-trans isomerase (PPIase) (Park et al., 1992) belonging to the family of immunophilins and is found in all tissues with particularly high expression in the brain (Steiner et al., 1992). FKBP12 has been found to have versatile biological functions, including (A) binding to FK506 and thus inhibiting calcineurin, a Ca^{2+}-calmodulin-dependent protein phosphatase (Liu et al., 1991), (B) serving as a subunit of two major calcium-release channels of the endoplasmic reticulum, the ryanodine receptor (RyR) and the Ins(1,4,5)P_3 receptor (IP_3R) (Brillantes et al., 1994; Cameron et al., 1995), (C) regulating $[Ca^{2+}]i$ by stabilizing the closed conformation of the calcium channels, and (D) interacting with the type-1 receptor of transforming growth factor β (TGFβ) and down-regulating its signaling (Wang et al., 1996). Moreover, the possible involvement of FKBP12 in the development of morphine tolerance and dependence has been suggested. After chronic morphine treatment, microarray analyses have shown that FKBP12 is up-regulated in the locus coeruleus, but this

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finding has not been verified by quantitative RT-PCR (McClung et al., 2005). Our current study demonstrates that this protein directly interacts with the carboxyl tail of rat OPRM1. The direct association of FKBP12 with OPRM1 modulates receptor phosphorylation and thereby regulates the morphine-induced PKCε activation.

Materials and methods

Materials

Anti-OPRM1 antibody anti-μC was kindly provided by Dr. Lee-yuan Liu-Chen (Temple University School of Medicine, Philadelphia, PA, USA) (Huang et al., 2008). Morphine was supplied by the National Institute on Drug Abuse. FK506 was purchased from A.G. Scientific, Inc. (San Diego, CA, USA). Cell-permeable calcineurin autoinhibitory peptide was purchased from Millipore (Billerica, MA, USA). All of the other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Yeast two-hybrid screening

The cDNA fragment encoding the carboxyl tail of rat OPRM1 (OPRM1CT, amino acids 340-398) was amplified by PCR using appropriate primers with EcoRI restriction sites on both ends of the nucleotide sequence of the primers. The fragment was ligated in frame to the corresponding sites in the yeast shuttle vector pGBKT7 (Clontech, Mountain View, CA, USA) to create a plasmid encoding the GAL4BD-OPRM1CT fusion protein. This plasmid was transformed into yeast strain AH109. The expression of the bait protein in yeast was confirmed by immunoblotting with myc antibody (BD Biosciences, Franklin Lakes, NJ, USA).

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The Matchmaker II two-hybrid system (Clontech) was used to screen a mouse brain cDNA library (complexity 3.5×10^6 total recombinants) constructed in fusion with GAL4AD in pACT2 with OPRM1CT. Approximately 2.6×10^6 transformants were screened, and the Ade^+/His^+-positive clones were tested for α-galactosidase activity. The plasmids recovered from the positive clones were transformed into XL1-blue bacteria to select colonies containing only the target plasmids. The protein-protein interactions were verified in yeast by re-mating each positive target plasmid in pACT2 transformed into yeast strain Y187 with the pGBKT7 or pGBKT7-OPRM1CT vectors in yeast strain AH109. Nonspecific interactions were detected by the mating of the target plasmids with the pGBKT7 vector.

GST fusion protein pull-down assay

GST fusion proteins with OPRM1CT (GST-OPRM1CT), the δ-opioid receptor C tail (amino acids 322-372, GST-OPRD1CT), and the κ-opioid receptor C tail (amino acids 334-381, GST-OPRK1CT) were generated by PCR using appropriate primers with BamHI restriction sites on both ends of the nucleotide sequence of the primers. The resulting PCR products were first subcloned into pCR2.1, subcloned into the corresponding BamHI site of pGEX-2T (Pharmacia LKB, Gaithersburg, MD, USA) with proper orientation, and transformed into XL1-blue bacteria. The GST-OPRM1CTP353A was generated by PCR using GST-OPRM1CT as templates.

The GST-fusion proteins were prepared by growing the bacteria overnight at 37°C in LB broth with ampicillin (100 μg/ml) and inducing with 5 mM isopropyl β D-thiogalactopyranoside for 5 h. The cells were pelleted by centrifugation at 12,000 × g for 1 min, resuspended in PBS with 1% Triton X-100 and the Complete™ protease inhibitor cocktail (Roche, Indianapolis, IN, USA) at

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4ºC, sonicated twice with a microprobe set at a maximum power, and centrifuged again at 12,000 × g and 4°C for 15 min. The supernatant containing the GST-fusion proteins was incubated with 50% slurry of reduced glutathione-agarose for 3 h at 4°C. The beads were washed with PBS containing protease inhibitors at 4ºC and incubated with cell extracts of HEK293 cells transiently transfected with myc-FKBP12 overnight at 4°C. The beads were then washed with PBS at 4ºC, eluted with SDS sample buffer, separated on SDS-PAGE, and transferred onto an Immobilon^{TM}-P transfer membrane (Millipore). The membranes were immunoblotted with mouse anti-myc antibody (1:500, Millipore) and goat anti-mouse IgG conjugated to alkaline phosphatase (1:5000, Bio-Rad, Hercules, CA, USA) and then detected by ECF (GE Healthcare, Piscataway, NJ, USA).

Cell culture

HEK293 cells were maintained in Eagle's minimum essential medium (MEM) with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The transfection of HEK293 cells with pcDNA3 plasmids containing HA-tagged OPRM1 or mutant receptor with Pro^{353} mutated to Ala (OPRM1P353A) and the establishment of stably transfected cells were performed as described previously (24). The DNA transfections were performed using the Effectene reagent (Qiagen, Valencia, CA, USA). The stably transfected cells were maintained in the same medium supplemented with 200 μg/ml G418.

Confocal imaging

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HEK293 cells stably expressing OPRM1 were transiently transfected with myc-FKBP12. Forty-eight hours after transfection, the cells were washed twice with PBS at 4°C and fixed with Lana's fixative solution for 30 min. The cells were then washed with PBS three times and blocked in blocking buffer (PBS with 5% normal donkey serum). OPRM1 was visualized by staining with rabbit anti-HA (Convance, Princeton, NJ, USA; 1:1,000) and Alexa 488-conjugated goat-anti-rabbit antibody (1:1,000) (Invitrogen, Carlsbad, CA, USA). The cells were then permeabilized with 0.3% Triton X-100, and FKBP12 was identified by staining with mouse anti-myc (Millipore; 1:1,000) and Alexa 594-conjugated goat-anti-mouse antibody (1:1,000). The confocal images were captured with a BD CARV II Confocal Imager and a Leica DMIRE2 fluorescence microscope.

Co-immunoprecipitation

The cells were plated onto poly-L-lysine-coated 100-mm dishes. After agonist treatment, the cells were extracted with cell lysis buffer (0.1% Triton X-100, 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10% glycerol, 10 mM EDTA, 10 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and 1× protease inhibitor cocktail from Sigma). For the immunoprecipitation of the receptor with overexpressed FKBP12, the cells were transfected with myc tagged FKBP12. Forty-eight hours after transfection, the cells were treated with 10 μM FK506 or not and then lysed. After centrifugation at 12,000 × *g* for 5 min, mouse anti-myc (Millipore), anti-HA (Covance), or rabbit anti-calcineurin (Millipore) antibodies and protein A/G agarose (Pierce, Rockford, IL, USA)

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were added to the supernatants, and the mixture was rotated overnight. The beads were then washed five times with cell lysis buffer and extracted with SDS sample buffer. Approximately equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Antibodies specific for myc, PKCε (Cell Signaling, Danvers, MA, USA), FKBP12 (Proteintech, Chicago, IL, USA), HA, and calcineurin were used for the immunoblotting. The blots were developed by ECL (Pierce) and analyzed using the Image J (NIH) software.

CoralHue fluo-chase assay

The coding sequence with or without the stop codon of FKBP12 was amplified by PCR and ligated into the phmKGN-MC, phmKGC-MC, phmKGN-MN, or phmKGC-MN expression vectors (MBL International, Woburn, MA, USA). The coding sequence without the stop codon of OPRM1 was amplified by PCR and ligated into the phmKGN-MN or phmKGC-MN expression vectors. HEK293 cells were transiently cotransfected with mKGN and mKGC fusion plasmids using Fugene HD (Roche) for 24 h. To obtain the CoralHue fluo-chase image, the cells were fixed in Lana's fixative solution for 30 min and then washed with PBS three times. The confocal images were captured with a BD CARV II Confocal Imager and a Leica DMIRE2 fluorescence microscope. To perform the CoralHue fluo-chase assay in a microplate, the cells were harvested and washed once with PBS. The cells were then resuspended in Dulbecco's PBS (PBS with 0.1% glucose, 0.01% $CaCl_2$, and 0.01% $MgCl_2$) and distributed in a 96-well microplate. The readings were collected using a Fusion Microplate reader (Perkin Elmer, Waltham, MA, USA) with an excitation wavelength of 485 ± 10 nm and an emission wavelength

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of 520 ± 10 nm. The readings of untransfected cells were subtracted from all of the sample readings.

Molecular docking and molecular dynamics (MD) simulations

The crystal structure of FKBP12 in solution was retrieved from the protein data bank (PDB) (ID: 2PPN) and rendered by the Molecular Operating Environment (MOE, Montreal, Canada). Because there was no structure with a high sequence similarity to the C-terminal domain (amino acids 329-398) of rat OPRM1, its crystal structure was constructed using an online program (http://zhanglab.ccmb. med.umich.edu/I-TASSER; Roy et al., 2010; Roy et al., 2012; Zhang, 2008). Because the sequence between amino acids 329 to 352 is the same as the sequence between amino acids 419 to 442 of mouse OPRM1 (PDB ID: 4DKL), the constructed structure of the OPRM1 C-terminal domain was superposed to the known structure of mouse OPRM1, and the root-mean-square deviation (RMSD) was approximately 1 Å, which indicates that the modeled structure of the OPRM1 C-terminal domain is reasonable. To dock FKBP12 with the OPRM1 C-terminal domain, the binding patterns of FKBP12 with other proteins (PDB ID: 1B6C, 2RSE, 3MDY, and 3H9R) were analyzed, and the key residues of FKBP12 in the interactions were identified to be Asp^{37}, Gln^{53}, and Glu^{54}. The molecular docking was then performed using the PatchDock program and refined using FireDock (http://bioinfo3d.cs.tau.ac.il/PatchDock/; Duhovny D, 2002; Schneidman-Duhovny et al., 2005). The ten best docking results were further evaluated by the MOE program, and two docking models were identified as complex models. These two docking models were further evaluated by MD simulations to determine their conformational stability. The MD simulations of these complex models were performed for 20 ns

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using the Amber 12 program (Case, 2012) on a GPU-based workstation (Hewlett-Packard, Palo Alto, CA, USA). The Generalized Born Salvation Model (Tsui and Case, 2000) for macromolecular simulations was used instead of explicit water. The temperature was set to 300 K. To characterize the stability of both models, the RMSD and root mean square fluctuation (RMSF) were calculated. The distances between Ser^{375}-Thr^{376} of the OPRM1 C-terminal domain to the nearest residues of FKBP12 were then calculated.

Knockdown of FKBP12

HEK293 cells stably expressing OPRM1 were transfected with the reported short interfering RNA (siRNA) corresponding to the target sequence GCTTGAAGATGGAAAGAAA of the FKBP12 gene (Giordano et al., 2008) (Qiagen, Valencia, CA, USA) or a scrambled sequence as the control at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were analyzed as indicated. The effect of siRNA on protein expression was determined by Western blot.

Receptor phosphorylation assay

Cells cultured in 100-mm dishes were incubated with 1 μM morphine or etorphine for 20 min at 37°C. The reactions were terminated on ice. The cells were washed with ice-cold PBS at 4ºC and subsequently lysed in 0.5 ml of lysis buffer (0.5% Triton X-100, 10 mM Tris pH 7.4, 150 mM NaCl, and 25 mM KCl with 0.1 mM phenylmethylsulfonyl fluoride, Complete™ protease inhibitor cocktail (Roche), 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 0.1 mM sodium vanadate as phosphatase inhibitors). After centrifugation at 12,000 × *g* for 5 min, the supernatant was immunoprecipitated with 1 μg of mouse anti-HA (Covance) and Protein G

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agarose beads (Invitrogen) at 4°C overnight. The beads were then washed six times with cell lysis buffer and extracted with SDS sample buffer. Approximately equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The phosphorylated OPRM1 receptor was detected by anti-phosphoSer^{375} OPRM1 antibody (OPRM1phosphoSer^{375}) (Cell Signaling) and normalized to the total amount of immunoprecipitated receptor. The intensity of the individual bands was determined using the ImageQuant analysis software (GE Healthcare).

PKCε activity assay

After various treatments, the cells were lysed in lysis buffer containing 1% Triton X-100, 10 mM Tris, pH 7.5, 120 mM NaCl, 25 mM KCl, and Complete^{TM} protease inhibitor cocktail. The cell lysate was centrifuged at 800 × *g* for 5 min. The supernatant was incubated with 1 μg of anti-PKCε polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Then, 20 μl of protein G/A agarose beads were added, and the supernatant was incubated for an additional 3 h. The agarose beads were washed five times with lysis buffer, and the PKCε activity in the immunoprecipitates was then measured using the HTScan PKCε Kinase Assay Kit (Cell Signaling).

Measurement of the activation of extracellular signal-regulated kinases (ERK1/2)

Cells in 35-mm dishes were washed twice with PBS at 4°C and 0.1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 0.1% Triton X-100, 50 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM sodium pyrophosphate, 10 mM sodium vanadate, and Complete^{TM} protease inhibitor cocktail). After

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centrifugation, the supernatant was transferred to a new tube, and SDS sample buffer was added. Approximately equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The amount of phosphorylated ERK1/2 was monitored using a monoclonal antibody for phosphorylated ERK1/2 (Cell Signaling) and was normalized to the amount of total ERK1/2 detected by total ERK antibodies (Cell Signaling).

Statistical Analyses

The data are presented as the means ± S.E. of at least three independent experiments. The blots and images are representative from at least three independent experiments. Unpaired Student's *t* test (two-tailed) was performed for the statistical comparisons.

Results

FKBP12 interacts with OPRM1

The yeast two-hybrid screening of approximately 2.6×10^6 transformants from a mouse brain cDNA library using OPRM1CT as the bait led to the identification of two positive colonies encoding a 91-residue protein corresponding to FKBP12 amino acids 19 to 109 with 100% identities. The specificity of the interaction of OPRM1CT with FKBP12 was verified by retransforming FKBP12 cDNA into yeast strain Y187 and then mating it with yeast AH109, which contained either the original bait pGBKT7/OPRM1CT vector or the pGBKT7 vector. The interaction between OPRM1CT and FKBP12 specifically allowed the transformants to grow on medium lacking leucine, tryptophan, histidine, and adenine (SD/-Leu/-Trp/-His/-Ade). The yeast

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cells with the bait vector alone were unable to survive on SD/-Leu/-Trp/-His/-Ade medium in the presence of FKBP12 (data not shown).

To further confirm the specificity of the interaction between the OPRM1 C tail and FKBP12 among the opioid receptors, an *in vitro* GST-fusion protein pull-down assay was performed. When the GST fusion proteins with OPRM1CT (Fig. 1A, lane 2), the δ-opioid receptor C tail (amino acids 322-372, OPRD1CT, Fig. 1A, lane4), or the κ-opioid receptor C tail (amino acids 334-381, OPRK1CT, Fig. 1A, lane 5) were incubated with extracts of cells expressing FKBP12, FKBP12 was pulled down efficiently by GST-OPRM1CT and to a slight extent by GST-OPRD1CT but not by GST-OPRK1CT. This observed difference was not the result of different amounts of GST fusion proteins used in the pull-down assays, as indicated by the Coomassie blue staining of the fusion proteins.

To study whether the interaction of OPRM1 with FKBP12 occurs in mammalian cells, HEK293 cells stably expressing HA-tagged rat OPRM1 were transiently transfected with myc-tagged FKBP12. The colocalization of OPRM1 and FKBP12 was then detected through immunofluorescence studies. OPRM1 and FKBP12 were observed to be colocalized mainly in the cell membrane (Fig. 2A). This interaction between OPRM1 and FKBP12 in mammalian cells was demonstrated further by immunoprecipitation with a myc antibody (Fig. 2B). The proximal association of OPRM1 with FKBP12 was investigated through CoralHue fluo-chase analysis. CoralHue fluo-chase analysis enables the detection of protein-protein interactions in live cells by the formation of a fluorescent complex of complementary fluorescent protein fragments fused to the interacting proteins (Kerppola, 2006a; Kerppola, 2006b). We constructed the various fusion

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protein constructs by fusing OPRM1 to a fragment of fluorescent protein (CoralHue® monomeric Kusabira-Green (mKG)) and by fusing FKBP12 to the complementary fragment of mKG according to the manufacturer's instructions, i.e., OPRM1 was fused to the N-terminal fragment (mKGN) and FKBP12 was fused to the complementary C-terminal fragment (mKGC) and *vice versa*. The fusion of the proteins was either at the N terminus (MN) or the C terminus (MC) of the mKGN or mKGC. Preliminary co-expression studies in HEK293 cells indicated that the interaction between OPRM1 and FKBP12 could be demonstrated only by the fusion of both OPRM1 and FKBP12 to the N terminus of the mKG fragments, which means that the mKG fragments were fused to the C terminus of OPRM1 and FKBP12. Furthermore, the fluorescent signals were similar for the following pairs: OPRM1-mKGN/FKBP12-mKGC and OPRM1-mKGC/FKBP12-mKGN (data not shown). Thus, the OPRM1-mKGN/FKBP12-mKGC pair was used for further studies. When OPRM1-mKGN and FKBP12-mKGC were co-expressed in HEK293 cells, fluorescent signals could be detected both by confocal imaging (Fig. 2C, left panel) and by a fluorescence plate reader (Fig. 2D), whereas the co-expression of OPRM1-mKGN with mKGC vector did not exhibit fluorescence (Fig. 2C, middle panel, Fig. 2D). These data support a direct interaction between OPRM1 and FKBP12 in live cells.

To further demonstrate that OPRM1 is associated with FKBP12 endogenously, OPRM1 was immunoprecipitated from the mouse hippocampus using an anti-OPRM1 antibody (anti-μC) that was developed by immunizing rabbits with antigen containing the last 15 amino acid residues of the receptor (Huang et al., 2008). Anti-μC could immunoprecipitate endogenous OPRM1 in the hippocampus, whereas the signal was minimal in the cerebellum, which expresses little to no

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OPRM1 (Fig. 2E). Furthermore, the amount of FKBP12 immunoreactivity within the hippocampal immunoprecipitated anti-μC complex was greater than that observed from cerebellum immunoprecipitates (Fig. 2E), which indicates that OPRM1 interacts with FKBP12 endogenously.

FKBP12 directly binds to the isoleucylprolyl (position 352-353) motif of the OPRM1 C tail

The interaction sites of FKBP12 and its isoform FKBP12.6 with other proteins have been demonstrated to involve a peptidylprolyl residue, and the first peptidyl residue is always a hydrophobic amino acid, such as leucyl, isoleucyl, and valyl. The Val-Pro dipeptide in RyR1 (position 2461-2462) and RyR3 (position 2322-2323), the Ile-Pro dipeptide at position 2427-2428 in RyR2, the leucylprolyl residue in IP_3R1 (amino acids 1400-1401), and the type-1 receptor of TGFβ (amino acids 193-194) have been shown to be crucial residues for FKBP binding, and the binding of FKBP to these proteins can regulate their activation (Cameron et al., 1997; Charng et al., 1996; Gaburjakova et al., 2001; Marx et al., 2000; Van Acker et al., 2004). These specific peptidylprolyl epitopes structurally resemble FK506 (Cameron et al., 1997), which explains the ability of FK506 to attenuate the interaction between the peptidylpropyl residue and FKBP12.

The addition of 10 μM FK506 to the mixture used in the GST pull-down assay blocked the binding of GST-OPRM1CT with FKBP12 (Fig. 1B, lane 4). The blockade of the interaction of OPRM1 with FKBP12 by FK506 was also observed intracellularly, as shown in Fig. 2B. These results suggest that the Pro residue(s) in the C tail of OPRM1 must be involved in the binding of

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FKBP12 to OPRM1. There are four Pro residues in the carboxyl tail of rat OPRM1 (position 353, 374, 396, and 398), and Pro at 353 and 398 is paired with Ile or Leu. These residues are conserved in human, mouse, rat, and other mammalian species. Truncation mutation studies with the GST-OPRM1CT sequence indicated that the interaction with FKBP12 remained even if the sequence from Ser^{356} to Pro^{398} was deleted (GST-OPRM1CT355TGA, Fig. 1A, lane 3, Fig. 1B, lane 5), which suggests that Pro^{353} may be the residue in the OPRM1 carboxyl tail sequence involved in the receptor-FKBP12 interaction. Thus, we constructed a GST-OPRM1CT fusion protein in which Pro^{353} was mutated to Ala (GST-OPRM1CTP353A). The P353A mutation almost completely eliminated the interaction of the C tail of OPRM1 with FKBP12 (Fig. 1B, lane 8), which indicates that the isoleucylprolyl (position 352-353) motif is critical for the binding of OPRM1 to FKBP12. To confirm that Pro^{353} is involved in the OPRM1 interaction with FKBP12 in mammalian cells, cells stably expressing OPRM1 with Pro^{353} mutated to Ala (OPRM1P353A) were established. As shown in Fig. 2B, the mutated receptor was unable to be co-immunoprecipitated with myc-FKBP12 by the myc antibody. As expected, the CoralHue fluo-chase analysis revealed that OPRM1P353A abolished the binding of OPRM1 to FKBP12 in live cells (Fig. 2C, right panel, Fig. 2D). Thus, Pro^{353} is the residue within the OPRM1 carboxyl tail sequence that interacts with FKBP12.

FKBP12 regulates the phosphorylation at Ser^{375} of OPRM1

Because FKBP12 interacts with Pro^{353} of the OPRM1 carboxyl tail, which is in proximity to the Ser/Thr residues involved in the agonist-induced OPRM1 phosphorylation (El Kouhen et al.,

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2001), the interaction between FKBP12 and the receptor may cause steric hindrance and affect receptor phosphorylation. Thus, we first performed molecular docking analysis and MD simulations to investigate this possibility and to locate which phosphorylation sites are most likely affected. The molecular docking of the OPRM1 C-terminal domain with FKBP12 yielded two docking models that most likely represent the interaction between the OPRM1 carboxyl tail domain and FKBP12 (Fig. 3A, 3B).

Throughout the 20-ns MD simulations, the RMSDs of both models increased rapidly and fluctuated roughly during the first 8 ns and then became stable over the remaining 12 ns (Fig. 3C), which indicates that both models were sufficiently robust and that the confirmations changed little in the last 12 ns of the simulations. The RMSF, which is another indicator of model stability, measures the average atomic mobility of the backbone atoms (N, Cα, and C atoms) during MD simulations. For both models, the RMSFs of FKBP12 showed small fluctuations at approximately 50 Å (Fig. 3D), whereas the RMSFs of the C-terminal domain of OPRM1 remained stable only for those residues from Asp^{340} to Arg^{382} (Fig. 3E). These data demonstrate that the interaction region was stable in the simulations, which shows good correlation with the above-described experimental results.

The influence of the interaction on the phosphorylation sites of OPRM1 was then explored. As shown in Fig. 3A and 3B, both models displayed a pocket formed by FKBP12 in which $Ile^{352}Pro^{353}$ of the OPRM1 carboxyl tail domain was docked. The phosphorylation sites of the OPRM1 C-terminal domain--Ser^{363} and Thr^{370}, were buried inside the structure of the C tail. Another phosphorylation site (Thr^{379}) was far away from FKBP12. Ser^{375} was the closest site to

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FKBP12, and this residue is located next to Thr^{376}. In the MD simulations of model 2, the distance between Ser^{375}-Thr^{376} of the OPRM1 C-terminal tail domain and FKBP12 was reduced from 17 Å to 15 Å after 10 ns and then remained stable for the remaining 10 ns (Fig. 3F), which suggests that these two residues are more likely affected during dynamic processes. The unchanged distance in model 1 suggests that this model is markedly more stable (Fig. 3F). The short distance between Ser^{375} and FKBP12 and the stable complex structure of this model suggests that the phosphorylation of Ser^{375} and most likely Thr^{376} may be affected by the interaction between FKBP12 and the OPRM1 carboxyl tail.

To investigate whether the phosphorylation of Ser^{375} is indeed affected by the receptor-FKBP12 interaction, the morphine-induced phosphorylation of Ser^{375} was monitored as the cellular FKBP12 levels were manipulated. The transfection of vector or control siRNA did not significantly affect the cellular FKBP12 level, but the over-expression of FKBP12 markedly increased cellular the FKBP12 level, and the knockdown of FKBP12 with siRNA drastically decreased the endogenous FKBP12 level to 16 ± 3.6% of that observed in cells transfected with control siRNA (Fig. 4A). As shown in Fig. 4A and 4B, the over-expression of FKBP12 significantly decreased the OPRM1 phosphorylation at Ser^{375} by 25 ± 4.5% after a 20-min exposure to 1 μM morphine, whereas the knockdown of FKBP12 significantly increased the morphine-induced receptor phosphorylation by 44 ± 8.8%. To further investigate whether this effect is agonist-specific, the etorphine-induced receptor phosphorylation was examined. Similar to the above mentioned effects, the 1 μM etorphine-induced receptor phosphorylation at Ser^{375} could be inhibited by 34 ± 5.6% through the over-expression of FKBP12 and enhanced by 46 ±

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6.8% through the knockdown of FKBP12 (Fig. 4C and 4D). Furthermore, the morphine-induced phosphorylation at Ser^{375} of the OPRM1P353A mutant, which did not exhibit the receptor-FKBP12 interaction, was higher than that observed with the wild-type receptor (Fig. 4A and 4B), which indicates that the interaction between the receptor and FKBP12 modulates the phosphorylation at Ser^{375}.

FKBP12 modulates the morphine-induced PKCε activation and recruitment

Morphine has been shown to increase the phosphorylation of OPRM1 at the Ser^{375} residue only (Chu et al., 2008; Doll et al., 2011). Previous studies in our laboratory have shown that PKCε activation can be modulated by the phosphorylation state of the receptor (Chu et al., 2010; Zheng et al., 2008b). Blunting the agonist-induced receptor phosphorylation potentiates PKCε activation, whereas enhancing receptor phosphorylation attenuates PKCε activation (Zheng et al., 2011). Therefore, we investigated whether the changes in the agonist-induced receptor phosphorylation as a consequence of the OPRM1-FKBP12 interaction can influence the morphine-induced PKCε activation. As shown in Fig. 5A, in HEK293 cells stably expressing OPRM1, 1 μM morphine activated PKCε to a level that was 3.7 ± 0.65-fold of the basal activity. When the level of FKBP12 in these cells was knocked down by the transfection of FKBP12 siRNA, which results in an increase in receptor phosphorylation (Fig. 4), the morphine-induced PKCε activation was reduced to a level that was 1.3 ± 0.08-fold of the basal level; in contrast, a 3.3 ± 0.60-fold increase in PKCε activation was observed in cells transfected with control siRNA. Similarly, in HEK293 cells stably expressing the OPRM1P353A mutant, which is unable to

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interact with FKBP12, 1 μM morphine induced PKCε activation to a level that was 1.5 ± 0.41-fold of the basal level. Moreover, the over-expression of FKBP12 in HEK293 cells expressing wild-type OPRM1 increased the morphine-induced PKCε activation to a level that was 5.0 ± 0.71-fold of the basal level, although this increase was not statistically significant. In contrast, etorphine, which can induce a robust phosphorylation of OPRM1 at multiple sites and is unable to activate PKCε (Zheng et al., 2011), could activate PKCε to a level that was 1.7 ± 0.17-fold of the basal level in HEK293 cells over-expressing FKBP12 and stably expressing wild-type OPRM1 (Fig. 5B).

To further investigate whether the interaction between OPRM1 and FKBP12 can affect PKCε recruitment to the receptor complex (Chu et al., 2010), a co-immunoprecipitation experiment was performed. As expected, PKCε was recruited to the activated receptors in cells expressing wild-type OPRM1 (Fig. 5C and 5D). When the intracellular FKBP12 was knocked down, the recruitment of PKCε to the receptor was blocked (Fig. 5C and 5D). Similarly, the amount of PKCε recruited to the receptor complex was reduced significantly in OPRM1P353A-expressing cells compared with those expressing the wild-type receptor (Fig. 5C and 5D). These data indicate that FKBP12 mediates PKCε recruitment and activation through its direct interaction with OPRM1.

FKBP12 modulates PKCε-dependent ERK1/2 activation

ERK1/2 activation induced by morphine has been shown to be PKCε-dependent (Chu et al., 2008; Chu et al., 2010; Zheng et al., 2008b). Thus, to further demonstrate that FKBP12 can

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modulate morphine-mediated PKCε activation, the influence of FKBP12 on ERK1/2 activation was investigated. As shown in Fig. 6A and 6B, 1 μM morphine treatment for 10 min increased ERK1/2 phosphorylation to a level that was 2.0 ± 0.14-fold of the basal level in HEK293 cells stably expressing wild-type OPRM1. When the FKBP12 level was knocked down by FKBP12 siRNA, which resulted in an increase in agonist-induced receptor phosphorylation and a decrease in PKCε activation, the morphine-induced ERK1/2 activation was attenuated to a level that was 1.4 ± 0.05-fold of the basal level. In contrast, a 1.8 ± 0.08-fold increase was observed in cells transfected with control siRNA. In addition, this attenuation persisted after 1 h of morphine treatment, which suggests that the effect was not time-dependent (data not shown). The attenuation of ERK1/2 activation induced by morphine was also observed in HEK293 cells stably expressing OPRM1P353A (1.3 ± 0.21-fold increase), which interacts poorly with FKBP12 (Fig. 6A and 6B). These data indicate that FKBP12 plays a critical role in morphine-induced ERK1/2 activation by regulating the interactions between OPRM1 and FKBP12 and subsequently PKCε activation.

FKBP12 mediates the recruitment of calcineurin to the receptor complex

Further analysis of the receptor complex revealed that 1 μM morphine treatment increases the amount of FKBP12 recruited to the receptor complex, whereas the recruitment of FKBP12 was absent in OPRM1P353A-expressing cells (Fig. 7A and 7B). Because calcineurin binds FKBP12 and has been shown to mediate PKCε activation in cardiomyocytes (Vincent et al., 2006), calcineurin within the OPRM1 complex was also examined. As shown in Fig. 7A and 7B, 1 μM

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morphine induced the recruitment of calcineurin by wild-type OPRM1 receptors, wheres the recruitment of calcineurin was absent in cells expressing the mutant OPRM1P353A and significantly reduced when the FKBP12 level was knocked down by siRNA treatment. To further demonstrate that calcineurin was localized within the receptor-FKBP12 complex, cellular proteins co-immunoprecipitated with the calcineurin antibody were examined. As summarized in Fig. 7C and 7D, calcineurin, FKBP12, and PKCε formed a complex in the absence of OPRM1 activation. After morphine treatment, the amount of OPRM1, FKBP12, and PKCε co-immunoprecipitated with calcineurin increased significantly in OPRM1-expressing cells, whereas the amount of FKBP12 and PKCε was not altered in cells expressing the mutant OPRM1P353A receptor.

Because it is a phosphatase, we examined whether calcineurin affects receptor phosphorylation utilizing its autoinhibitory peptide. As shown in Fig. 8A and 8B, treatment with the cell-permeable calcineurin autoinhibitory peptide significantly increased the receptor phosphorylation at Ser^{375}, which demonstrates that calcineurin contributes to the decrease in receptor phosphorylation mediated by the association of FKBP12 with the receptor. Taken together, these data show that FKBP12 regulates the receptor phosphorylation and that this phosphorylation results in the recruitment and the activation of PKCε within the OPRM1 signaling complex.

Discussion

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Using the carboxyl tail of rat OPRM1 as the bait in the yeast two-hybrid system, FKBP12 was demonstrated to directly interact with OPRM1. The interaction between FKBP12 and OPRM1 was further confirmed in mammalian cells by several methods. First, the direct interaction was observed in a GST-fusion protein pull-down assay, and FK506 can block the interaction. Second, a CoralHue fluo-chase assay showed that this direct interaction exists in living cells. Third, the endogenous interaction was demonstrated by the co-immunoprecipitation of FKBP12 with OPRM1 in the mouse hippocampus. The interaction was further shown to be mainly in the cell membrane through colocalization immunofluorescence studies.

The association of FKBP12 was shown to be selective for OPRM1 but not OPRD1 or OPRK1. The conserved FKBP12 binding site, a peptidylprolyl residue, is present in the carboxyl tail of OPRM1 ($Ile^{352}Pro^{353}$), which was confirmed to be critical for the receptor-FKBP12 interaction through our mutational analyses. The peptidylprolyl residue is absent in the C tails of OPRD1 and OPRK1, which may well explain the lack of an interaction of these receptors with FKBP12.

One of major functions of FKBP12 is to regulate $[Ca^{2+}]i$ by binding to two Ca^{2+} release channels of the sarcoplasmic and endoplasmic reticula, the RyR and the IP_3R. The activation of OPRM1 has been shown to induce $[Ca^{2+}]i$ release from the intracellular Ca^{2+} pool when the P2Y receptor is co-activated by ADP (Chu et al., 2008). Moreover, the mobilization of the intracellular Ca^{2+} pool by the P2Y receptor involves IP_3 generation (Liu et al., 2000). However, the agonist-induced $[Ca^{2+}]i$ release was not modified by the manipulation of the intracellular FKBP12 levels (data not shown). Utilizing molecular docking and MD simulations, we found that the steric hindrance incurred by the direct receptor-FKBP12 interaction may influence

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receptor phosphorylation, particularly the phosphorylation of the Ser^{375} residue. The phosphorylation sites of OPRM1 have been demonstrated to be Ser^{363}, Thr^{370}, and Ser^{375} residues in the carboxyl tail, and Thr^{370} and Ser^{375} have been found to be agonist-dependent phosphorylation sites (El Kouhen et al., 2001). Recently, Thr^{376} and Thr^{379} were found to be phosphorylation sites (Just et al., 2013). However, morphine is shown to induce the phosphorylation of Ser^{375} only, whereas other agonists, such as etorphine and DAMGO, can induce the phosphorylation of multiple sites (Chu et al., 2008; Doll et al., 2011). Using the commercial available antibody to phosphorylated Ser^{375}, the morphine- and etorphine-induced phosphorylation at Ser^{375} was confirmed to be regulated by FKBP12. Due to the limited availability of antibodies, it has not yet been clarified whether the interaction between the receptor and FKBP12 can affect other phosphorylation sites.

In addition to the probable steric hindrance suggested by our modeling studies (Fig. 3), FKBP12 possesses PPIase activity that can change the carboxyl tail conformation and thus affect the agonist-induced receptor phosphorylation. However, the activity-deficient mutant also reduces the overall FKBP12 affinity for its target (Timerman et al., 1995). Thus, further investigation is required to determine whether the PPIase activity of FKBP12 affects receptor phosphorylation. It is clear that the recruitment of calcineurin to the receptor complex by FKBP12 contributes to the phosphorylation state of OPRM1 (Fig. 8). It is likely that the decrease in receptor phosphorylation as a consequence of FKBP12-receptor interaction is attributed to both the inhibition of GRK activity due to its proximity to the Ser^{375} residue and the recruitment of calcineurin to the receptor complex.

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A previous study indicated that the receptor phosphorylation at the Ser^{375} residue in the carboxyl tail is an important determinant of the agonist-dependent PKCε signaling pathway (Zheng et al., 2011). Our data clearly demonstrate that the receptor-FKBP12 association and the subsequent morphine-induced receptor phosphorylation at the Ser^{375} residue correlate well with PKCε activation and association with the receptor complex. Furthermore, because other agonists, such as etorphine, do not activate PKCε, as observed in the current and previous studies (Chu et al., 2008; Zheng et al., 2008b), the over-expression of FKBP12, which inhibited receptor phosphorylation, resulted in PKCε activation. These results not only substantiate our previous observation that the signaling pathways selected by an individual agonist can be regulated by the receptor phosphorylation state but also indicate that FKBP12 may be the key element underlying the interplay between receptor phosphorylation and PKC activation.

Although our study cannot preclude the possibility of PKCε activation induced by other signaling molecules, our co-immunoprecipitation studies demonstrate that the association of FKBP12 with OPRM1 directly mediates PKCε recruitment to the receptor signaling complexes. FKBP12, calcineurin, and PKCε form a complex in the absence of OPRM1 activation, which is consistent with other studies that showed a basal interaction between FKBP12, calcineurin, and PKCε (Cardenas et al., 1994; Husi et al., 1994; Vincent et al., 2006). The dissociation of FKBP12 from the receptor by knocking down the cellular FKBP12 level or using the FKBP12 association-deficient mutant receptor abolished the recruitment of calcineurin and PKCε to the receptor. These data strongly indicate that FKBP12 serves as an adaptor for the recruitment of PKCε and calcineurin. The fact that morphine can regulate the synaptic GluA1 subunit level of

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the AMPA receptor via its activation of calcineurin (Kam et al., 2010) suggests functions of these molecules in OPRM1 signaling beyond those that were explored in the current study.

In summary, the current study delineated a mechanism in which the interaction between FKBP12 and OPRM1 inhibits the agonist-induced receptor phosphorylation and further facilitates the morphine-induced PKCε activation. The association of the receptor with FKBP12 may underlie the interplay between receptor phosphorylation and agonist-dependent signaling. In view of the fact that FKBP12 is up-regulated in morphine-tolerant rats, as shown through microarray analyses (McClung et al., 2005), the interaction between FKBP12 and OPRM1 may be enhanced to reinforce PKC activation, regardless of whether the up-regulation of FKBP12 is adaptive to or causative of morphine tolerance. In this respect, interference with the FKBP12 association with OPRM1 may lessen the chronic effects of morphine, including tolerance development.

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Authorship Contributions

Participated in research design: Qiu, Loh, Chen, Law

Conducted experiments: Qiu, Zhao, Wang, Xu, Huie, Jiang, Yan

Performed data analysis: Qiu, Zhao, Wang, Law

Wrote or contributed to the writing of the manuscript: Qiu, Zhao, Loh, Chen, Law

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References

Bailey CP, Llorente J, Gabra BH, Smith FL, Dewey WL, Kelly E and Henderson G (2009) Role of protein kinase C and μ-opioid receptor (MOPr) desensitization in tolerance to morphine in rat locus coeruleus neurons. *European Journal of Neuroscience* **29**(2):307-318.

Bohn LM, Lefkowitz RJ and Caron MG (2002) Differential mechanisms of morphine antinociceptive tolerance revealed in beta arrestin-2 knock-out mice. *J Neurosci* **22**(23):10494-10500.

Brillantes AB, Ondrias K, Scott A, Kobrinsky E, Ondriasova E, Moschella MC, Jayaraman T, Landers M, Ehrlich BE and Marks AR (1994) Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **77**(4):513-523.

Cameron AM, Nucifora FC, Jr., Fung ET, Livingston DJ, Aldape RA, Ross CA and Snyder SH (1997) FKBP12 binds the inositol 1,4,5-trisphosphate receptor at leucine-proline (1400-1401) and anchors calcineurin to this FK506-like domain. *J Biol Chem* **272**(44):27582-27588.

Cameron AM, Steiner JP, Roskams AJ, Ali SM, Ronnett GV and Snyder SH (1995) Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca2+ flux. *Cell* **83**(3):463-472.

Cardenas ME, Hemenway C, Muir RS, Ye R, Fiorentino D and Heitman J (1994) Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands. *EMBO J* **13**(24):5944-5957.

Case DA, Darden, T.A., Cheatham III, T.E., Simmerling, C.L., Wang, J., Duke, R.E., Luo, R., Walker R.C, Zhang, W., Merz, K.M., Roberts, B., Hayik, S.,Roitberg, A., Seabra, G., Swails, J., Goetz, A.W., Kolossváry, I., Wong, K.F., Paesani, F., Vanicek, J., Wolf, R.M., Liu, J., Wu, X., Brozell, S.R., Steinbrecher, T., Gohlke, H., Cai, Q., Ye, X., Wang, J., Hsieh, M.-J., Cui, G., Roe, D.R., Mathews, D.H., Seetin, M.G., Salomon-Ferrer, R., Sagui, C., Babin, V., Luchko, T., Gusarov, S., Kovalenko, A., and Kollman, P.A. (2012) *AMBER 12*, University of California, San Francisco.

Charng MJ, Kinnunen P, Hawker J, Brand T and Schneider MD (1996) FKBP-12 recognition is dispensable for signal generation by type I transforming growth factor-beta receptors. *J Biol Chem* **271**(38):22941-22944.

Chu J, Zheng H, Loh HH and Law PY (2008) Morphine-induced mu-opioid receptor rapid desensitization is independent of receptor phosphorylation and beta-arrestins. *Cell Signal* **20**(9):1616-1624.

Chu J, Zheng H, Zhang Y, Loh HH and Law PY (2010) Agonist-dependent mu-opioid receptor signaling can lead to heterologous desensitization. *Cell Signal* **22**(4):684-696.

Doll C, Konietzko J, Poll F, Koch T, Hollt V and Schulz S (2011) Agonist-selective patterns of micro-opioid receptor phosphorylation revealed by phosphosite-specific antibodies. *Br J Pharmacol* **164**(2):298-307.

Duhovny D NR, Wolfson HJ (2002) Efficient Unbound Docking of Rigid Molecules, in *Proceedings of the 2'nd Workshop on Algorithms in Bioinformatics(WABI)* (al Ge ed) pp 185-200, Springer Verlag, Rome, Italy.

El Kouhen R, Burd AL, Erickson-Herbrandson LJ, Chang CY, Law PY and Loh HH (2001) Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid receptor internalization. *J Biol Chem* **276**(16):12774-12780.

Gaburjakova M, Gaburjakova J, Reiken S, Huang F, Marx SO, Rosemblit N and Marks AR (2001) FKBP12 binding modulates ryanodine receptor channel gating. *J Biol Chem* **276**(20):16931-16935.

Giordano A, Romano S, Mallardo M, D'Angelillo A, Cali G, Corcione N, Ferraro P and Romano MF (2008) FK506 can activate transforming growth factor-beta signalling in vascular smooth muscle cells and promote proliferation. *Cardiovasc Res* **79**(3):519-526.

Huang P, Chen C, Xu W, Yoon SI, Unterwald EM, Pintar JE, Wang Y, Chong PL and Liu-Chen LY (2008) Brain region-specific N-glycosylation and lipid rafts association of the rat mu opioid receptor. *Biochem Biophys Res Commun* **365**(1):82-88.

Husi H, Luyten MA and Zurini MG (1994) Mapping of the immunophilin-immunosuppressant site of interaction on calcineurin. *J Biol Chem* **269**(19):14199-14204.

Just S, Illing S, Trester-Zedlitz M, Lau EK, Kotowski SJ, Miess E, Mann A, Doll C, Trinidad JC, Burlingame AL, von Zastrow M and Schulz S (2013) Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. *Mol Pharmacol* **83**(3):633-639.

Kam AY, Liao D, Loh HH and Law PY (2010) Morphine induces AMPA receptor internalization in primary hippocampal neurons via calcineurin-dependent dephosphorylation of GluR1 subunits. *J Neurosci* **30**(45):15304-15316.

Kerppola TK (2006a) Complementary methods for studies of protein interactions in living cells. *Nat Methods* **3**(12):969-971.

Kerppola TK (2006b) Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. *Nat Protoc* **1**(3):1278-1286.

Kieffer BL (1999) Opioids: first lessons from knockout mice. *Trends Pharmacol Sci* **20**(1):19-26.

Liu DM, Katnik C, Stafford M and Adams DJ (2000) P2Y purinoceptor activation mobilizes intracellular Ca2+ and induces a membrane current in rat intracardiac neurones. *J Physiol* **526 Pt 2**:287-298.

Liu J, Farmer JD, Jr., Lane WS, Friedman J, Weissman I and Schreiber SL (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**(4):807-815.

Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblit N and Marks AR (2000) PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* **101**(4):365-376.

Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dolle P, Tzavara E, Hanoune J, Roques BP and Kieffer BL (1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* **383**(6603):819-823.

McClung CA, Nestler EJ and Zachariou V (2005) Regulation of gene expression by chronic morphine and morphine withdrawal in the locus ceruleus and ventral tegmental area. *J Neurosci* **25**(25):6005-6015.

Park ST, Aldape RA, Futer O, DeCenzo MT and Livingston DJ (1992) PPIase catalysis by human FK506-binding protein proceeds through a conformational twist mechanism. *J Biol Chem* **267**(5):3316-3324.

Rezvani A, Huidobro-Toro JP, Hu J and Way EL (1983) A rapid and simple method for the quantitative determination of tolerance development to opiates in the guinea-pig ileum in vitro. *J Pharmacol Exp Ther* **225**(2):251-255.

Roy A, Kucukural A and Zhang Y (2010) I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* **5**(4):725-738.

Roy A, Yang J and Zhang Y (2012) COFACTOR: an accurate comparative algorithm for structure-based protein function annotation. *Nucleic Acids Res* **40**(Web Server issue):W471-477.

Schneidman-Duhovny D, Inbar Y, Nussinov R and Wolfson HJ (2005) PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic Acids Res* **33**(Web Server issue):W363-367.

Smith FL, Javed R, Elzey MJ, Welch SP, Selley D, Sim-Selley L and Dewey WL (2002) Prolonged reversal of morphine tolerance with no reversal of dependence by protein kinase C inhibitors. *Brain Research* **958**(1):28-35.

Steiner JP, Dawson TM, Fotuhi M, Glatt CE, Snowman AM, Cohen N and Snyder SH (1992) High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature* **358**(6387):584-587.

Timerman AP, Wiederrecht G, Marcy A and Fleischer S (1995) Characterization of an exchange reaction between soluble FKBP-12 and the FKBP.ryanodine receptor complex. Modulation by FKBP mutants deficient in peptidyl-prolyl isomerase activity. *J Biol Chem* **270**(6):2451-2459.

Tsui V and Case DA (2000) Theory and applications of the generalized Born solvation model in macromolecular simulations. *Biopolymers* **56**(4):275-291.

Van Acker K, Bultynck G, Rossi D, Sorrentino V, Boens N, Missiaen L, De Smedt H, Parys JB and Callewaert G (2004) The 12 kDa FK506-binding protein, FKBP12, modulates the Ca(2+)-flux properties of the type-3 ryanodine receptor. *J Cell Sci* **117**(Pt 7):1129-1137.

Vincent F, Duquesnes N, Christov C, Damy T, Samuel JL and Crozatier B (2006) Dual level of interactions between calcineurin and PKC-epsilon in cardiomyocyte stretch. *Cardiovasc Res* **71**(1):97-107.

Wang T, Li BY, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, Martin J, Manganaro T and Donahoe PK (1996) The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. *Cell* **86**(3):435-444.

Whistler JL, Chuang HH, Chu P, Jan LY and von Zastrow M (1999) Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* **23**(4):737-746.

Zhang Y (2008) I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* **9**:40.

Zheng H, Chu J, Qiu Y, Loh HH and Law P-Y (2008a) Agonist-selective signaling is determined by the receptor location within the membrane domains. *Proceedings of the National Academy of Sciences* **105**(27):9421-9426.

Zheng H, Chu J, Zhang Y, Loh HH and Law PY (2011) Modulating {micro}-opioid receptor phosphorylation switches agonist-dependent signaling as reflected in PKC{epsilon} activation and dendritic spine stability. *J Biol Chem* **286**(14):12724-12733.

Zheng H, Loh HH and Law P-Y (2008b) -Arrestin-Dependent {micro}-Opioid Receptor-Activated Extracellular Signal-Regulated Kinases (ERKs) Translocate to Nucleus in Contrast to G Protein-Dependent ERK Activation. *Mol Pharmacol* **73**(1):178-190.

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Footnotes

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Figure Legends

Fig. 1 Analysis of the interaction between FKBP12 and OPRM1 through a GST pull-down assay. (A) HEK293 cells stably expressing full-length myc-tagged FKBP12 were lysed, immobilized to a GST fusion protein containing OPRM1CT, OPRD1CT, OPRK1CT, or mutated OPRM1CT355TGA resins, and immunoblotted with an antibody to myc. The bacterially expressed GST fusion proteins were verified by staining the blot with Coomassie blue. (B) HEK293 cells stably expressing full-length myc-tagged FKBP12 were lysed, immobilized to a GST fusion protein containing OPRM1CT or the mutants OPRM1CT355TGA and OPRM1CTP353A, and immunoblotted with an antibody to myc. 10 μM FK506 was added 10 min before the addition of the GST fusion proteins.

Fig. 2 Intracellular and endogenous interaction between FKBP12 and OPRM1. (A) Colocalization of OPRM1 and FKBP12. HEK293 cells stably expressing HA-OPRM1 were transiently transfected with myc-FKBP12 and then immunostained with anti-HA and anti-myc antibodies. (B) Immunoprecipitation of OPRM1 with FKBP12. HEK293 cells stably expressing HA-OPRM1 (as a negative control) and stably co-expressing HA-OPRM1 or HA-OPRM1P353A and myc-FKBP12 were lysed. A set of cells were treated with 10 μM FK506 for 20 min before lysis. The lysate samples were immunoprecipitated with myc antibody. The precipitates were immunoblotted with antibodies to either HA or myc. (C) Confocal image of CoralHue fluo-chase assay. HEK293 cells were transiently transfected with OPRM1-mKGN and either

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FKBP12-mKGC or vector mKGC or with OPRM1P353A-mKGN and FKBP12-mKGC. Twenty-four hours after transfection, the cells were fixed, and the fluorescence was detected with a BD CARV II Confocal Imager and a Leica DMIRE2 fluorescence microscope. The existence of cells is shown in the lower panel. (D) CoralHue fluo-chase assay using a microplate reader. HEK 293 cells were transfected as described above. Twenty-four hours after transfection, the cells were washed and collected in Dulbecco's PBS and distributed in a 96-well microplate. The readings were collected using a Fusion Microplate reader. **, $P < 0.01$ *versus* OPRM1-mKGN+mKGC. (E) Co-immunoprecipitation of FKBP12 with OPRM1 in a mouse brain region with little to no endogenous OPRM1 expression (cerebellum) or in a mouse brain region with high endogenous OPRM1 expression (hippocampus). Hippocampus and cerebellum were removed from mouse brain and homogenized in lysis buffer. The lysate was immunoprecipitated with an anti-μC antibody and immunoblotted with a FKBP12 antibody.

Fig. 3 Modeled complex of OPRM1 C-terminal domain and FKBP12. (A-B) The constructed OPRM1 C-terminal domain (red) was docked into the crystal structure of FKBP12 (PDB ID: 2PPN, purple) with a redered hydrophobic surface. A: model 1, B: model 2. (C) RMSD of models 1 and 2 during the 20-ns MD simulations. (D-E) RMSF of FKBP12 (D) and the OPRM1 C-terminal domain (E) in models 1 and 2. (F) The distances between $Ser^{375}Thr^{376}$ of the OPRM1 C-terminal domain and the nearest residues of FKBP12 through the 20-ns MD simulations.

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Fig. 4 FKBP12 modulates the agonist-induced OPRM1 phosphorylation at Ser^{375}. (A-B) Morphine-induced receptor phosphorylation on Ser^{375} influenced by FKBP12. HEK293 cells stably expressing OPRM1 were transfected with FKBP12, the PcDNA3 vector, FKBP12 siRNA, or control siRNA. Forty-eight hours after transfection, the cells were treated with 1 μM morphine for 20 min. HEK293 cells stably expressing OPRM1P353A were treated with 1 μM morphine for 20 min. The receptor phosphorylation on Ser^{375} was then determined (A) and quantified (B) as described in "Materials and methods". *, $P < 0.05$. (C-D) Etorphine-induced receptor phosphorylation on Ser^{375} is influenced by FKBP12. HEK293 cells stably expressing OPRM1 were transfected as described above and treated with 1 μM etorphine for 20 min. The receptor phosphorylation on Ser^{375} was then determined (C) and quantified (D) as described in "Materials and methods". *, $P < 0.05$.

Fig. 5 FKBP12 modulates PKCε activation and recruitment to receptor. (A) HEK293 cells stably expressing OPRM1 were transfected with FKBP12 siRNA or myc-FKBP12 and their respective control siRNA or vector PcDNA3. Forty-eight hours after transfection, the cells were treated with 1 μM morphine for 5 min; HEK293 cells stably expressing OPRM1P353A were treated with 1 μM morphine for 5 min. The PKCε activity was then measured. *, $P < 0.05$, **, $P < 0.01$. (B) HEK293 cells stably expressing OPRM1 were transfected with myc-FKBP12 or vector PcDNA3 as described above and treated with 1 μM etorphine for 5 min. The PKCε activity was then measured. *, $P < 0.05$. (C-D) HEK293 cells stably expressing OPRM1 or mutant OPRM1P353A were treated with 1 μM morphine for 5 min. HEK-OPRM1 cells were transfected

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with FKBP12 siRNA or control siRNA, and 48 h after transfection, the cells were treated with 1 μM morphine for 5 min. The OPRM1 signaling complexes were immunoprecipitated with HA antibody and immunoblotted with a PKCε antibody (C). The relative densities of immunoprecipitated PKCε were quantified by normalizing to the total amount of immunoprecipitated receptors (D). *, $P < 0.05$.

Fig. 6 FKBP12 modulates PKCε-dependent ERK1/2 activation. HEK293 cells stably expressing OPRM1 were transfected with FKBP12 siRNA or myc-FKBP12 and their respective control siRNA or vector PcDNA3. Forty-eight hours after transfection, the cells were treated with 1 μM morphine for 10 min, and the phosphorylated ERK1/2 was then determined (A) and quantified (B) as described in "Materials and methods". *, $P < 0.05$, **, $P < 0.01$ *versus* cells without agonist treatment or as denoted.

Fig. 7 The association of FKBP12 with OPRM1 mediates calcineurin recruitment to the receptor signaling complex. (A-B) HEK293 cells stably expressing OPRM1 or mutant OPRM1P353A were treated with 1 μM morphine for 5 min. The OPRM1 signaling complexes were immunoprecipitated with HA antibody and immunoblotted with calcineurin and FKBP12 antibodies (A). The relative densities of the immunoprecipitated calcineurin and FKBP12 were quantified by normalizing to the total amount of immunoprecipitated receptors (B). **, $P < 0.01$. (C-D) HEK293 cells stably expressing OPRM1 or mutant OPRM1P353A were treated with 1 μM morphine for 5 min. The cell lysates were then immunoprecipitated with calcineurin

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antibody and immunoblotted with PKCε and FKBP12 antibodies (C). The relative densities of immunoprecipitated PKCε and FKBP12 were quantified (D) by normalizing to the total amount of immunoprecipitated calcineurin. *, $P < 0.05$ *versus* OPRM1-expressing cells.

Fig. 8 Calcineurin plays a role in the regulation of receptor phosphorylation. HEK293 cells stably expressing OPRM1 were treated or not treated with 40 μM calcineurin autoinhibitory peptide for 2 h. The cells were then treated with 1 μM morphine for 20 min. The receptor phosphorylation at Ser^{375} was determined (A) and quantified (B) as described in "Materials and methods". *, $P < 0.05$.

Figure 1

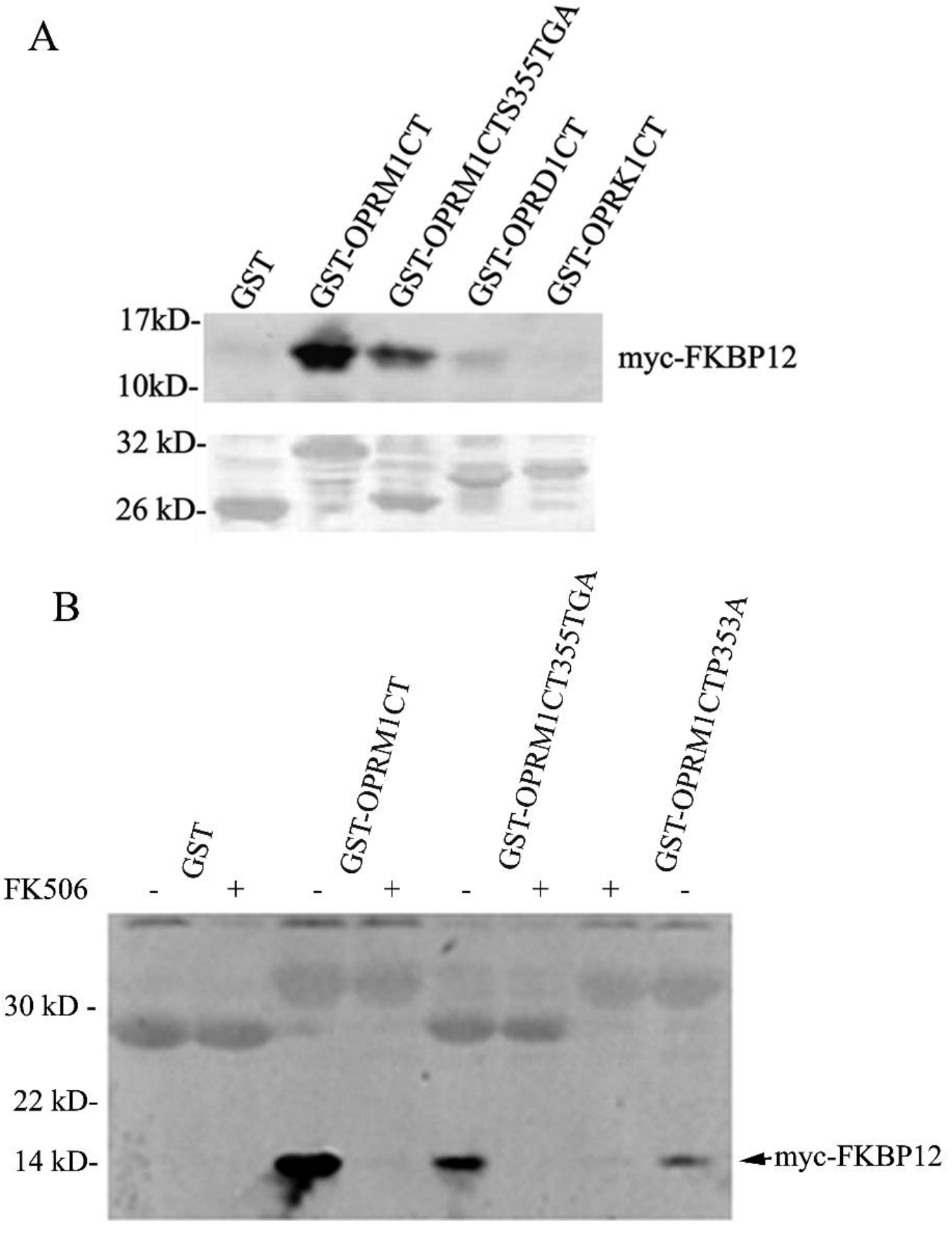

A
GST
GST-OPRM1CT
GST-OPRM1CTS355TGA
GST-OPRD1CT
GST-OPRK1CT
17kD-
10kD-
myc-FKBP12
32 kD-
26 kD-
B
GST
GST-OPRM1CT
GST-OPRM1CT355TGA
GST-OPRM1CTP353A
FK506
- + - + - + + -
30 kD -
22 kD-
14 kD-
myc-FKBP12

Figure 2

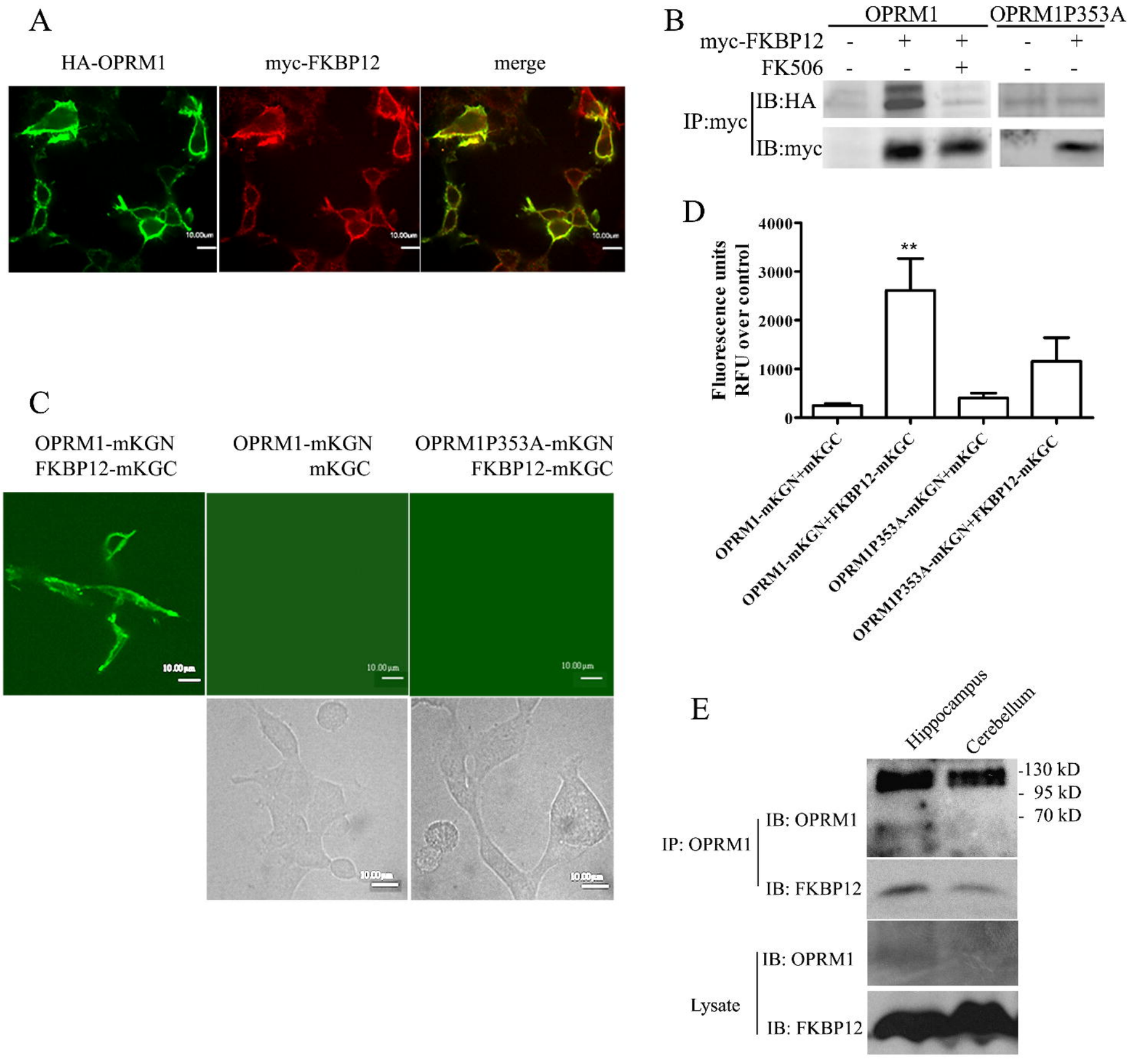

A
HA-OPRM1
myc-FKBP12
merge
B
OPRM1
OPRM1P353A
myc-FKBP12 - + + - +
FK506 - - + - -
IP:myc
IB:HA
IB:myc
C
OPRM1-mKGN
FKBP12-mKGC
OPRM1-mKGN
mKGC
OPRM1P353A-mKGN
FKBP12-mKGC
D
Fluorescence units
RFU over control
4000
3000
2000
1000
0
**
OPRM1-mKGN+mKGC
OPRM1-mKGN+FKBP12-mKGC
OPRM1P353A-mKGN+mKGC
OPRM1P353A-mKGN+FKBP12-mKGC
E
Hippocampus
Cerebellum
-130 kD
- 95 kD
- 70 kD
IP: OPRM1
IB: OPRM1
IB: FKBP12
Lysate
IB: OPRM1
IB: FKBP12

Figure 3

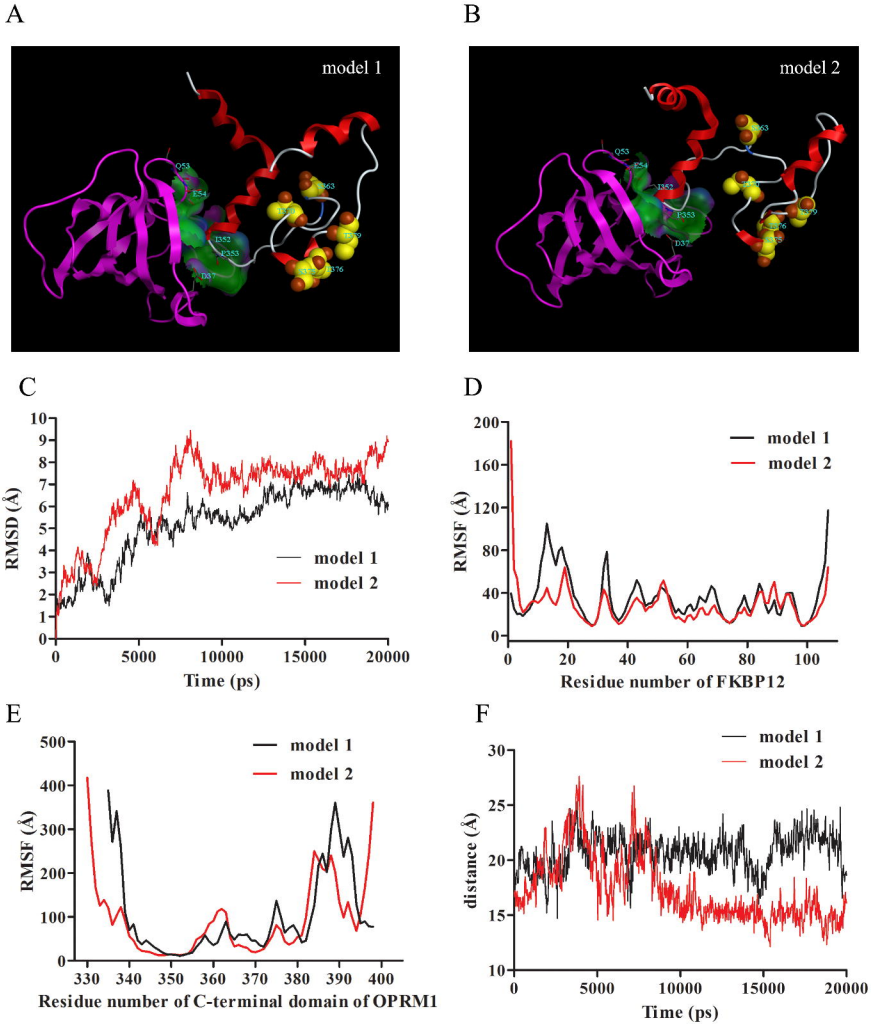

A
model 1
B
model 2
C
RMSD (Å)
Time (ps)
model 1
model 2
D
RMSF (Å)
Residue number of FKBP12
model 1
model 2
E
RMSF (Å)
Residue number of C-terminal domain of OPRM1
model 1
model 2
F
distance (Å)
Time (ps)
model 1
model 2

Figure 4

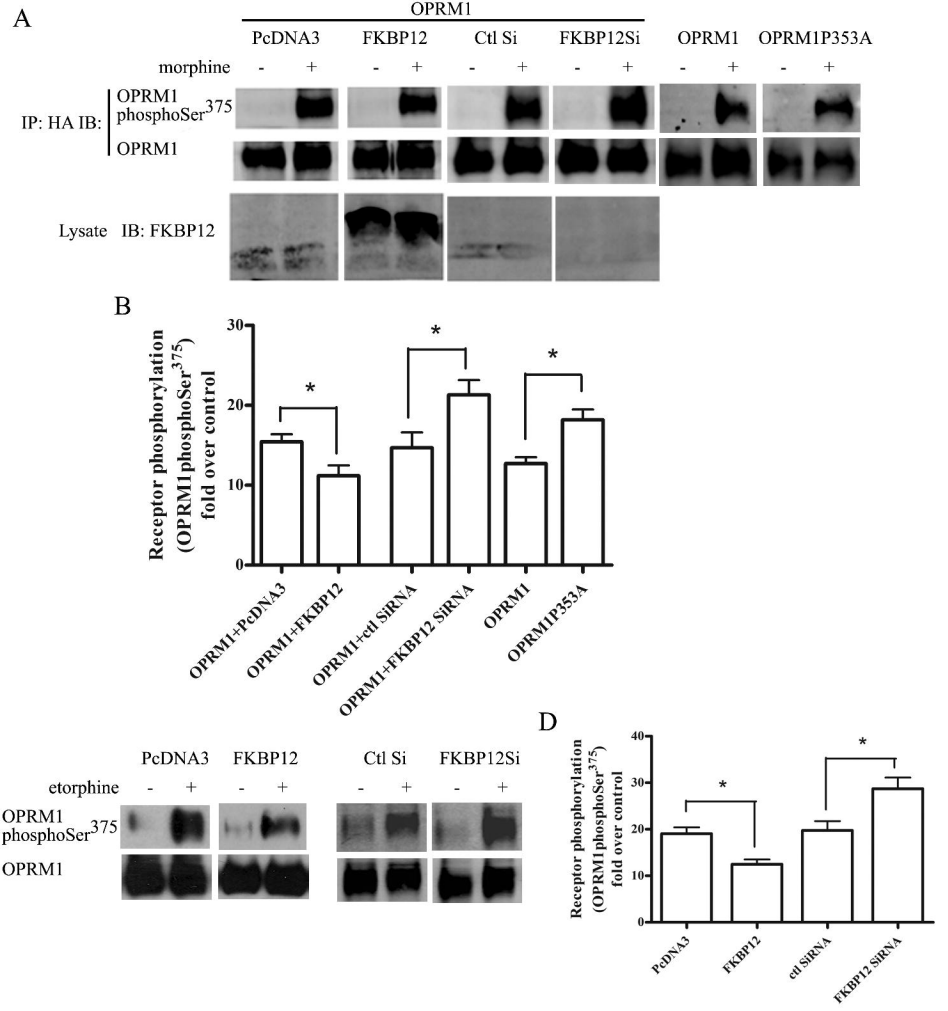

A
OPRM1
PcDNA3
FKBP12
Ctl Si
FKBP12Si
OPRM1
OPRM1P353A
morphine
IP: HA IB:
OPRM1 phosphoSer375
OPRM1
Lysate IB: FKBP12
B
Receptor phosphorylation (OPRM1phosphoSer375) fold over control
OPRM1+PcDNA3
OPRM1+FKBP12
OPRM1+ctl SiRNA
OPRM1+FKBP12 SiRNA
OPRM1
OPRM1P353A
C
PcDNA3
FKBP12
Ctl Si
FKBP12Si
etorphine
IP: HA IB:
OPRM1 phosphoSer375
OPRM1
D
Receptor phosphorylation (OPRM1phosphoSer375) fold over control
PcDNA3
FKBP12
ctl SiRNA
FKBP12 SiRNA

Figure 5

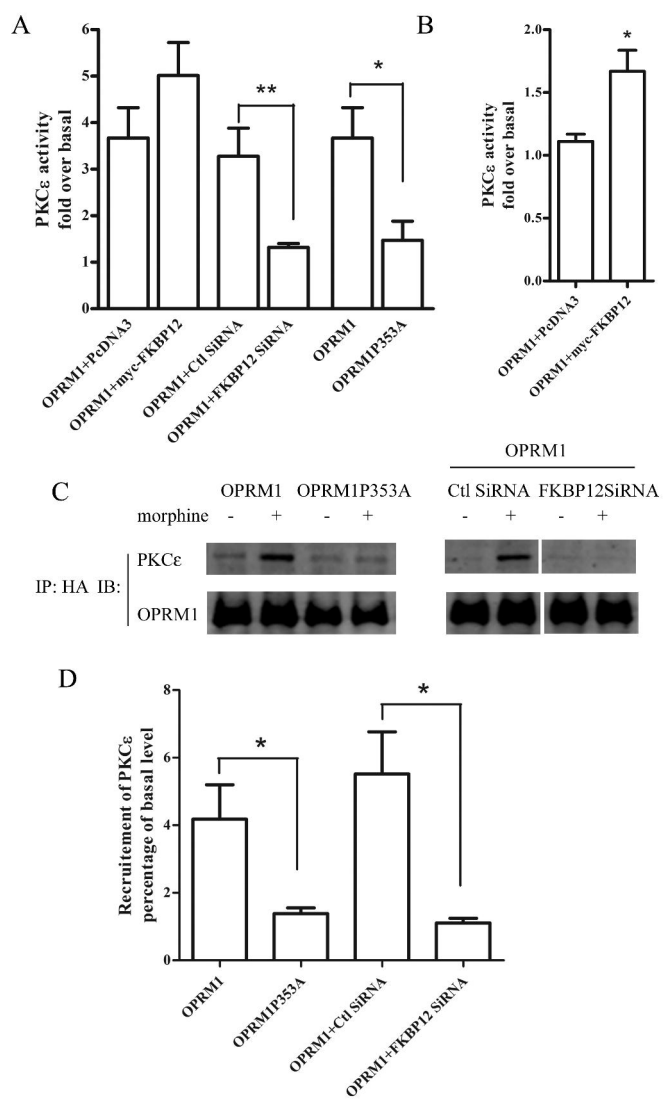

A
PKCε activity fold over basal
OPRM1+PcDNA3
OPRM1+myc-FKBP12
OPRM1+Ctl SiRNA
OPRM1+FKBP12 SiRNA
OPRM1
OPRM1P353A
B
PKCε activity fold over basal
OPRM1+PcDNA3
OPRM1+myc-FKBP12
C
OPRM1
OPRM1
OPRM1P353A
Ctl SiRNA
FKBP12SiRNA
morphine
IP: HA IB:
PKCε
OPRM1
D
Recruitement of PKCε percentage of basal level
OPRM1
OPRM1P353A
OPRM1+Ctl SiRNA
OPRM1+FKBP12 SiRNA

Figure 6

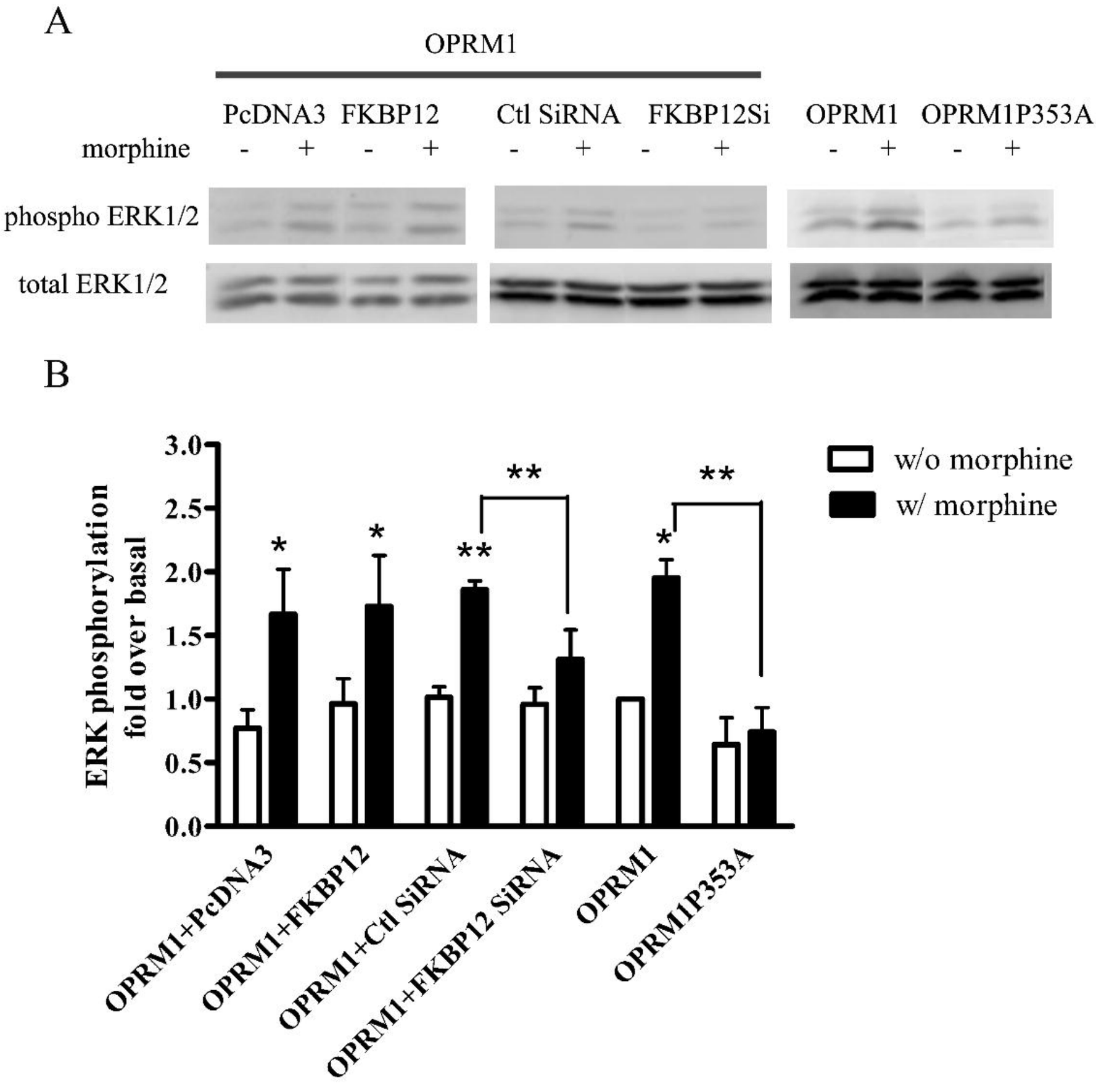

A
OPRM1
PcDNA3
FKBP12
Ctl SiRNA
FKBP12Si
OPRM1
OPRM1P353A
morphine
- + - + - + - + - + - +
phospho ERK1/2
total ERK1/2
B
w/o morphine
w/ morphine
ERK phosphorylation fold over basal
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
OPRM1+PcDNA3
OPRM1+FKBP12
OPRM1+Ctl SiRNA
OPRM1+FKBP12 SiRNA
OPRM1
OPRM1P353A

Figure 7

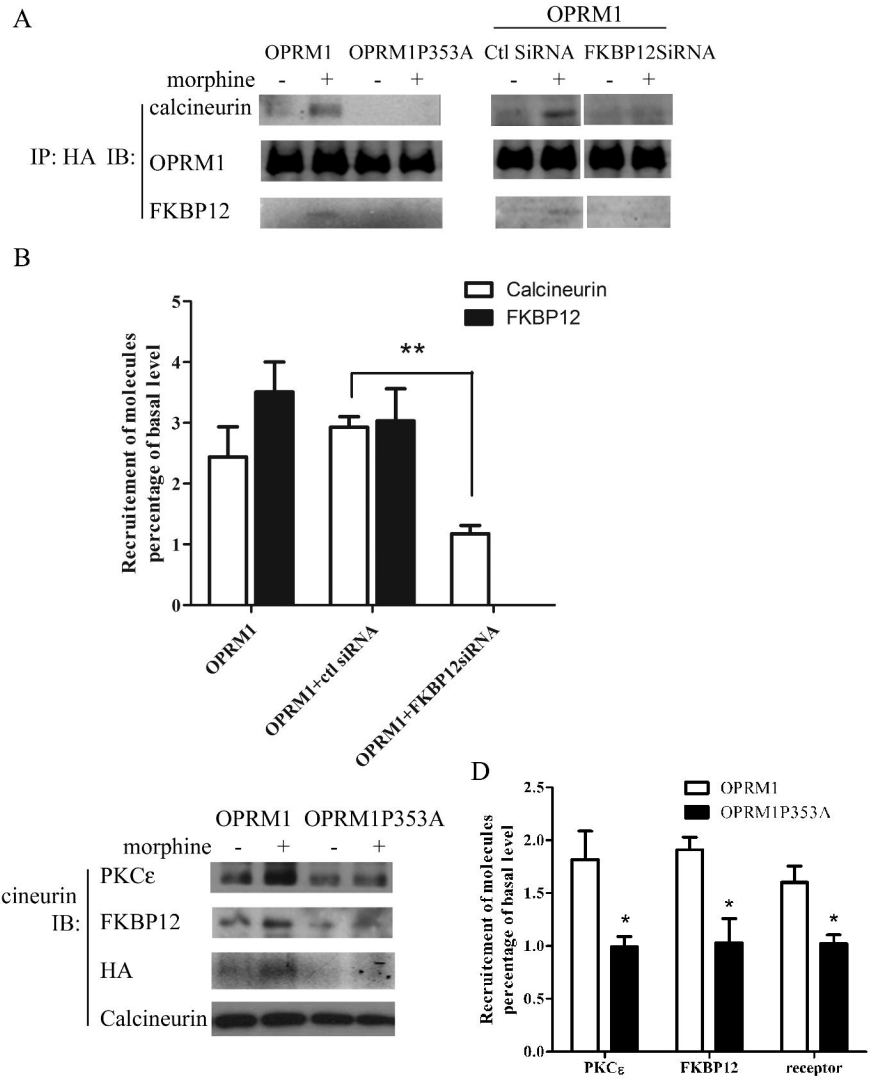
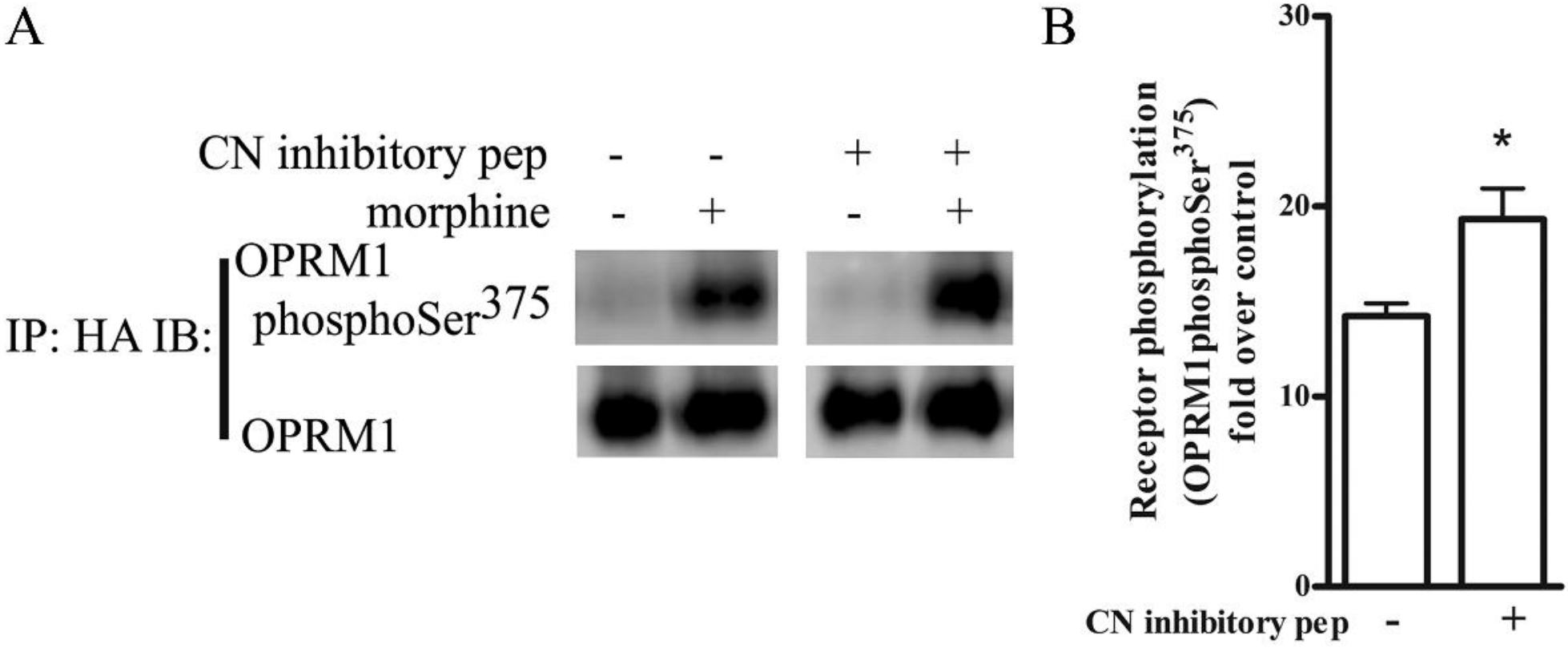

A
OPRM1
OPRM1 OPRM1P353A Ctl SiRNA FKBP12SiRNA
morphine - + - + - + - +
IP: HA IB:
calcineurin
OPRM1
FKBP12
B
Calcineurin
FKBP12
Recruitement of molecules percentage of basal level
**
OPRM1
OPRM1+ctl siRNA
OPRM1+FKBP12siRNA
C
OPRM1 OPRM1P353A
morphine - + - +
IP:calcineurin
IB:
PKCε
FKBP12
HA
Calcineurin
D
OPRM1
OPRM1P353A
Recruitement of molecules percentage of basal level
PKCε
FKBP12
receptor

Figure 8


A
CN inhibitory pep - - + +
morphine - + - +
IP: HA IB:
OPRM1
phosphoSer375
OPRM1
B
Receptor phosphorylation
(OPRM1phosphoSer375)
fold over control
30
20
10
0
*
CN inhibitory pep - +