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The Mu-Opioid Receptor and the NMDA Receptor Associate in PAG Neurons: Implications in Pain Control

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The capacity of opioids to alleviate inflammatory pain is negatively regulated by the glutamate-binding *N*-methyl-D-aspartate receptor (NMDAR). Increased activity of this receptor complicates the clinical use of opioids to treat persistent neuropathic pain. Immunohistochemical and ultrastructural studies have demonstrated the coexistence of both receptors within single neurons of the CNS, including those in the mesencephalic periaqueductal gray (PAG), a region that is implicated in the opioid control of nociception. We now report that mu-opioid receptors (MOR) and NMDAR NR1 subunits associate in the postsynaptic structures of PAG neurons. Morphine disrupts this complex by protein kinase-C (PKC)-mediated phosphorylation of the NR1 C1 segment and potentiates the NMDAR–CaMKII, pathway that is implicated in morphine tolerance. Inhibition of PKC, but not PKA or GRK2, restored the MOR–NR1 association and rescued the analgesic effect of morphine as well. The administration of *N*-methyl-D-aspartic acid separated the MOR–NR1 complex, increased MOR Ser phosphorylation, reduced the association of the MOR with G-proteins, and diminished the antinociceptive capacity of morphine. Inhibition of PKA, but not PKC, CaMKII, or GRK2, blocked these effects and preserved morphine antinociception. Thus, the opposing activities of the MOR and NMDAR in pain control affect their relation within neurons of structures such as the PAG. This finding could be exploited in developing bifunctional drugs that would act exclusively on those NMDARs associated with MORs.

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INTRODUCTION

The glutamate *N*-methyl-D-aspartate receptor (NMDAR) is essential to the function of the nervous system, and therefore its de-regulation contributes to the pathophysiology of many neurological disorders. These include neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis (Lipton, 2006), and mood disorders, such as schizophrenia and depression (Mechri *et al*, 2001; Maeng and Zarate, 2007). The persistent activation of NMDARs is also responsible for the neural changes accompanying different variants of neuropathic pain, including nerve injury-induced neuropathy, diabetic neuropathy, chronic inflammatory pain, cancer pain, and post-herpetic pain. In these conditions of persistent pain, mu-opioid receptor (MOR)-activating opioids do not provide efficacious relief (Chapman *et al*, 1994; Sigtermans *et al*, 2009). Thus, to treat neuropathic pain in the clinic, opioid doses must be substantially increased beyond those effective against nociceptive pain, and the relief is, in most cases, only partial. Neuropathic pain is characterized by tactile allodynia and hyperalgesia, which remit with drug treatments that block NMDAR function, for example, ketamine, methadone, and memantine (Mizoguchi *et al*, 2009). Although drugs that block NMDAR channel permeation improve or prolong opioid analgesia in humans, because of their lack of selectivity for those receptors involved in nociception, they produce a series of unacceptable drawbacks, such as drowsiness, hallucinations, and even coma (Palmer, 2001). Therefore, opioids are usually combined with antidepressants, anticonvulsants, or sodium channel blockers to increase their clinical effectiveness (reviewed by Mizoguchi *et al*, 2009). Certainly, a better understanding of the cross-talk that operates between MOR and NMDAR to regulate nociception would improve the efficacy and selectivity of therapies used to treat debilitating neuropathic pain.

The observation that MOR-acting opioids are of limited efficacy in neuropathic pain is supported by pharmacological and molecular studies indicating that morphine analgesia is

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under negative functional regulation by the NMDAR–neural nitric oxide synthase (nNOS) cascade (Inoue *et al*, 2003; Rodríguez-Muñoz *et al*, 2008). However, the relationship between MORs and NMDARs is bidirectional, and tolerance to morphine develops as a consequence of MOR-induced potentiation of NMDAR–calcium and calmodulin-dependent kinase-II (CaMKII) activity (Trujillo, 2002; Garzón *et al*, 2008). Indeed, MOR-acting opioids regulate glutamate-activated NMDAR currents in different areas of the nervous system, including the thalamus (Narita *et al*, 2008), nucleus coeruleus (Koyama and Akaike, 2008), brainstem medulla (Chen and Huang, 1991; Kow *et al*, 2002), and spinal dorsal horn neurons (Rusin and Randic, 1991). Most relevantly, acute opioid administration increases NMDAR function in neurons with opioid receptors (Martin *et al*, 1997; Przewlocki *et al*, 1999).

Anatomical studies have shown that MORs and NMDARs colocalize in many regions of the CNS, including patches within spiny neurons of the caudate putamen, the habenular nucleus, the spinal cord dorsal horn, the shells of the nucleus accumbens, and neurons of solitary tract nucleus. Importantly, these two receptors colocalize on single neurons within the CNS (reviewed by Trujillo, 2002), and at the ultrastructural level both receptors show convincing colocalization (Glass *et al*, 2009). Specifically, the midbrain periaqueductal gray (PAG) is densely innervated by glutamatergic projections from the forebrain, and there is robust MOR–NMDAR colocalization in the dendrites and somata of ventrolateral PAG neurons (Commons *et al*, 1999; Narita *et al*, 2008). The PAG is of physiological relevance to the nociceptive modulation network that operates both at the supraspinal level and through dorsal horn interneurons (Mansour *et al*, 1988; Marinelli *et al*, 2002). Deep brain stimulation targeting the PAG produces analgesia through the action of endogenous opioids on PAG MORs (Barbaro, 1988). This therapy has successfully been used to treat intractable pain in humans, including phantom limb pain, post-herpetic neuralgia, and diverse neuropathies (Bittar *et al*, 2005; Owen *et al*, 2007).

Therefore, the current literature convincingly demonstrates that the MOR and the NMDAR coexist at certain postsynapses and that both receptors show an electrophysiological interaction in individual neurons. Therefore, MOR–NMDAR cross-regulation in pain control could be consequence of their presence in the same neuronal compartment without ruling out that direct interaction occurs. This last possibility is of special interest because it would provide a suitable substrate to develop more specific regulators of MOR–NMDAR function. With this in mind, we assessed whether MORs and NMDARs establish an association in PAG synaptosomes and analyzed the manner in which their agonists, morphine and NMDA, and a series of Ser/Thr kinases regulate this relationship.

MATERIALS AND METHODS

Preparation and Solubilization of the PAG Synaptoneurosome-Enriched Fraction. MOR Immunoprecipitation and Co-Precipitation of Associated Proteins

Procedures involving mice strictly followed the guidelines of the European Community for the Care and Use of Laboratory Animals (Council Directive 86/609/EEC) and Spanish Law (RD 1201/2005) regulating animal research. The experimental protocols were reviewed and approved by the Committee for Animal Experimentation at the CSIC.

Experimental tissue was obtained from male albino CD1 mice (Charles River, Barcelona, Spain) weighing 22–27 g. For immunoprecipitation studies, the PAG from eight mice was typically pooled. The assays were repeated at least twice on samples that had received an identical opioid treatment and were collected at the same interval after opioid administration. The methods used to prepare the PAG synaptosomal fraction have been described elsewhere (Rodríguez-Muñoz *et al*, 2007b). The affinity-purified IgGs against the extracellular domains of the MOR 2EL (205–216: MATTKYRQGSID; GenScript, NJ, USA) and of the NMDAR NR1 subunit (483–496: KFGTQERVNNSNKK; Sigma-Genosys, Cambridge, UK) 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 then immunoprecipitated from solubilized membranes and resolved by SDS/PAGE as described previously (Garzón *et al*, 2005a). The separated proteins were then transferred onto 0.2 μm PVDF membranes and probed with the selected antibodies in DecaProbe chambers (PR 150; Hoefer-GE, Barcelona, Spain).

To assess the morphine-induced Ser phosphorylation of MORs, the existing protein interactions were disrupted under denaturing conditions prior to immunoprecipitation (Rodríguez-Muñoz *et al*, 2007b). Afterwards, MORs were detected with the MOR CT antibody and phosphoserines were detected with a mouse monoclonal antibody (IgM, 1 : 1000; Calbiochem; clone 1C8 525281).

Detection of Signaling Proteins

The specificity and efficacy of the antibodies used in immunoprecipitation assays from mouse brain synaptosomes have been addressed elsewhere (Garzón *et al*, 2005b; Rodríguez-Muñoz *et al*, 2011b; Garzón *et al*, 2011). These antibodies fulfill the recommended criteria for use in western blotting as well (Saper and Sawchenko, 2003). All the antibodies (see Supplementary information) were diluted in TBS + 0.05% Tween-20 (TTBS) and incubated with the PVDF membranes for 24 h at 6 °C. The secondary antisera were incubated for 2–3 h and visualized by using the Immobilon Western Chemiluminiscent HRP substrate (Millipore; #WBKLS0100). Chemiluminescence was recorded with a ChemiImager IS-5500 (Alpha Innotech, San Leandro, CA, USA). Densitometry was performed by using the Quantity One Software (Bio-Rad) and expressed as the mean of the integrated volume after subtracting the background (average optical density of the pixels within the object area/mm^2).

Expression of C-Terminal Sequences of MOR1, MOR1C, NR1(C0–C1–C2), and NR1(C0–C2) Splice Variants

The KRX/pFN2A-MOR1 (C-terminus), KRX/pET151-MOR1C (C-terminus), KRX/pFN2A-NR1 (segments C0–C2), and NR1 (segments C0–C1–C2) strains were grown to an optical density of 0.5–0.6. IPTG (Promega; #V3955) and

Rhamnose (Promega; #L5701) were added at a final concentration of 1 mM and 0.1%, respectively. After overnight induction at room temperature, the cells were collected by centrifugation and the pellets were kept at −20 °C. The proteins were purified under native conditions using Ni-NTA-agarose columns (Invitrogen; Probond Purification System; #K850-01), or on glutathione–Sepharose 4B columns (Amersham Biosciences; #27-4570) previously equilibrated with 30 bed volumes of: 50 mM Tris-HCl (pH 8), 1 mM EDTA, 150 mM NaCl, 0.5% Tween-20, and 1% Triton X-100. The retained fusion proteins were cleaved on the column by using the ProTEV protease (Promega; #V605A), collected, and concentrated in a centrifugal filter device (10 000 nominal molecular weight limit; Amicon Microcon YM-10 #42407; Millipore). The TEV protease was removed by immobilization on affinity resins (Amersham Biosciences; #17-0575-01).

Evaluation of MOR–NMDAR1 Interactions

The interaction of the NR1 C-terminal sequence C0–C1–C2 (100 nM) or its clipped C0–C2 sequence (100 nM) with C-terminus MOR1 or MOR1C variants (100 nM) was studied. The NR1 proteins were incubated with 100 nM GST protein (GenScript, USA; Z02039. negative control) or with GST-MOR sequences in 450 μl of HBS-EP buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% P20) and mixed by rotation for 30 min at room temperature. After the incubation, glutathione–Sepharose was added to these protein mixtures. The pellets obtained by centrifugation were washed three times and solubilized in 2 × Laemmli buffer. The presence of NR1 C-terminal sequences was analyzed by western blotting using an antibody against the C2 segment (NMDAR1 C2, 1 : 500, ab6485).

Phosphorylation of the NR1 subunit of the NMDAR (C0–C1–C2, 100 nM) was performed in a 50-μl reaction mixture containing 60 mM NaHEPES (pH 7.5), 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 μM Na-orthovanadate, 1 mM DTT, and 250 μM ATP. The reaction was performed at room temperature in the presence of 30 nM protein kinase-Cγ (PKCγ), and it was terminated after 20 min by the addition of the PKC inhibitor Gö7874 (Calbiochem; #365252) at a concentration of 5 μM. The influence of PKC phosphorylation of NR1 C0–C1–C2 on its binding to the MOR was then determined as described above.

SPR Analysis

Interactions were determined by using a BIACORE X (GE Healthcare). NR1 C-terminal sequences C0–C1–C2 or C0–C2 (50 μg/ml) were coupled to Channel-2 of CM5 sensor chips (GE; BR-1000-14) by amine coupling at pH 7.0 (GE; BR-1000-50); Channel-1 acted as the blank. The sensor surface was equilibrated with HBS-EP buffer (GE; BR-1001-88). After passing the MOR1 C-terminal sequence (75 μl) over the sensor surface, the sensorgrams were collected at 25 °C with a flow rate of 5 μl/min. The CM5 sensor chip was regenerated after each cycle with two 15-μl pulses of 10 mM glycine given at a 30-s interval (pH 2.5; GE; BR-1003-56). Increasing analyte concentrations were studied, and the results were plotted by using the BIAevaluation software (v 4.1).

Cell Culture and Transfection

Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml streptomycin, 100 μg/ml penicillin, and 10% (v/v) fetal bovine serum at 37 °C and in an atmosphere of 5% CO_2. The cells were transfected for 48 h at ~70% confluence by using Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer. The cells were further incubated for 18–36 h prior to testing for transgenic expression.

BiFC Analysis

The plasmid pPD49.83 was used to generate two cloning vectors for Bimolecular Fluorescence Complementation (BiFC) analysis. The constructs containing the heat-shock promoter, hsp-16.41, an Myc or hemagglutinin tag for detection of BiFC fusion proteins, a multiple cloning site for sub-cloning the gene of interest, a linker sequence, and the N-terminal fragment of Venus truncated at residue 173 (VN173) or the C-terminal fragment of Venus starting at residue 155 (VC155) were a gift from Dr Chang-Deng Hu at Purdue University (USA). Full-length murine NR1 (C0–C1–C2) and MOR were sub-cloned in-frame into the pCE-BiFC-VN173 or pCE-BiFC-VC155 plasmid by using standard cloning strategies. Fragments were amplified by PCR using the following primers: NR1: 5′-AGGA/AGCTTAGCACCATG CACCTGCTGACATTC-3′ (forward)/5′-CGT/CTAGAGCGT CTCTGCTCTCCCTATGAC-3′ (reverse) for the pCE-BiFC-VN173 and MOR 5′-CCGG/TCGACGCAAGCATTCAGAAC CAAGGACA-3′ (forward)/5′-GGTAC/CGGATGGCGTGGGA CCCAGTTTG-3 (reverse) for pCE-BiFC-VC155 plasmid. Samples were imaged on glass-bottom plates (MatTek, USA) by confocal microscopy by using a Leica DMIII 6000 CS confocal fluorescence microscope equipped with a TCS SP5 scanning laser.

Animals, Icv Injection, and Evaluation of Antinociception

Male albino CD-1 mice weighing 22–25 g were used. The response of the animals to nociceptive stimuli was determined by the warm water (52 °C) tail-flick test. Baseline latencies ranged from 1.5 to 2.2 s, and they were not significantly affected by the kinase inhibitors used or their solvent. A cut-off time of 10 s was used to minimize the risk of tissue damage. Antinociception was expressed as a percentage of the maximum possible effect (MPE = 100 × (test latency−baseline latency)/(cut-off time−baseline latency)). The animals were lightly anaesthetized with ether and morphine sulphate (Merck, Darmstadt, Germany). Then, 15 and 180 pmol NMDA (Tocris; #0114), 1 nmol Gö7874 (Calbiochem; #365252), 15 nmol KN93 (Calbiochem; #422711), 5 and 15 nmol PKA inhibitor 6-22 amide (Sigma-Aldrich; #P6062), or 20 and 100 nmol βARK1 inhibitor (Calbiochem; #182200) were injected in a volume of 4 μl into the lateral ventricle. Saline was likewise administered as a control. Antinociception was assessed at different time intervals thereafter.

The development of morphine-induced acute opioid tolerance was monitored 24 h after giving a priming dose

of 10 nmol morphine intracerebroventricularly. Thus, a dose–effect curve of morphine was constructed by determining analgesia 30 min after the injection of the opioid. This interval corresponds to morphine analgesic peak effect.

Statistical Significance

ANOVA followed by the Student–Newman–Keuls test (SigmaStat; SPSS Science Software, Erkrath, Germany) was performed and significance was defined as $P<0.05$.

RESULTS

MOR and NMDAR Associate in Mouse Neuronal Tissue

NMDARs, which control a cation channel that is highly permeable to Ca^{2+}, are formed by a tetramer consisting of a pair of NR1 subunits associated with at least one type of NR2 (A, B, C, and D) or NR3 (A and B) subunit (Mori and Mishina, 1995). To determine the existence of an association between MORs and NMDARs, we first analyzed whether they could be co-immunoprecipitated from mouse brain synaptosomes. In neuronal membranes, the cytosolic regions of these two receptors interact with third partner proteins; thus, in the immunoprecipitation analysis, we used antibodies directed to the extracellular domains of the MOR and NR1 subunits. Because MOR variants show differences in their cytosolic C-terminus (Pan, 2005), our approach would not distinguish among them. The MOR co-precipitated with NR1 subunits but showed little or no co-precipitation with NR2/3 subunits. This pattern was observed in the PAG, cerebral cortex, striatum, and dorsal spinal cord, but co-precipitation was almost absent from the cerebellum (Figure 1a). Using an antibody against the NR1 extracellular peptide sequence, we determined that the NR1 subunit co-precipitated with MOR1 and MOR1C variants (Figure 1b). NR1 subunits show variability in their distal C-terminus and are classified as C2 and C2′ (Zukin and Bennett, 1995). The C-terminus of the NR1 subunit is composed of C0–C1–C2(C2′) regions, variants 011/111 and 010/110 respectively; however, some NR1 lack the C1 segment, C0–C2(C2′), variants 001/101 and 000/100 respectively. An *ex vivo* analysis of the NR1 subunits co-precipitated with the MORs indicated the presence of both C2 and C2′ variants (Figure 1c).

The MOR–NR1 association withstood solubilization by sonication–Nonidet-p40 or RIPA buffer (Pierce; 89900), although it was destroyed by 1% SDS solubilization buffer. Because SDS at this concentration prevents interactions between proteins, the presence of the ionic detergent was reduced with octylthioglucoside. The anti-MOR antibody subsequently captured the MOR without the associated NR1 subunit (Figure 2a). This observation suggests that the MOR associates (directly or indirectly) with the NR1 subunit in the synaptic membrane, and when separated the mutual affinity shown by these two proteins is probably occluded by the presence of third partner proteins. Therefore, MOR–NR1 co-precipitation is not a result of their interaction during the solubilization procedure, and certainly suggests a functional role for this relationship.

Given the MOR–NR1 association in synaptosomal membranes, we next addressed whether these receptors

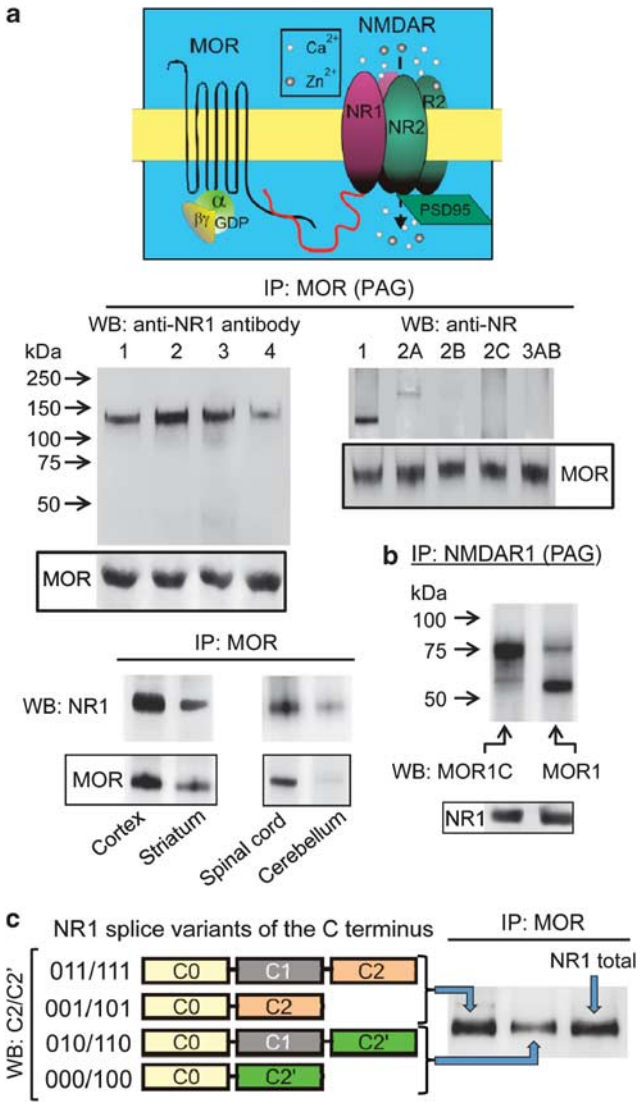

Figure 1 MOR1 and NMDAR associate in the nervous system. Reciprocal co-precipitation of MOR and NR1 subunits from various areas of mouse CNS system tissue. (a) Solubilized synaptosomes were incubated with biotinylated IgG directed to the second extracellular loop of the MOR (2EL). After being recovered with streptavidin–sepharose, the MOR-containing complexes were processed to remove the IgG before immunodetection of MOR1 and associated proteins (see section Materials and methods). For the PAG assay, the anti-NR1 antibodies used were the following: Lane-1, Abcam ab1880; lane-2, Abcam ab28669; lane-3, Sigma-Genosys (482-456); lane-4, Abnova PAB12221. The antibodies used for detection of PAG NR subunits were as follows: NR1, Abcam ab1880; NR2A, Abcam ab14596; NR2B, Abcam ab14400; NR2C, Abcam ab110 ; and NR3AB, Abcam ab2639. The Abcam ab1880 antibody was used to detect MOR-associated NR1 derived from the other structures. (b) The Sigma-Genosys (482-456) antibody directed against the NR1 extracellular peptide sequence co-precipitated MOR1 (MOR CT antibody) and MOR1C (Neuromics; #RA20001) variants. (c) To determine the NR1 variants associated with the MOR membrane, proteins were solubilized and the MOR was immunoprecipitated (IP) with affinity-purified IgG anti-MOR (2EL). The NR1 subunits that co-precipitated with MORs were recognized with antibodies directed to the NMDAR1 (ab1880), NMDAR1 C2 (ab6485), and NMDAR1 C2′ (ab6486) regions of the NR1 subunit C-terminus.

physically interact in living cells. The BiFC approach is used for the detection of protein direct interactions in the normal cellular environment (Shyu *et al*, 2008). Thus, CHO cells

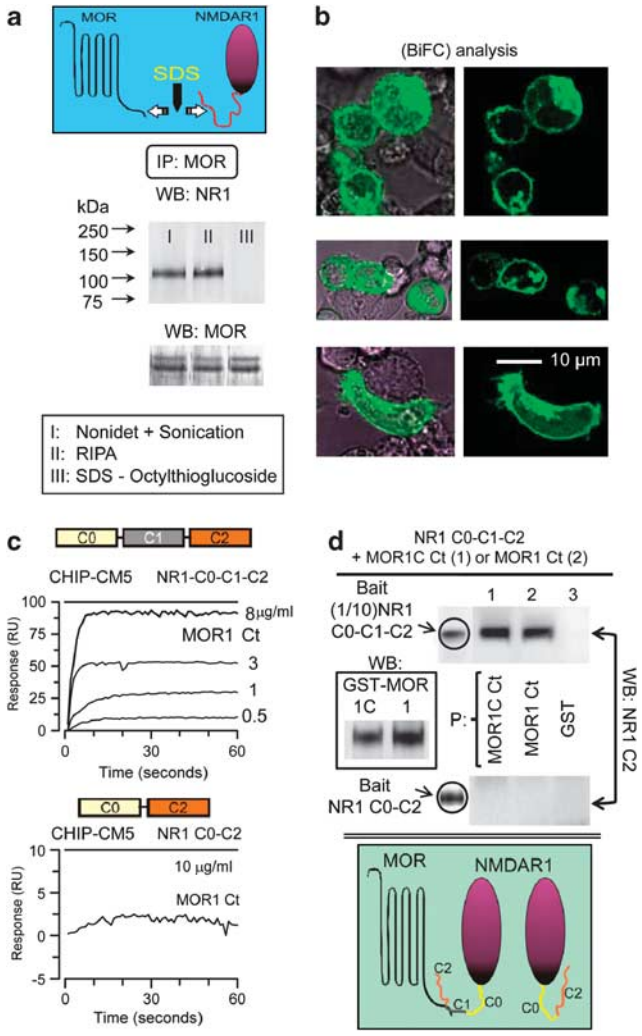

Figure 2 Direct physical interaction of MORs with NMDARs: involvement of the C terminal sequences. (a) The membranes were solubilized and the MOR was then immunoprecipitated (MOR 2EL antibody) to study the association between MOR and NR1 (MOR CT and ab1880 antibodies). Further details are provided under section Materials and methods. (b) BiFC analysis of the interaction between MOR1 and NR1. CHO cells were transiently co-transfected with cDNAs encoding $NR1^{VN173}$ and $MOR1^{VC155}$ (0.3 μg). The confocal fluorescent signals are obtained when two molecules of $NR1^{VN173}$ and $MOR1^{VC155}$ associate. Left: Phase field and fluorescent images are combined. Right: Fluorescent image of positive cells in the field. (c) SPR analysis of the MOR–NR1 interaction. The MOR1 C-terminal sequence (287–399) interacts with the NR1 C-terminus containing the C1 region. The sensorgrams were constructed with MOR1 Ct in the fluid phase at the concentrations (μg/ml) indicated. The NR1 C0–C2 produced no signal with the MOR1 C-terminus. (d) *In vitro* pull-down assays. NR1 C0–C1–C2 or NR1 C0–C2 C-terminus variants were incubated with either the GST–MOR1C C-terminus (337–439) (lane-1) or the GST–MOR1 C-terminus (287–399) (lane-2). Both MOR1 C-terminal sequences bind to and co-precipitate with NR1 C0–C1–C2 but not NR1 C0–C2. GST did not bind to the NR1 C1 region (lane-3). P, captured and precipitated with glutathione–Sepharose; WB, anti-GST or anti-NR1 C2 region antibody.

were transfected with a mix (1 : 1) of plasmids expressing MOR1 coupled to VC155 and NR1 (C0–C1–C2) coupled to VN173 at the corresponding C-termini. The physical interaction of the carrier proteins allows the VC155 and VN173 fragments to couple and form a stable fluorescent complex. Numerous cells showed the fluorescent signal, indicating that MOR and NR1 can form a heterodimer *in vivo* (Figure 2b). Because these VC and VN fragments are not fluorescent on their own, the cells that did not fluoresce were likely not transfected or were singly transfected with either the MOR1–VC155 or the NR1–VN173 construct.

Dopamine-D1 receptors and group-I metabotropic glutamate receptor-5a (mGlu5a) form complexes with NR1 subunits through their respective C-termini (Fiorentini *et al*, 2003; Perroy *et al*, 2008). Accordingly, we determined the role of these protein regions in the interaction between MOR and NR1 (Supplementary Figure 1). Using surface plasmon resonance (SPR) analysis, which detects protein interactions during co-incubation, we observed a robust interaction between the MOR1 C-terminus and the C1 region of the NR1 C-terminus (Figure 2c). *In vitro* co-incubation assays confirmed this pattern; the MOR1 and the MOR1C C-termini bound to the NR1 cytosolic sequence C0–C1–C2 but not NR1 C0–C2 (Figure 2d). Ser residues in the C1 sequence, that is, Ser890, Ser896, and Ser897, are implicated in the regulation of NMDAR function by PKC and PKA (Hisatsune *et al*, 1997; Tingley *et al*, 1997). The MOR1–NR1 direct binding was greatly diminished by the action of PKCγ on this critical C1 domain (Figure 3). The analysis of the NR1 C-terminal sequence, C0–C1–C2 (DNASTAR; Protean v8.0.2), revealed a cluster of positively charged residues at the end of the C1 segment (889–898: SSFKRRRSSK) that could interact with the negative regions in the C-terminus of MOR1 and MOR1C. Phosphorylation of these NR1 C1 Ser residues reduces the positive charge, thereby weakening the association between MOR and NMDAR1. The isoelectric point shifts from 11.72–9.98 (P), 7.61 (P-P), and 6.8 (P-P-P) (ExPASy's Compute pI/Mw).

Pharmacological Recovery of Opioid Analgesia from Tolerance

Intracerebroventricular (icv) administration of morphine to mice produces a dose-dependent antinociceptive effect that reaches a maximum at about 30 min after injection. The administration of a 10-nmol dose brings about a profound decrease in the response to successive doses of morphine that is a result of acute analgesic tolerance. The lower 3-nmol dose produces no single-dose tolerance, indicating that the effects of morphine have to reach a certain threshold before desensitization is achieved (Rodríguez-Muñoz *et al*, 2007a). This single-dose (acute) tolerance can be detected 3 h after administration, but is clearly observed when the analgesic dose–response curve is assessed 6 h after the animals receive the initial priming dose of 10 nmol morphine. Therefore, this process requires time to develop (Rodríguez-Muñoz *et al*, 2007b). In our assay, the apparent ED_{50} of icv morphine was 3.75 nmol per mouse (95% confidence limits: 2.85–4.90) for the control mice and >10 nmol per mouse for mice pre-treated (24 h) with 10 nmol morphine. We then determined whether inhibition of the Ser and Thr kinases PKC, PKA, and G-protein receptor kinase-2 (GRK2), could restore morphine analgesic potency from the acute tolerant state. The mice received the priming dose of 10 nmol morphine. Twenty-four hours later, they were injected intracerebroventricularly with the PKA inhibitor 6-22 amide at 5 or 15 nmol, the PKC inhibitor

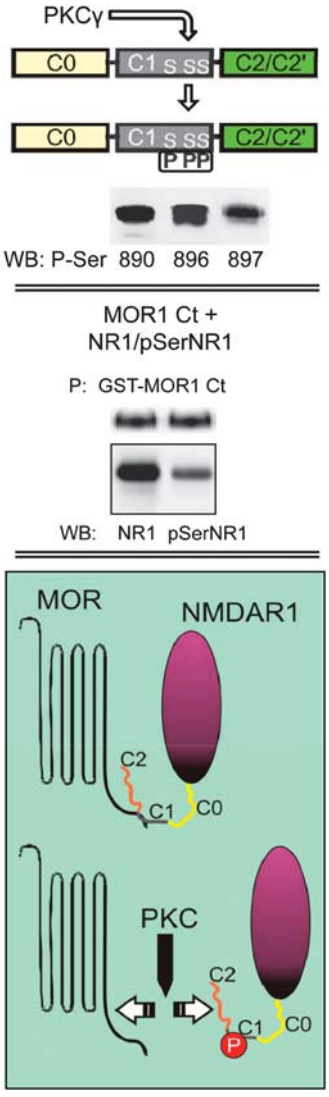

Figure 3 Influence of PKC on the association of MOR1 and NR1 C-termini. The NR1 C0–C1–C2 sequence (100 nM) was exposed to PKCγ activity (30 nM). Western blot (WB) analysis revealed phosphorylation of Ser 890, Ser896, and Ser897 located in the NR1 C1 segment. The GST–MOR1 C-terminus (100 nM) was then incubated with 100 nM NR1 C0–C1–C2 that had or had not been exposed to active PKCγ. The GST–MOR1 Ct was then precipitated (P) with glutathione–Sepharose and the associated NR1 was evaluated by WB by using the anti-NR1 C2 antibody.

Gö7874 at 1 nmol, and ,the GRK2 inhibitor iβARK1 at 20 or 100 nmol. The effect of increasing opioid test doses was assessed 30 min after the inhibitor injection. The dose of 20 nmol iβARK1 reverses antinociceptive tolerance in DAMGO-tolerant mice. However, doses up to 100 iβARK1 fail in recovering morphine from antinociceptive tolerance (Sánchez-Blázquez *et al*, 2008; Hull *et al*, 2010). On the other hand, the PKA inhibitor 6-22 amide at 5 nmol is effective in restoring morphine analgesia from NMDA antagonism (Figure 7a). Therefore, given the suitability of the doses used for the kinase inhibitors, we concluded that tolerant mice recovered their responses to morphine after PKC inhibition but not after PKA or GRK2 inhibition (Figure 4a).

Effect of Morphine on the Association between MOR and NR1: Role of PKC

For those cases in which receptor dimerization has been proposed, the formation of the complex usually affects the properties of each individual partner, particularly the capacity of agonists to promote receptor internalization. Therefore, we first determined the effect of *in vivo* administration of morphine on the stability of the PAG MOR–NMDAR association when analyzed *ex vivo*. In agreement with previous reports (reviewed by Rodríguez-Muñoz *et al*, 2007b), a single icv dose of 10 nmol morphine promoted no significant changes in cell-surface MORs. At the intervals at which morphine analgesia declined, but not before, the association of NR1 subunits with the MOR diminished (Figure 4b). The NR1 subunits associated with MORs showed no Ser phosphorylation, but in the membrane morphine increased the activating phosphorylation of NR1 Ser890 and NR2A tyrosine-1325, as well as CaMKII autophosphorylation at Thr286 (Figure 4c). Morphine also diminished the association of the MOR with the post-synaptic marker PSD95 (Figure 5a). These changes were accompanied by increases in MOR phosphorylation, decreases in MOR coupling to the regulated G-proteins (Figure 5b), and the onset of acute tolerance (Rodríguez-Muñoz *et al*, 2007b). A second dose of 10 nmol morphine given 24 h after the priming dose produced only weak analgesic effects, indicating that acute tolerance had developed (Figure 4). Under these circumstances, morphine further reduced the number of MOR–NR1 complexes (Figure 5b). All these molecular changes brought about by the icv dose of 10 nmol morphine were prevented by the co-administration of the MOR-selective antagonist Cys^2, Tyr^3, Orn^5, Pen^7-amide (CTOP; 0.6 nmol per mouse) (Gulya *et al*, 1988) (Supplementary Figure 2).

Inhibition of PKC before the administration of this priming dose of morphine prevented the Ser phosphorylation of the MOR, and, consequently, the receptor maintained its control over G-protein-mediated transduction. In these circumstances, morphine did not diminish the MOR–NR1 association, and no analgesic tolerance developed (Figures 4 and 6a). In mice that had been rendered tolerant to morphine, administration of Gö7874 restored MOR function and morphine regained its analgesic potency within a few minutes. This positive effect was related to a series of molecular changes, such as reformation of MOR–NR1 complexes, dephosphorylation of MORs, and recovery of MOR regulation over G-proteins. Thus, the mice showed a full analgesic response to the second dose of morphine, but without separation of Gö7874-reformed MOR–NR1 complexes or internalization of MORs (Figure 6b). Therefore, morphine-induced analgesic acute tolerance is mostly mediated by PKC activity on a series of signaling proteins. Inhibition of this kinase resets the system to the parameters observed before the administration of the morphine priming dose and prevents the opioid from inducing changes related to MOR desensitization.

We previously reported that iβARK1, a GRK2 inhibitor, does not affect the development of morphine tolerance (Sánchez-Blázquez *et al*, 2008). We have now determined that GRK2 inhibition did not prevent morphine from inducing MOR–NR1 separation. Moreover, iβARK1 did not

reverse analgesic tolerance or promote MOR–NR1 re-association (Figures 4 and 6c). Similarly, inhibition of PKA did not rescue morphine tolerance or stimulate the re-grouping of MOR with the NR1 subunit.

NMDAR Activation Reduces Morphine Analgesia and Disrupts MOR–NR1 Association by a PKA-Dependent Mechanism

The icv administration of NMDA, an agonist that acts at the glutamate-binding site on the NR2 subunit, resulted in the loss of the capacity of morphine to produce antinociception (Figure 7a). The NMDAR inhibitors MK801 (Tocris; #0924) (Wong *et al*, 1986) and D-AP5 (Tocris; #0106) (Olverman *et al*, 1984) injected intracerebroventricularly at 0.3 nmol 10 min before NMDA preserved morphine analgesia effects. Inhibition of PKC (1 nmol Gö7874) or GRK2 (100 nmol iβARK1) did not alter the negative effect of NMDA on morphine analgesia. Moreover, the inhibitor of CaMKII activity, KN93 (Calbiochem; #422711), used at 15 nmol, dose that prevents morphine acute tolerance (Sánchez-Blázquez *et al*, 2008; Garzón *et al*, 2011), also failed against the antagonism of NMDA on morphine analgesia. However, in mice treated with the PKA inhibitor, NMDA did not alter morphine normal analgesic effect. Although NMDA did not promote a substantial reduction of MOR or NR1 subunits in the PAG synaptosomal preparation, it greatly increased the Ser phosphorylation of the MOR and the uncoupling of the MOR from the regulated G-proteins. These desensitizing changes were accompanied by reductions in MOR–NR1 co-precipitation (Figure 7b). The direct effect of NMDA on the NMDAR promoted some activating autophosphorylation of CaMKII, although it hardly affected PKC-mediated Ser phosphorylation of the NR1 subunit or Src-mediated tyrosine phosphorylation of NR2A/B (Figure 7c). These NMDA-induced changes on MOR and NMDAR signaling were all prevented by the NMDAR antagonists MK801 (Supplementary Figure 3) or D-AP5 (not shown). The inhibition of GRK2, CaMKII, or PKC did not modify the NMDA-induced MOR–NR1 disruption. On the contrary, inhibition of PKA preserved the MOR–NR1 association (Figure 7d).

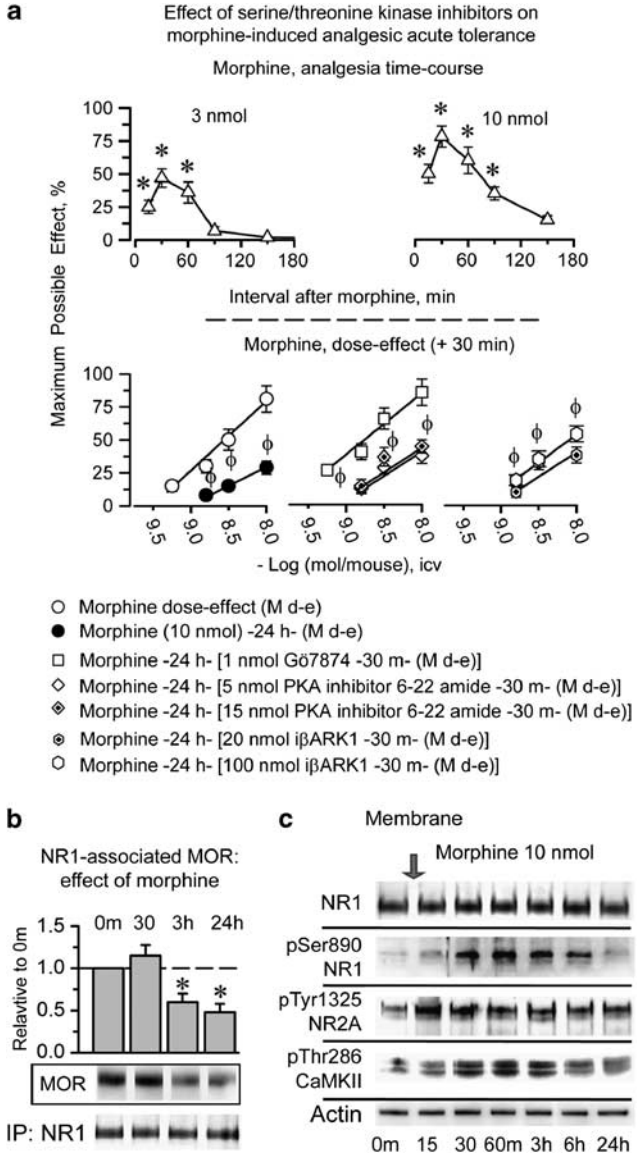

Figure 4 Effect of PKC, PKA, and GRK2 inhibition on morphine-induced acute analgesic tolerance. (a) Mice were intracerebroventricularly injected with 3 or 10 nmol morphine and antinociception was assessed by the warm water (52 °C) tail-flick test. Each point is the mean ± SEM from groups of eight mice. The administration of a priming dose of 10 nmol, but not 3 nmol, morphine promoted acute analgesic tolerance. This was demonstrated by constructing a dose–effect curve for morphine 24 h after the mice received the priming dose. The pharmacological rescue from morphine-induced single-dose tolerance was studied. The PKC inhibitor Gö7874 (1 nmol), the PKA inhibitor 6-22 amide (5 and 15 nmol), and the GRK2 inhibitor iβARK1 (20 and 100 nmol) were injected intracerebroventricularly 30 min before constructing the morphine dose–effect curve. *Significantly different from the basal latencies obtained before the icv morphine injection. $^{\phi}$Significantly different from the value obtained in the absence of the priming dose of morphine (ANOVA–Student–Newman–Keuls test; $p<0.05$). (b) Morphine (10 nmol per mouse) reduced the association of NR1 subunits with MORs. The NR1 subunits were immunoprecipitated (IP) with the anti-NR1 antibody (ab1880) and the associated MORs were then evaluated by western blotting (anti-MOR CT). Representative blots are shown. NR1 was used as a loading control. The immunosignals (average optical density of the pixels within the object area/mm^2; Quantity One Software, Bio-Rad) were expressed relative to the levels of MORs observed in control mice that had not received morphine (given an arbitrary value of 1). Each data point represents the mean of three assays performed on PAG samples obtained from independent groups of mice. The data are presented as the mean ± SEM (Sigmaplot v11). *Significantly different from the value of 0 m (controls that did not receive morphine) (ANOVA–Student–Newman–Keuls test; $p<0.05$). (c) Phosphorylations related to NMDAR–CaMKII activity were studied in the PAG membranes obtained from morphine-treated mice. The mice were administered 10 nmol morphine intracerebroventricularly. The animals were then divided into groups of six mice and killed at the post-opioid intervals indicated. The presence of NR1 and activating phosphorylations of NR1, NR2A, and CaMKII was determined in the PAG synaptosomal membrane. The assay was repeated three times on samples derived from different groups of mice and the results were comparable.

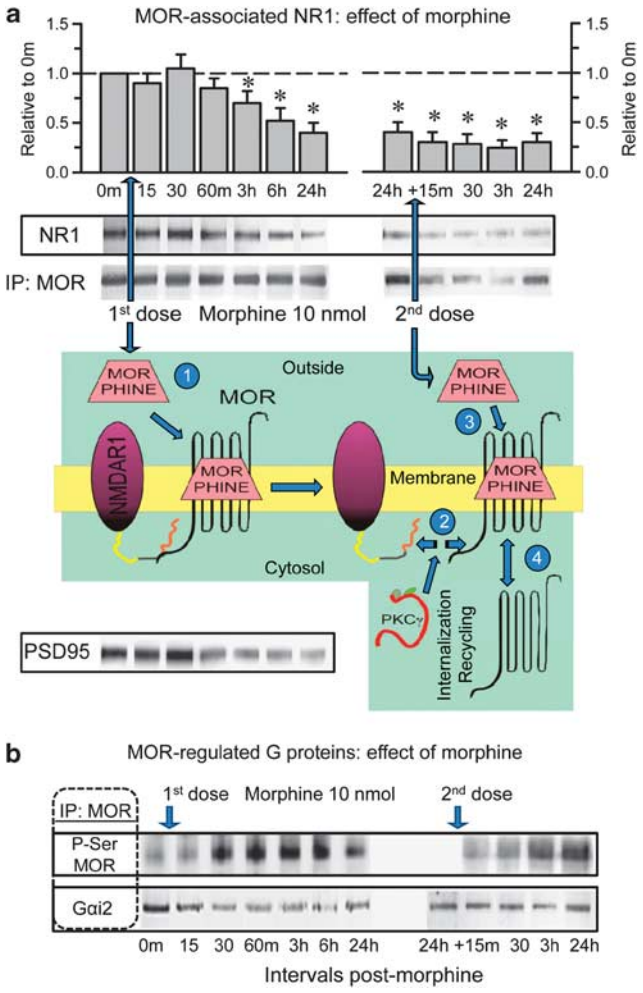

Figure 5 Effect of repeated morphine administration on MOR-associated proteins. Groups of mice received an initial icv dose of 10 nmol morphine. They then received a second dose of 10 nmol morphine or saline 24 h later. The mice were then killed at the indicated time points. For each interval studied, the PAG structures from six mice were pooled for the *ex vivo* determinations. (a, b) The MOR was immunoprecipitated (IP) with the 2EL antibody and the associated NR1, PSD95, and Gαi2 proteins were evaluated. The MOR Ser phosphorylation status was determined with the 1C8 clone after applying denaturing conditions to remove associated proteins that could mask the findings (see section Materials and methods). *Significantly different from the value of 0 m (controls that did not receive morphine). Details are as in Figure 4b.

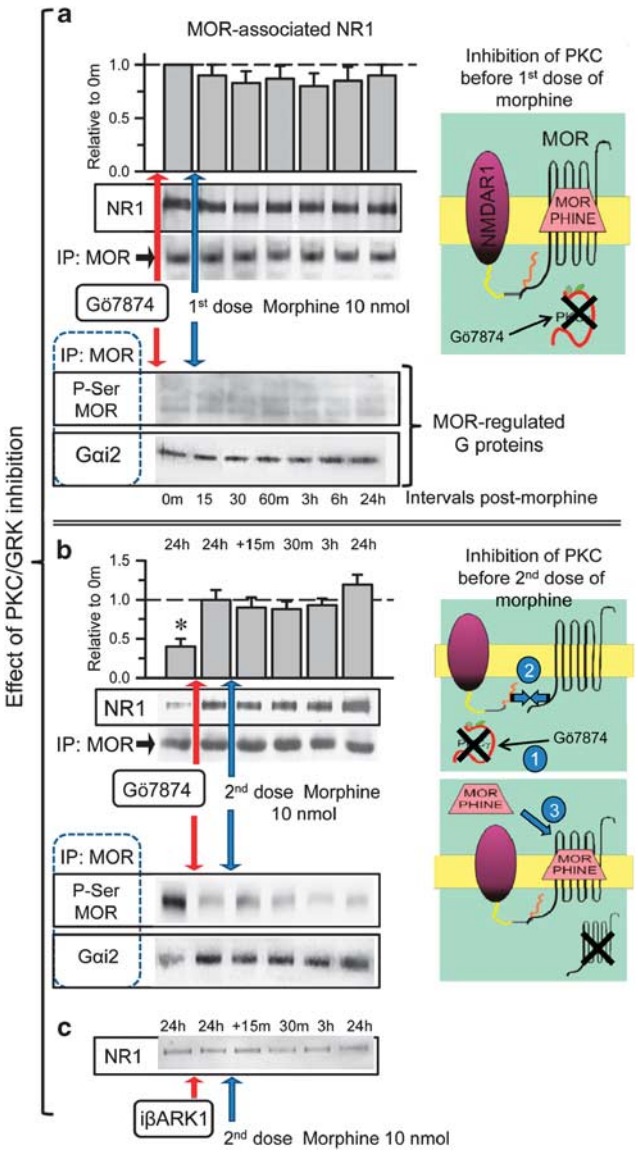

Figure 6 Morphine promotes MOR–NR1 separation by a PKC-mediated mechanism. (a) The PKC inhibitor Gö7874 (1 nmol) was injected intracerebroventricularly to mice 30 min before the administration of 10 nmol morphine. At the intervals indicated, groups of six mice each were killed and the PAG was obtained for the *ex vivo* analysis. MOR was immunoprecipitated (IP) to determine its Ser phosphorylation and association with NR1 and Gαi2 subunits. (b) Mice that had received a priming dose of 10 nmol icv morphine were injected with 1 nmol icv Gö7874 24 h later. After 30 min, the mice received a second test dose of 10 nmol icv morphine. *Significantly different from the value of 0 m (controls that did not receive morphine; Figure 7a). Details are as in panel a. (c) Using the protocol in panel b, the mice were injected with 100 nmol icv iβARK1 30 min before the morphine test dose. Further details are provided under section Materials and methods and the legend to Figure 5.

DISCUSSION

We have observed that metabotropic MORs and glutamate-driven ionotropic NMDARs are associated in different areas of the mouse CNS and that PKC and PKA regulate their relation. During recent years, different laboratories have convincingly described the functional cross-regulation of MORs and NMDARs in pain control. Moreover, anatomical studies, ultrastructural analyses, and electrophysiological data have suggested that the opposing interaction of MORs and NMDARs could be achieved in the same neuron within a common cellular compartment. Although it is complicated to ascertain the direct physical association of these two receptors in *in vivo* studies we have, however, provided *ex vivo* and *in vitro* data demonstrating that such an interaction is certainly possible. Notwithstanding, just the finding of the association between MORs and NMDARs is of relevance to the pharmacology of pain, particularly to the clinical management of opioid-resistant neuropathic pain. At the molecular level, various signaling proteins have been implicated in the bidirectional MOR–NMDAR regulation (Trujillo, 2002; Garzón *et al*, 2008). In the framework of this MOR–NMDAR association, without

ruling out the possibility of their physical interaction, those regulatory mechanisms have increased importance and provide the rationale to identify markers associated with increased nociception or therapeutic targets to control these dysfunctions.

The *ex vivo* analyses provided little information on the precise NR2/3 composition of MOR-associated NMDARs. However, our study provides information on the manner in which the MOR could interact with the NR1 subunit. *In vitro*, this association occurs through the respective C-termini; the MOR binds to NR1 subunits carrying the C1 region but shows no interaction with NR1 subunits lacking the C1 segment. Moreover, the MOR co-precipitated with the PSD95, indicating that this receptor associates with the NR1 splice variant that contains the sequence for anchoring to PSD95 in the C2′ segment (Kornau *et al*, 1995). Therefore, MOR associates with NR1 subunits with a C-terminus composed of C0–C1–C2(C2′) regions. Interestingly, the few GPCRs that have been reported to associate with NMDARs interact with this NR1-containing C1 region. Indeed, the C-termini of dopamine-D1 receptor and of group-I metabotropic glutamate receptor (mGlu5a) interact with the NR1 C1 terminal sequence (Fiorentini *et al*, 2003; Pei *et al*, 2004; Perroy *et al*, 2008). The binding of Ca^{2+}-calmodulin to this cytosolic region of NR1 negatively regulates calcium flux through NMDARs. Morphine stimulates the separation of the PAG MOR–NMDAR complex probably through PKC acting on the C1 segment of the NR1 C-terminus (Chakravarthy *et al*, 1999), and thus enhances NMDAR calcium permeation by preventing this inhibitory binding of Ca^{2+}-calmodulin.

Whereas, GRK2/3 controls the effects of MOR agonists, such as [D-Ala^2, *N*-MePhe^4, Gly-ol^5] encephalin (DAMGO), which are strong inducers of receptor internalization (Hull *et al*, 2010), PKC, and to some degree PKA as well, has systematically been implicated in the development of tolerance to the analgesic effects of morphine, which promotes low levels of MOR internalization (reviewed by Garzón *et al*, 2008). In the CNS, PKC through potentiation of NMDAR function negatively regulates morphine analgesia but not that of DAMGO (Bilsky *et al*, 1996) (Lu *et al*, 1999; Sánchez-Blázquez *et al*, 2009). Therefore, morphine was used in this study because its antinociception is regulated by PKC and NMDAR activity. Our recent studies indicate that, through MOR–G$\beta\gamma$–PI3K–Akt–nNOS, morphine stimulates the production of nitric oxide (NO) (Sánchez-Blázquez *et al*, 2010), which releases zinc ions from endogenous stores to recruit PKCγ and Raf-1 to the HINT1 protein at the MOR C-terminus (Rodríguez-Muñoz *et al*, 2008; Rodríguez-Muñoz *et al*, 2011a). Then, PKCγ

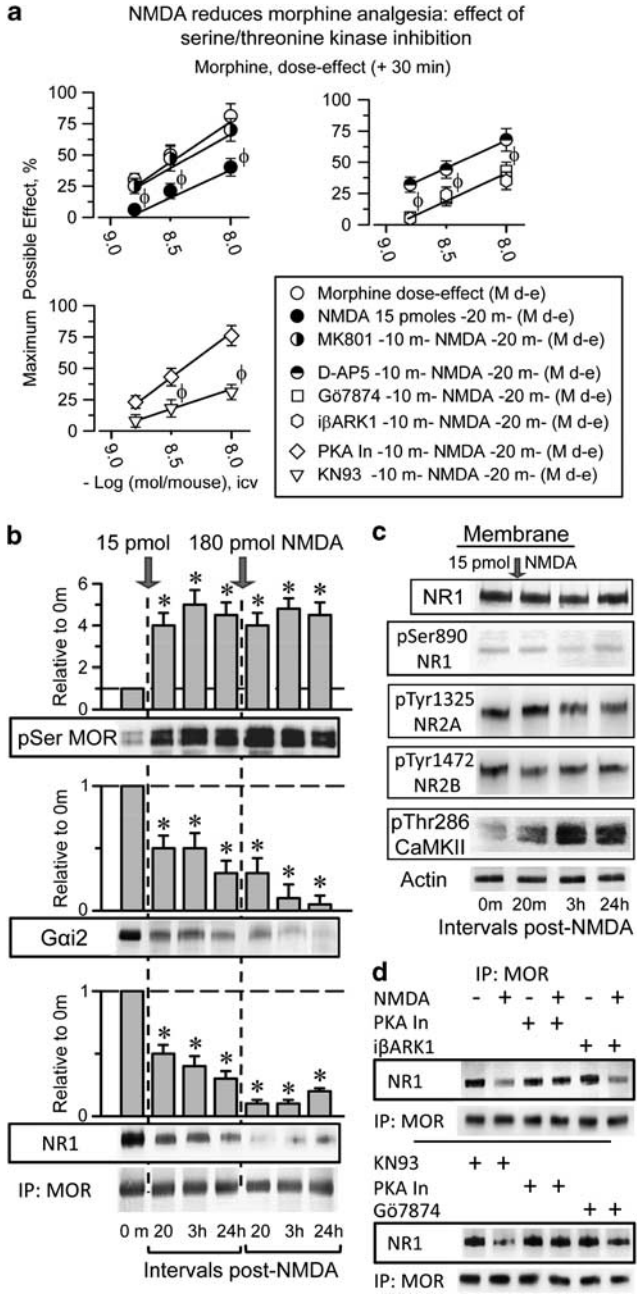

Figure 7 Icv NMDA desensitizes the associated MOR and separates the MOR from the NR1 subunit. (a) The injection of 15 pmol icv NMDA diminished the analgesic potency of morphine. The NMDAR antagonists MK801 and D-AP5 (0.3 nmol), the PKC inhibitor Gö7874 (1 nmol), the PKA inhibitor 6-22 amide (PKA In, 5 nmol), the CaMKII inhibitor KN93 (15 nmol), and the GRK2 inhibitor iβARK1 (100 nmol) were injected intracerebroventricularly 10 min before NMDA. Then, morphine was intracerebroventricularly injected 20 min after NMDA and analgesia was evaluated 30 min after opioid injection. $^{\phi}$Significantly different from the corresponding value of morphine analgesia obtained in the absence of the icv NMDA injection (ANOVA–Student–Newman–Keuls test; $p<0.05$). (b) Mice were injected with 15 and 180 pmol icv NMDA and groups of six mice each were killed at the post-NMDA intervals indicated. The PAG was removed and pooled for the *ex vivo* studies. The MOR was immunoprecipitated (MOR 2EL antibody) and its Ser phosphorylation (1C8 clone) was evaluated. The influence of NMDA on the association of Gαi2 subunits and of NMDAR NR1 subunits was also determined. (c) Protein phosphorylation related to NMDAR–CaMKII activity was studied in PAG membranes obtained from NMDA-treated mice. (d) Effect of Ser and Thr kinase inhibitors on NMDA-evoked separation of the MOR–NR1 complex. The mice received the inhibitors and 10 min later 15 nmol NMDA. The mice were killed 20 min after receiving NMDA, and the PAG was removed and pooled for the *ex vivo* assays. Control mice received saline instead of the inhibitor or NMDA. The MOR was immunoprecipitated (IP) and the associated NR1 was measured. Further details are provided under section Materials and methods and the legend to Figure 4.

causes MOR–NR1 separation and produces the sustained potentiation of NMDAR calcium currents by activating Src (Sánchez-Blázquez *et al*, 2009), and also the Raf-1–ERK1/2 cascade (Rodríguez-Muñoz *et al*, 2011a). Afterwards, NMDAR-regulated CaMKII promotes MOR phosphorylation and its uncoupling from regulated G-proteins (Sánchez-Blázquez *et al*, 2008, and references therein). The MOR–NMDAR association indicates that those concatenated processes are confined within the close environment of both receptors. PKA has also been implicated in the phosphorylation of NR1 C1 Ser897 (Tingley *et al*, 1997); however, our results indicate that morphine recruits PKC to separate both receptors in PAG neurons. In agreement with our observation, PKC phosphorylation of NR1 Ser890 promotes the dispersion of membrane NR1 subunits, which reorganize when they are de-phosphorylated. This is not observed when Ser896 or Ser897 is phosphorylated (Tingley *et al*, 1997).

The PKC-mediated separation of MORs from NMDARs is observed when the analgesic efficacy of morphine declines. This phenomenon starts about 3 to 6 h after the injection of icv morphine. Twenty-four hours later, some of the MORs in the synaptosomal membrane remain separated from NMDARs. This timing reasonably agrees with the PKC-mediated activation of ERK1/2 at MORs, which leads to the enhancement of NMDAR function (Rodríguez-Muñoz *et al*, 2011a). After MOR separates from the NMDAR, but not before, the administration of a second dose of morphine promoted the GRK2-mediated phosphorylation of MOR Ser375, and these receptors then underwent internalization/recycling (Rodríguez-Muñoz *et al*, 2007b; present study). Therefore, NR1 separation from the MOR C-terminus permits GRK2 to act on Ser/Thr residues required for internalization of the receptor. The recycling of the MOR did not help morphine to recover full analgesic potency, but brought no further tolerance even after additional administrations of the opioid (Rodríguez-Muñoz *et al*, 2007b). Thus, MOR signaling was kept reduced by the effect of PKC-activated NMDARs, and accordingly in morphine-tolerant mice the inhibition of PKC caused MOR dephosphorylation and re-established MOR coupling to G-proteins. The inhibition of PKC facilitated NR1 C1 dephosphorylation and probably also facilitated the inhibitory binding of Ca^{2+}-calmodulin to this region. Thus, morphine triggers a PKC activity, which last longer than its analgesic effect. This durable activation of PKC seems to be mediated by the morphine-induced, long-lasting enhancement of NMDAR function (Sánchez-Blázquez *et al*, 2009; Rodríguez-Muñoz *et al*, 2011b). The NMDAR/nNOS-released zinc binds to the regulatory domain of conventional PKC isoforms and contributes to their activation. Then, zinc ions promote PKC translocation from the soluble phase to the membrane, enhances their affinity for phorbol esters or diacylglycerol, and as high zinc stabilizes their binding to the regulatory domain, PKC activation persists for long intervals (Zalewski *et al*, 1990; Rodríguez-Muñoz *et al*, 2008; Garzón *et al*, 2011). Indeed, this is not a singular event; in the NMDAR environment the long-term activation of PKA has also been reported this time related to transient inhibition of the protein Ser/Thr phosphatase calcineurine (Malleret *et al*, 2001). As a whole, PKC inhibition reset the MOR–NMDAR system, reduced NMDAR function, and restored the capacity of morphine to produce antinociception in mice that had been rendered tolerant to morphine. Similarly, NMDAR antagonists or CaMKII inhibitors also reverse morphine tolerance, but GRK2 inhibition does not (Sánchez-Blázquez *et al*, 2008; Hull *et al*, 2010). These observations suggest that the priming dose of morphine triggers a late negative feedback through MOR-stimulated NMDARs and causes acute morphine tolerance that diminishes the analgesia achieved with successive doses. Although NMDARs are not involved in the regulation of analgesia produced by single doses of morphine or fentanyl in rodents, combinations of NMDAR antagonists with opioids seem to be effective to control pain in humans (Redwine and Trujillo, 2003, and references therein). It is therefore possible that MOR-associated NMDARs are silent in most situations, but when the nociceptive signal reaches a certain threshold, as in situations of neuropathic pain, NMDAR activity negatively affects the signaling capacity of the MOR and then NMDAR antagonism is beneficial to opioid analgesia.

The pharmacological activation of NMDARs also caused the separation of MOR and NR1 subunits, and, importantly, stimulated the phosphorylation and uncoupling of MORs. Under these circumstances, NMDA administration greatly diminished the efficacy of morphine in controlling nociception, similar to what is observed in situations of neuropathic pain in which there is an NMDAR hyper-function (Chapman *et al*, 1994; Celerier *et al*, 2000). There is a general agreement that PKA enhances NMDAR function in pain-suffering arthritic rats (Bird *et al*, 2005; Fu *et al*, 2008). We found that NMDA-induced antagonism of morphine antinociception could be prevented by inhibiting PKA but not PKC. Therefore, PKA may be responsible for the dissociation of NR1 subunits from MORs, which occurs as a result of NMDAR activation leading to MOR Ser phosphorylation and uncoupling from G-proteins. PKA located in the PSD–NMDAR complex is activated through Ca^{2+}-calmodulin-dependent adenylyl cyclase and then potentiates NMDAR-dependent calcium currents (Chetkovich and Sweatt, 1993). PKA does not affect NMDARs that contain NR1 subunits lacking the C1 region (Westphal *et al*, 1999). Therefore, the C1 segment of the NR1 subunit, which carries the inhibitory site for Ca^{2+}-calmodulin binding, is a common target for PKC and PKA phosphorylation. Then, NMDA-activated PKA enhances the NMDAR calcium fluxes necessary for CaMKII or nNOS activation. Therefore, this mechanism would bring about a reduction in morphine-induced antinociception, in addition to the possibility of a direct effect of PKA on MOR cytosolic residues (Chakrabarti *et al*, 1998). In situations in which NMDARs, through PKA, and MORs, through PKC, contribute to opioid tolerance, the simultaneous inhibition of PKC and PKA would effectively restore the antinociceptive effect of morphine (reviewed by Garzón *et al*, 2008).

In summary, this study has shown that in the mouse PAG the MOR and the NMDAR form a PKC/PKA-regulated association, and that these receptors could physically interact. This association is altered by the functional antagonism that exists between MOR and NMDAR signaling in pain control. Thus, morphine and NMDA recruit PKC and PKA, respectively, to promote functional changes in the associated receptor-activating or inactivating phosphorylations.

This MOR–NMDAR association appears to be of relevance in the control of nociception and provides a significant conceptual advance as to how the NMDAR exerts its negative regulation on MOR function. This finding could be useful in the clinical management of pain states refractory to opioid treatment; for example, through the selectivity provided by bifunctional drugs that binding to the MOR reach and antagonize the function of the associated NMDAR.

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DISCLOSURE

The authors declare that, except for the income received from our primary employer 'Ministerio de Ciencia y Tecnología', no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)