Cellular/Molecular

Morphine- and CaMKII-Dependent Enhancement of GIRK Channel Signaling in Hippocampal Neurons

Rounak Nassirpour,[1,2] Laia Bahima,[1] Arnaud L. Lalive,[3] Christian Lüscher,[3,4] Rafael Luján,[5] and Paul A. Slesinger[1,2]

[1]Peptide Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037, [2]Biology Graduate Program, Department of Biology, University of California, San Diego, La Jolla, California, 92093, [3]Department of Basic Neurosciences, Medical Faculty, CH-1211 Geneva, Switzerland, [4]Clinic of Neurology, Department of Clinical Neurosciences, Geneva University Hospital, CH-1211 Geneva, Switzerland, and [5]Department Ciencias Médicas, Facultad de Medicina, Universidad de Castilla-La Mancha, 02006 Albacete, Spain

G-protein-gated inwardly rectifying potassium (GIRK) channels, which help control neuronal excitability, are important for the response to drugs of abuse. Here, we describe a novel pathway for morphine-dependent enhancement of GIRK channel signaling in hippocampal neurons. Morphine treatment for ~20 h increased the colocalization of GIRK2 with PSD95, a dendritic spine marker. Western blot analysis and quantitative immunoelectron microscopy revealed an increase in GIRK2 protein and targeting to dendritic spines. *In vivo* administration of morphine also produced an upregulation of GIRK2 protein in the hippocampus. The mechanism engaged by morphine required elevated intracellular Ca^{2+} and was insensitive to pertussis toxin, implicating opioid receptors that may couple to Gq G-proteins. Met-enkephalin, but not the μ-selective (DAMGO) and δ-selective (DPDPE) opioid receptor agonists, mimicked the effect of morphine, suggesting involvement of a heterodimeric opioid receptor complex. Peptide (KN-93) inhibition of CaMKII prevented the morphine-dependent change in GIRK localization, whereas expression of a constitutively activated form of CaMKII mimicked the effects of morphine. Coincident with an increase in GIRK2 surface expression, functional analyses revealed that morphine treatment increased the size of serotonin-activated GIRK currents and Ba^{2+}-sensitive basal K^+ currents in neurons. These results demonstrate plasticity in neuronal GIRK signaling that may contribute to the abusive effects of morphine.

Introduction

A balance of excitatory and inhibitory signals is essential for normal processing in the brain. To accomplish this, neurons implement mechanisms of homeostatic plasticity that adjust the synaptic strength in a neuronal network (Pozo and Goda, 2010). Three primary signaling pathways influence the activity of the network; these include fast excitatory and inhibitory signals, mediated by ionotropic receptors (i.e., NMDAR, AMPAR, $GABA_A$-R) (Pozo and Goda, 2010), and slow inhibitory signals (Isaacson et al., 1993; Scanziani, 2000), mediated by G-protein-coupled receptors and G-protein-activated inwardly rectifying potassium (GIRK or Kir3) channels. In the hippocampus, several different inhibitory neurotransmitters, such as GABA, serotonin, adenosine, and somatostatin, stimulate their cognate G-protein-coupled receptors (GPCRs) and activate GIRK channels, leading to hyperpolarization of the neuron's membrane potential (Lüscher et al., 1997; Leaney, 2003). Loss of GIRK signaling leads to impairments in learning and memory tasks, changes in the response to drugs of abuse and alcohol, whereas excessive GIRK activity may interfere with neuronal signaling, such as in Down's syndrome (Lüscher and Slesinger, 2010). The pathways for regulating the expression of neuronal GIRK channels are not well understood.

In the hippocampus, the $GABA_B$ receptor-dependent slow IPSC (sIPSC) occurs with repetitive stimulation, suggesting that GABA released into the synaptic cleft pools and diffuses away to perisynaptically localized $GABA_B$ receptors coupled to GIRK channels (Isaacson et al., 1993; Scanziani, 2000). Recent anatomical studies of GIRK channels and $GABA_B$ receptors, however, suggest a more complex arrangement (Lujan et al., 2009). Electron microscopic (EM) immunohistochemical experiments confirm that $GABA_B$ receptors and GIRK channels are expressed in dendritic shafts but also reveal that $GABA_B$ receptors and GIRK channels are colocalized in dendritic spines (Kulik et al., 2003, 2006; Koyrakh et al., 2005). The differential expression of GIRK channels in dendritic shafts and spines raises the possibility that alterations in synaptic plasticity may involve changes in the localization of GIRK channels in hippocampal neurons. Consistent with this, a pathway for potentiating the GIRK-sIPSC in hippocampal neurons has been described that requires elevations in intracellular calcium and activation of CaMKII (Huang et al., 2005), similar to NMDAR-dependent long-term potentiation (LTP) of AMPAR-mediated EPSCs (Kessels and Malinow, 2009).

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Correspondence should be addressed to Paul A. Slesinger, Peptide Biology Laboratory, the Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037. E-mail: slesinger@salk.edu.

R. Nassirpour's present address: Senior Scientist, Exploratory Drug Safety Research and Development, Pfizer Inc., 1 Burtt Road, Andover, MA 01810.

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Here, we investigated whether morphine alters GIRK signaling in hippocampal neurons. Opioids have been shown to modulate excitatory and inhibitory synaptic transmission in the hippocampus (Salmanzadeh et al., 2003; Guo et al., 2005; Liao et al., 2005; Bao et al., 2007; McQuiston, 2007). Furthermore, morphine administration increases the activity of CaMKII *in vivo* (Lou et al., 1999; Wang and Wang, 2006), suggesting that GIRK channels are a potential downstream target of morphine (Huang et al., 2005). Thus, morphine might enhance GIRK signaling in hippocampal neurons via activation of CaMKII. Using a combination of biochemical, histological, and electrophysiological approaches, we demonstrate that morphine treatment for ~20 h upregulates GIRK signaling in cultured hippocampal neurons, with a preferential localization in the dendritic spines. We further show that Gq G-proteins and CaMKII are involved in this form of GIRK channel plasticity.

Materials and Methods

Hippocampal neuronal cultures. Primary cultures of hippocampal neurons were prepared from Sprague Dawley rat pups [postnatal day (P)0–P2] using a modification of a previously described method (Brewer et al., 1993). All procedures were approved by the Salk Institute's Institutional Animal Care and Use Committee. For each set of cultures, the hippocampi from four rats were isolated, dissociated with papain (Worthington), and the cells plated at 50,000 cells cm^2 in Neurobasal-A medium supplemented with B27 and glutamax (Invitrogen). The media was replaced the day after plating and cells were fed twice weekly thereafter. Hippocampal neurons were plated onto 12 mm glass coverslips (Warner Instruments) coated with 0.1 mg/ml poly-D-lysine (Sigma). All cells were grown at 37°C and in 5% CO_2 for 10–14 or 21 d *in vitro* (DIV). Approximately three to four preps per week were used for the drug studies, and each drug study was repeated several times with different cultures and combined in the final analysis for the colocalization studies.

For overexpression experiments, neurons in culture at 20 DIV were infected with Sindbis viruses expressing actin-YFP (a gift from Dr. Gentry Patrick, University of California, San Diego, La Jolla, CA) or αCaMKII(1-290)-EGFP (ΔCaMKII-GFP) (a gift from Dr. Jose Esteban, Universidad Autónoma de Madrid, Madrid, Spain) and processed 20 h later for confocal imaging. Whole-cell patch-clamp recordings were made 20–30 h after infection. Recordings from infected (determined by the GFP fluorescence) and uninfected cells were performed from the same infected cultures.

Systemic morphine administration. Thirteen Sprague Dawley rats weighing 40–50 g (~3 weeks old), which had *ad libitum* access to food and water, were used for the study. A single morphine injection was shown previously to increase activity of CaMKII in rats (Lou et al., 1999). Rats were injected with a single subcutaneous injection of morphine sulfate (1, 10, or 20 mg/kg; Sigma) or an equal volume of normal saline. Rats were anesthetized and killed 5 h after different treatments. The brains were rapidly removed and dissected on ice. The hippocampi were homogenized in ice-cold strong lysis buffer (Citosignal). The homogenate was centrifuged at 14,000 rpm for 10 min at 4°C and the resultant supernatant subjected to Western blot analysis as described.

Immunocytochemistry and confocal imaging. At 20 DIV, cultures were incubated with morphine sulfate salt pentahydrate (Sigma) at 100 μM for ~20 h. For experiments with pertussis toxin (PTX; 250 ng/ml; Sigma), PTX was added to the culture medium and incubated for 6 h at 37°C in the presence of morphine. KN-92 or KN-93 (10 μM; Calbiochem) was added to the culture medium during the last 2 h of the morphine treatment. DMSO (Sigma) was used as the vehicle control for the CaMKII inhibitor experiments. Naloxone (100 μM, Sigma), DAMGO (100 μM; Tocris Bioscience), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), picrotoxin (50 μM, Sigma), GABA (100 μM), and DL-2-amino-5-phosphonovaleric acid lithium salt (APV; 100 μM; Sigma), tetrodotoxin (TTX; 2 μM), or α-methyl-(4-carboxyphenyl)glycine (300 μM) was applied for 20 h in the presence and absence of morphine. After washing, 21 DIV neurons were fixed with −20°C methanol and permeabilized with 0.2% Triton-X in PBS. Blocking solution containing 2% bovine serum albumin (Jackson ImmunoResearch), 5% goat serum (Sigma), and 0.2% Triton X (Sigma) in PBS was then applied for 1 h at room temperature. Neurons were incubated with rabbit anti-GIRK2 (1:200; Alomone), goat anti-NMDA (1:50; NMDAε1/NR2a; Santa Cruz Biotechnology), guinea pig anti-μOR (1:1000; Neuromics), and mouse anti-PSD95 (1:300; Affinity BioReagents) antibodies overnight at 4°C. Cells were washed and incubated with Alexa 488- or 647-conjugated secondary antibodies (1:300; Invitrogen) for 1 h at room temperature. The specificity of the GIRK2 primary antibody was investigated by antigen competition. In addition, the background due to nonspecific staining of secondary antibodies was investigated and minimized. Primary and secondary antibodies were diluted in blocking solution. The coverslips were mounted on microscope slides using Progold antifading reagent (Invitrogen) and imaged by laser confocal microscopy (63×, oil-immersion objective, HeNe and Argon laser) using a Leica TCS SP2 AOBS microscope. For comparison of images, the pinhole, exposure time, resolution, and gain were kept constant. The images were equally scaled in NIH ImageJ.

Colocalization was visualized by merging red–green channels into RGB and using ImageJ RGB Colocalization plug-in (with default values) to determine the Pearson's correlation coefficient. Under low power (192 × 192 μm), a neuron's soma was located and the dendritic field surrounding the soma was divided into four or five fields at higher magnification (40–60 by 40–60 μm at full resolution, 1024 × 1024). The proximal and distal dendrites were not clearly distinguishable due to the overlapping dendrites from different neurons. To compare different preparations of cultures and different experimental conditions, we converted the Pearson's coefficient to a colocalization ratio, where the Pearson's coefficient for the experimental groups was normalized to the untreated control group. For actin-YFP experiments, one set of infected cultures was treated with morphine for 20 h and another set from the same neuronal prep was used as control. Isolated dendrites from both the control and morphine-treated cultures were studied for colocalization using the YFP fluorescence as the green channel and the endogenous PSD95 or GIRK2 as the red channel. For the ΔCaMKII-GFP neurons, isolated infected and uninfected dendrites were used to quantify colocalization between PSD95 and GIRK2. For these images, GFP was captured at 488 nm, PSD95 at 568 nm, and GIRK2 at 647 nm (bleed through was minimized before images were obtained). For the purposes of the RGB colocalization quantification, GIRK2 was used as the red channel and PSD95 as the green channel. To restrict the colocalization analysis to the infected actin-YFP or ΔCaMKII-GFP dendrites, a region of interest (40–60 μm long) was drawn around the infected or uninfected dendrite. For all analyses, $p < 0.05$ was considered statistically significant (SigmaStat 3.0; Systat Software). Data are presented as mean ± SEM and evaluated for statistical significance using unpaired Student's *t* test for groups of two or one-way ANOVA for multiple groups (*post hoc* Bonferroni test), using untreated cultures as a control (SigmaStat 3.0).

Western blot. Primary hippocampal neurons were plated on six-well plates at 1.5×10^6 cells per well. At 10–13 DIV, neurons were incubated with morphine sulfate salt pentahydrate at 100 μM for 20 h and then cells were washed twice in PBS, removed with a cell scraper, homogenized in strong lysis buffer (Citosignal) by pipetting up and down, and incubated on ice for 1 h. Lysates were centrifuged (12,000 rpm, 15 min, 4°C) and supernatants were collected. Protein levels were quantified using BCA protein assay reagent (Pierce Biotechnology). Total protein (10 μg) was heated for 10 min at 75°C and separated on an SDS-PAGE gel, transferred to nitrocellulose membrane, and blocked 1 h at room temperature (RT) in 5% milk in Tris-buffered saline (TBS-T). Membranes were incubated with primary GIRK2 antibody (1:200; APC-006; Alomone), primary $GABA_{B2}$ antibody (1:2000; clone N81/2; NeuroMab), primary NMDA antibody (1:250; sc-1468; Santa Cruz Biotechnology), or primary GAPDH antibody (1:15,000; AM4300; Ambion) for 1 h at RT or overnight (o/n) at 4°C. After three washes of 5 min with TBS-T, the membranes were exposed to the corresponding HRP-conjugated antibodies (1:3000; Santa Cruz Biotechnology) for 1 h at RT. After extensive washes with TBS-T and TBS, the immunoblots were visualized by ECL development (Pierce). Optical density measurements were made using NIH ImageJ software.

Surface biotinylation. Primary hippocampal neurons were plated on 3 cm dishes at 1.5×10^6 cells per dish (12 dishes). At 11–14 DIV, neuron cultures were cooled on ice and washed two times (2 × 3 ml) with

ice-cold ACSF containing Mg^{+2}, pH 7.4, 10 mM HEPES-free acid, 145 mM NaCl, 2.5 mM KCl, 10 mM glucose, 1 mM MgCl, 2 mM CaCl (290–320 mOsm). Then, neurons were incubated with 1 mg/ml Sulfo-NHS-SS-biotin (catalogue #21331; Pierce) in ACSF for 20 min on ice. Unreacted biotinylation agent was quenched by washing four times with 100 mM ice-cold glycine in ACSF and then 20 min mild shaking at 4°C. Cultures were harvested in modified radioimmunoprecipitation assay (RIPA) buffer (pH7.4, 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.5%SDS, 0.5% deoxycholic acid, and protease inhibitors) and incubated on ice for 1 h. The homogenates were centrifuged at 20,000 g for 15 min at 4°C. The volume of the resultant supernatant was brought to 800 μl by adding RIPA buffer and incubated with 200 μl of 50% NeutraAvidin agarose (catalogue #29200; Pierce) o/n at 4°C. NeutraAvidin agarose mix was washed five times with RIPA buffer (spin/wash five times for 2 min at 5000 rpm), bound proteins were eluted with 4X NuPage sample buffer plus NuPAGE reducing agent DTT (Invitrogen) by heating the samples at 75°C for 30 min. Eluted surface proteins were separated from the beads by spinning at 12,000 rpm for 2 min and removing the supernatant. Twenty microliters of each sample were loaded and a Western blot was performed as described above.

Whole-cell patch-clamp electrophysiology. Patch-clamp recordings were made from hippocampal neurons (11–14 DIV), which had the morphological features of pyramidal neurons as previously described (Sodickson and Bean, 1996). Whole-cell patch-clamp recordings were performed using borosilicate glass electrodes (Warner) with resistances of 5–8 MΩ when filled with the intracellular solution contained the following (in mM): 20 NaCl, 130 KCl, 5.46 $MgCl_2$, 10 HEPES/NaOH, pH 7.4, and 5 EGTA/KOH, pH 7.4. On the day of recording, 2.56 mM K_2ATP was added with 300 μM Li_2GTP to the intracellular solution. Macroscopic membrane currents were recorded using an Axopatch 200B (Molecular Devices) amplifier, adjusted electronically for cell capacitance and series resistance (80–100%), filtered at 2 kHz with an eight-pole Bessel filter, digitized at 5 kHz with a Digidata 1200 series interface (Molecular Devices), and stored on a laboratory computer. The extracellular solution contained the following (in mM): 156 NaCl, 4 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, and 10 HEPES/NaOH, pH 7.4. A different external solution was perfused during recordings where NaCl was replaced with an equimolar concentration of KCl (140 NaCl, 20 KCl) and also contained 100 μM APV, 50 μM picrotoxin, and 2 μM TTX. This extracellular solution and other drugs were applied directly to the patched cell using a localized perfusion system. (±)-Baclofen (Sigma) was dissolved in water to make a 10 mM stock and then diluted directly into the extracellular solution. Data were acquired at room temperature (22–25°C) and analyzed with Clampfit 8.0 (Molecular Devices). Data are presented as mean ± SEM and evaluated for statistical significance using unpaired Student's t test, where $p < 0.05$ was considered significant (SigmaStat 3.0). All drugs used were obtained from Sigma unless otherwise stated.

Immunoelectron microscopy and quantification. The subcellular localization of GIRK2 in hippocampal cultures in control conditions and after treatment with morphine was analyzed using the pre-embedding immunogold method (Lujan et al., 1996). Briefly, hippocampal cultures were fixed using 4% paraformaldehyde plus 0.2% glutaraldehyde plus 15% picric acid in 0.1 phosphate buffer (PB), pH 7.4, during 4 h. Because these technical conditions are not suitable for studying GIRK2 expression in the postsynaptic density, we cannot make any conclusions about possible expression of GIRK2 along the postsynaptic density (PSD). Hippocampal cultures were incubated in 10% normal goat serum (NGS) diluted in TBS for 1 h at room temperature and then incubated for 24 h in anti-GIRK2 antibodies (Alomone) at a final protein concentration of 1–2 μg/ml diluted in TBS containing 1% NGS. After several washes in TBS, sections were incubated for 3 h in goat anti-rabbit IgG coupled to 1.4 nm gold (Nanoprobes) diluted 1:100 in TBS containing 1% NGS. The cultures were then washed in PBS and postfixed in 1% glutaraldehyde diluted in the same buffer for 10 min. They were washed in double-distilled water, followed by silver enhancement of the gold particles with an HQ silver kit (Nanoprobes). This processing was followed by treatment with osmium tetraoxide (1% in 0.1 M PB), block-staining with uranyl acetate, dehydration in graded series of ethanol, and embedding in Durcupan (Fluka) resin. Hippocampal cultures were cut at 70–90 nm on an ultramicrotome (Reichert Ultracut E; Leica) and collected on 200-mesh nickel grids. Staining was performed on drops of 1% aqueous uranyl acetate followed by Reynolds's lead citrate. Ultrastructural analyses were performed in a Jeol-1010 electron microscope.

Immunogold labeling was evaluated quantitatively to test differences in GIRK2 in control conditions and after treatment with morphine. Quantification was performed on three ultrathin sections randomly chosen from each of four different coverslips. Electron microscopic serial ultrathin sections were cut close to the surface of each block. In hippocampal culture neurons, randomly selected areas were captured at a final magnification of 35,000× and measurements covered a total section area of ~6000 mm^2 for each experimental group. Dendritic shafts, dendritic spines, and intracellular compartments were assessed for the presence of GIRK2 immunoparticles. In each reference area, the numbers of gold particles attached to the plasma membrane of dendritic spines, dendritic shafts, cell bodies, and membranous intracellular compartments were counted. Then the percentage of immunoparticles for GIRK2 at each subcellular compartment was calculated in control conditions and after treatment with morphine. In addition, to determine possible changes in density in the spines, the numbers of immunoparticles attached to the plasma membrane of dendritic spines ($n = 60$ spines) were counted and the length of the extrasynaptic membrane from all immunopositive spines was measured using a digitizing tablet and appropriate software (Sigma-Scan Pro; Jandel Scientific). Then, the density of immunoparticles for GIRK2 at dendritic spines, measured as number of immunoparticles per micron of spine plasma membrane, was calculated in control conditions and after treatment with morphine, and statistically compared using a Student's t test. The background labeling was evaluated in the same way calculating the density of GIRK2 over mitochondria, nuclei, and empty resin.

Results

Morphine-induced changes in dendritic GIRK2 channels

To investigate the role of morphine in regulating GIRK channel signaling, we used cultures of dissociated rat hippocampal neurons. These cultures have well developed dendritic spines (Papa et al., 1995; Kim and Sheng, 2004), are electrically active, have been used extensively to elucidate mechanisms of glutamatergic trafficking (Lin et al., 2009), and express GIRK channels, G-proteins, and a variety of GPCRs, including $GABA_B$ and 5-HT receptors (Andrade et al., 1986; Leaney, 2003). To identify dendritic spines, hippocampal neurons were immunostained with antibodies against PSD95 or NMDA receptors, two proteins localized specifically in the postsynaptic density of dendritic spines (Fig. 1) (Kim and Sheng, 2004). The extent of colocalization was quantified by deriving the Pearson's correlation coefficient for the two fluorophores (see Materials and Methods). NMDAR and PSD95 displayed a correlation coefficient of ~0.45, indicating colocalization of these two proteins in the dendritic spines (Fig. 1*A*,*D*). GIRK2 channels, on the other hand, overlapped significantly less with PSD95 or NMDA receptors (Fig. 1*B*,*C*). On average, the Pearson's correlation coefficient was ~0.1 for GIRK2 and NMDA, and ~0.1 for GIRK2 and PSD95 (Fig. 1*D*). Because the colocalization assay at the light microscopic level cannot distinguish between the spine head, neck, or perisynaptic membranes, we will refer to two general compartments, the dendritic shaft and the dendritic spine (PSD95 or NMDAR positive). The immunolabeling experiments show that GIRK2 does not colocalize strongly with two synaptic spine markers, suggesting GIRK2 channels are expressed predominantly in dendritic shafts under basal conditions.

Using this colocalization assay, we investigated the effect of a range of morphine concentrations (5–100 μM) on GIRK2 expression in hippocampal neurons (Fig. 2) and quantified the colocal-

ization of GIRK2 and PSD95 after ~20 h of morphine treatment. In previous *in vivo* studies, synaptic plasticity changed ~24 h following a single injection of a drug of abuse (e.g., cocaine, morphine) (Kauer and Malenka, 2007). An ~20 h exposure to 30 or 100 μM morphine appeared to increase the amount of GIRK2 immunostaining and significantly increased the colocalization with PSD95 nearly twofold (Fig. 2*A*,*B*). Removal of morphine for the last 2 h of a 20 h exposure restored GIRK2 colocalization with PSD95 to a level similar to untreated neurons (1.07 ± 0.05, $n = 41$). The competitive opioid receptor antagonist naloxone (100 μM) prevented the change in GIRK2 immunostaining with morphine (Table 1, Fig. 2*C*,*D*). Morphine also increased the colocalization between GIRK2 and NMDA receptor, another marker for dendritic spines, ~1.5-fold (1.52 ± 0.15, $n = 50$). Together, these results suggested that ~20 h exposure to morphine upregulates GIRK2 channels in the dendritic spines. Interestingly, this concentration of morphine (30–100 μM) maximally activates heterodimeric opioid receptors, which are less sensitive than monomeric opioid receptors (Levac et al., 2002; Johnson et al., 2006).

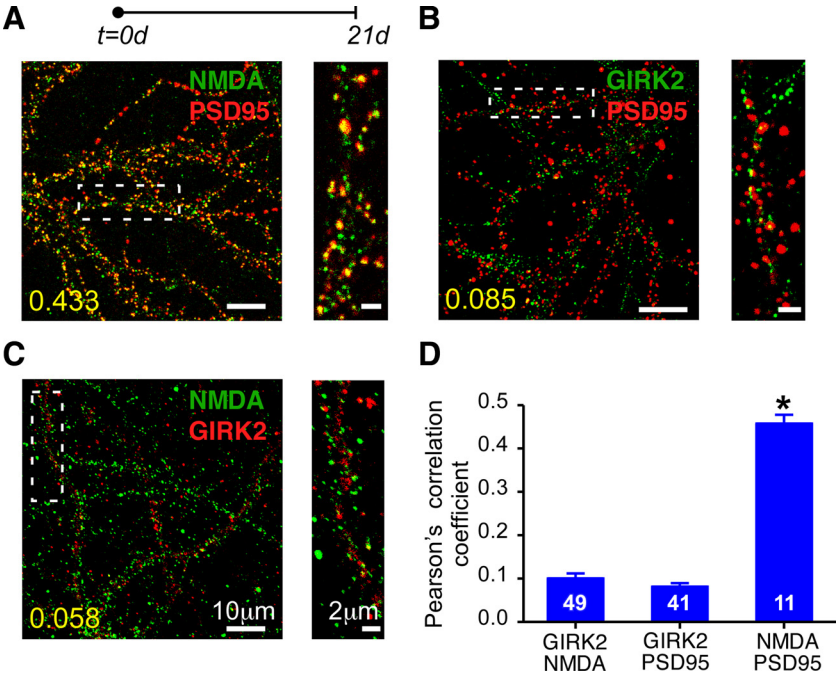

Figure 1. GIRK2 is predominantly expressed in the dendritic shaft of mature cultured hippocampal neurons and overlaps little with dendritic spine markers (PSD95 and NMDA receptors). ***A–B***, Hippocampal neurons (21 DIV) were immunostained for GIRK2 and a spine marker, NMDA receptors (***A***) or PSD95 (***B***). Little colocalization of GIRK2 with the dendritic spine markers was observed, suggesting GIRK2 channels are expressed mainly on the dendritic shaft. ***C***, Comparison of PSD95 and NMDA receptors. As expected, PSD95 and NMDA exhibited a high degree of colocalization (yellow). The extent of colocalization was determined by deriving the Pearson's correlation coefficient for red– green images. The Pearson's coefficient is shown for each image. ***A–C***, Right, Magnification of a single dendritic branch. ***D***, Bar graph showing the average (±SEM) Pearson's coefficient for GIRK2/NMDA, GIRK2/PSD95, and NMDA/PSD95, with the number of dendritic fields indicated.

Opioid receptors are expressed both presynaptically and postsynaptically in hippocampal neurons (Wimpey and Chavkin, 1991; Arvidsson et al., 1995; Ding et al., 1996; Drake and Milner, 1999; Stumm et al., 2004; McQuiston, 2007); thus, the signaling pathway underlying the morphine-induced change in GIRK2 expression could be complex depending on the target receptor (see Discussion). We first explored the effect of disinhibiting cultured hippocampal neurons; the $GABA_A$ antagonist bicuculline did not produce the same effect as morphine nor did bicuculline occlude the morphine-dependent change in GIRK2 expression (Table 1). This suggests that $GABA_A$ receptor activation is not involved. We also found the NMDA and AMPA receptor antagonists (AP5 and CNQX) did not prevent the morphine-dependent increase in colocalization between GIRK2 and PSD95 (Table 1), indicating that activation of these ionotropic receptors is not required. Last, inhibiting spontaneous electrical activity with 2 μM TTX did not prevent the morphine-dependent increase in colocalization between GIRK2 and PSD95 (Table 1), indicating that activity-dependent release of neurotransmitters or neuropeptides is not essential. These pharmacological studies lead us to propose that stimulation of postsynaptic opioid receptors underlies the morphine-induced change in GIRK2 colocalization with PSD95. Several studies have suggested that μ- and δ-opioid receptors may be expressed postsynaptically in pyramidal hippocampal neurons (Arvidsson et al., 1995; Bausch et al., 1995; Ding et al., 1996; Drake and Milner, 1999; Liao et al., 2005; Liao et al., 2007). Consistent with this conclusion, immunostaining for μ-opioid receptors revealed diffuse expression of μ-opioid receptors that also colocalized with GIRK2 channels (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Morphine treatment (100 μM for ~20 h), however, did not alter the colocalization of GIRK2 and μ-opioid receptor.

We next investigated the possible subtype of opioid receptor using selective agonists. The μ-selective alkaloid peptide DAMGO (1 μM) did not alter the colocalization of GIRK2 and PSD95 in neurons (Table 1). DPDPE (100 μM), a selective agonist for δ-opioid receptor, also did not significantly change the colocalization of GIRK2 and PSD-95 (Table 1). By contrast, hippocampal neurons exposed for ~20 h to 10 and 30 μM met-enkephalin, which stimulates μ- and δ-opioid receptors (Drake et al., 2007b), increased the colocalization ratio for GIRK2 and PSD95 (Fig. 2*F*), similarly to morphine. Most common opioid receptors couple to PTX-sensitive G-proteins. To investigate this, we examined the effect of PTX on the morphine-treated neurons. Surprisingly, PTX treatment (6 h) did not prevent the morphine-induced increase in colocalization of GIRK2 with PSD95 (Fig. 2*G*). Together, these results suggest that morphine activates postsynaptic opioid receptors using PTX-insensitive G-proteins (see Discussion).

Upregulation of GIRK2 in dendritic spines

The increase in colocalization between GIRK2 and PSD95 with morphine treatment suggested that GIRK2 channels were specifically upregulated in dendritic spines. To confirm this, hippocampal cultures were infected with Sindbis virus encoding actin-YFP (Fig. 3), which provides an independent assay for visualizing dendritic spines (Honkura et al., 2008). Morphine treatment (20 h) increased the colocalization of GIRK2 channels with actin-YFP, similar to the increase in colocalization between GIRK2 and PSD95 (Fig. 3*A*,*C*). To rule out that PSD95 was upregulated in the dendritic shaft, we also examined the effect of morphine on the colocalization PSD95 and actin-YFP (Fig. 3*B*,*C*). In contrast to GIRK2, the colocalization of PSD95 with

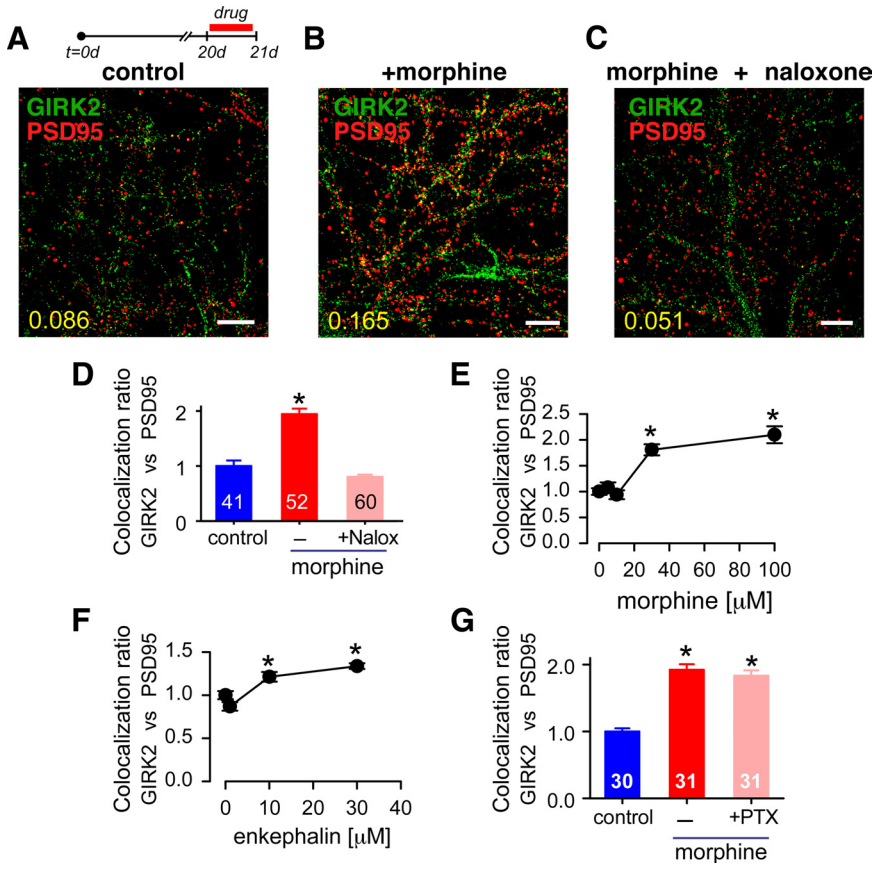

Figure 2. Stimulation of opioid receptors with morphine alters GIRK2 localization in hippocampal neurons. Hippocampal neurons (21 DIV) were exposed to 100 μM morphine for ~20 h and then immunostained for GIRK2 and PSD95. ***A–C***, Representative images show the pattern of GIRK2 and PSD95 expression. ***B***, Morphine increased the colocalization of GIRK2 with PSD95. ***C***, The nonselective opioid receptor antagonist, naloxone (100 μM), occluded the morphine-dependent increase in colocalization between GIRK2 and PSD95. Pearson's coefficient for individual images is shown in lower left. To compare the colocalization among different hippocampal neuronal preparations, a colocalization ratio was calculated (the Pearson's coefficient for experimental group was normalized to control). Scale bars, 10 μm. ***D***, Bar graph shows the mean colocalization ratio for GIRK2 and PSD95 for control, morphine, and morphine plus naloxone groups (mean ± SEM). ***E***, ***F***, Dose-response curves for morphine (***E***) and met-enkephalin (***F***) and the colocalization ratio for GIRK2/PSD95. ***G***, Bar graph shows the mean colocalization ratio for GIRK2 and PSD95 in control neurons, morphine-treated neurons, and PTX-treated neurons in morphine. Morphine increased the colocalization of GIRK2 and PSD95 in the presence of PTX, suggesting involvement of PTX-insensitive G-proteins. Numbers in bars show numbers of dendritic fields analyzed. *, $p < 0.05$ vs control, one-way ANOVA followed by Bonferroni *post hoc* test for significance.

actin did not change with morphine. Together, these findings suggest that morphine treatment upregulates GIRK2 expression in the dendritic spines.

To better resolve an increase of GIRK2 in the dendritic spine, we investigated the localization of GIRK2 in hippocampal neurons at the subcellular level using quantitative immunoelectron microscopy for GIRK2 channels (Fig. 4). Quantification of immunogold labeling of GIRK2 channels revealed high percentage of particles in intracellular compartments of dendritic shafts and soma of control hippocampal neurons, with less immunogold labeling in the spines (Fig. 4*A*, *C*). Analysis of gold particles on the plasma membrane showed nearly equivalent fraction of GIRK2 on the shaft and spine. In morphine-treated neurons, the percentage of immunogold GIRK2 labeling increased on the spine plasma membrane but decreased on the shaft and soma plasma membrane (Fig. 4*B*, *C*). Immunogold labeling of GIRK2 also increased in the somatic endoplasmic reticulum (Fig. 4*B*), suggesting an increase in transcription/translation of GIRK2. We also examined the effect of morphine on the density of labeling in the spine. Morphine significantly increased the expression in the spine plasma membrane to 4.59 ± 0.87 particles/μm versus control of 2.15 ± 0.69 particles/μm of spine plasma membrane (Student's *t* test, $p < 0.05$, $n = 60$ spines). These mean densities were significantly different from background mean density with no primary antibody (0.051 ± 0.01 particle/μm in control vs 0.058 ± 0.01 particle/μm in morphine-treated neurons; Student's *t* test, $p < 0.001$). The mean length of the spine, however, did not change with morphine treatment; 785 ± 216 nm (control) versus 790 ± 222 nm (plus morphine) ($n = 60$ spines), suggesting the morphological features of the spines did not change with morphine. The combined light- and electron-microscopic immunohistochemical experiments demonstrate that morphine treatment increases the expression of GIRK2 protein, with a marked ~2-fold increase on the plasma membrane of dendritic spines.

Enhanced basal and 5HT-induced GIRK currents with morphine

We next investigated the physiological consequences of the morphine-induced increase in GIRK2 expression in the dendritic spines of hippocampal neurons using whole-cell patch clamping. Because reliable GIRK current recordings from ~3-week-old cultured neurons were difficult to obtain, we examined younger (11–14 DIV) cultures of hippocampal neurons, which were more amenable to patch-clamp recordings. We first confirmed that the younger neurons contain PSD95-labeled dendritic spines (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Like 3-week-old neurons, morphine (100 μM) treatment for ~20 h increased the colocalization of GIRK2 and PSD95 nearly twofold in young hippocampal neuronal cultures (control, 1.00 ± 0.06, $n = 46$ vs morphine, 1.88 ± 0.08 $n = 43$ fields, $p < 0.05$; Student's *t* test) (Fig. 5*A*, *B*).

Whole-cell macroscopic current recordings from control hippocampal neurons revealed both $GABA_B$ and 5-HT receptor-activated currents, using 100 μM baclofen and 1 μM 5-HT, respectively (Fig. 5*D*). These receptor-induced currents exhibited inward rectification and a reversal potential near the potassium equilibrium potential (Fig. 5*D*, arrow), indicating activation of GIRK channels. A Ba^{2+}-sensitive, inwardly rectifying potassium current is also present in these neurons, which is likely carried by predominantly GIRK channels (Karschin et al., 1996; Chen and Johnston, 2005). To assess whether this Ba^{2+}-sensitive basal current was mediated entirely by GIRK channels, we attempted to measure the effect of tertiapin (100 nM), a toxin reported to inhibit GIRK channels (Jin and Lu, 1998). However, tertiapin-Q (200 nM) inhibited an inward current but also shifted the reversal potential, making it difficult to interpret the tertiapin-Q-sensitive GIRK currents (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Nonetheless, the morphine-dependent increase in GIRK2 expression is consistent with the

morphine-dependent increase in both the 5-HT-induced GIRK currents and Ba^{2+}-sensitive basal currents (Fig. 5*F*). Surprisingly, the amplitude of baclofen-activated GIRK currents decreased following morphine treatment (Fig. 5*F*).

We therefore investigated whether morphine can alter expression of $GABA_B$ receptors and GIRK2 channels. We used a biochemical assay to measure the surface and total proteins levels for GIRK2 and $GABA_{B2}$. Providing further support for the finding that morphine significantly increases the expression levels of GIRK2 channels in the dendrites, morphine treatment consistently increased levels of total GIRK2 protein (Fig. 5*G*). Biotinylation experiments revealed a dramatic increase in GIRK2 surface but not NMDA receptor expression in morphine-treated neurons (Fig. 5*H*). Conversely, the total protein levels of $GABA_{B2}$ subunit significantly decreased, suggesting the reduction in $GABA_B$-activated GIRK current might be due to a reduction in $GABA_B$ receptors. However, the surface labeling of $GABA_{B2}$ subunits was not significantly reduced (Fig. 5*H*), suggesting that $GABA_B$ receptors may have undergone other changes (see Discussion). We investigated whether *Girk2* mRNA changed with morphine. Using qRT-PCR, morphine treatment increased ~3-fold the levels of *Girk2* transcripts (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). Finally, we examined the effect of systemic morphine on GIRK2 expression in the hippocampus. We predicted that GIRK2 would be upregulated in the hippocampus. Rats were injected subcutaneously with 1, 10, or 20 mg/kg and examined for possible changes in total GIRK2 protein 5 h later, using quantitative Western analysis. A single morphine injection (10 mg/kg) significantly ($p < 0.05$) increased the levels of GIRK2 protein in the hippocampus (Fig. 5*I*). Interestingly, 20 mg/kg did not produce the same increase, suggesting the effect was not due to a generalized property of morphine (e.g., depressed respiration). Together, the molecular, biochemical, and electrophysiological data suggest that morphine treatment increases the expression levels of GIRK2 on the plasma membrane, leading to an enhancement of GIRK signaling.

Table 1. Effect of pharmacological treatments on the colocalization ratio of GIRK2 and PSD95 immunostaining in 21 DIV cultured hippocampal neurons

Treatment	Pharmacological targets	Colocalization ratio Mean ± SEM	N
Control		1.01 ± 0.05	94
Morphine	μ/δ/κ-opioid R agonist	1.51 ± 0.04*	66
DAMGO	μ-opioid R agonist	1.09 ± 0.04	20
DPDPE	δ-opioid R agonist	0.94 ± 0.06	19
Met-enkephalin	μ/δ-opioid R agonist	1.21 ± 0.06*	22
Bicuculline	$GABA_A$R antagonist	1.00 ± 0.07	10
AP5 + CNQX	Ionotropic glutamate R antagonists	1.00 ± 0.06*	30
GABA	$GABA_{A/B}$R agonist	1.12 ± 0.06	48
Morphine +			
Naloxone	μ/δ/κ-opioid R antagonist	0.80 ± 0.04	60
Gabazine	$GABA_A$R antagonist	1.60 ± 0.11*	42
SCH50911	$GABA_B$R antagonist	1.87 ± 0.10*	46
Gabazine + CGP 35348	$GABA_A$/$GABA_B$R antagonists	1.67 ± 0.23*	47
AP5 + CNQX	Ionotropic glutamate R antagonists	1.63 ± 0.06*	32
TTX	Na channel inhibitor	1.67 ± 0.08*	46
PTX	Gαi/o inhibitor	1.83 ± 0.08*	31

Neurons were treated with indicated drugs: morphine (100 μM), DAMGO (1 μM), DPDPE (100 μM), met-enkephalin (10 μM), bicuculline (30 μM), GABA (100 μM), naloxone (100 μM), gabazine (10 μM), gabazine (10 μM), CGP 35348 (10 μM), SCH50911 (50 μM), AP5 (100 μM), CNQX (10 μM), TTX (2 μM), and/or PTX (250 ng/ml). PTX was applied during the last 6 h of the morphine treatment. Values shown are the mean colocalization ratio (±SEM). N, Number of dendritic fields analyzed; R, receptor. *$p < 0.05$ versus control paired with drugs groups, one-way ANOVA followed by Bonferroni post hoc test for significance.

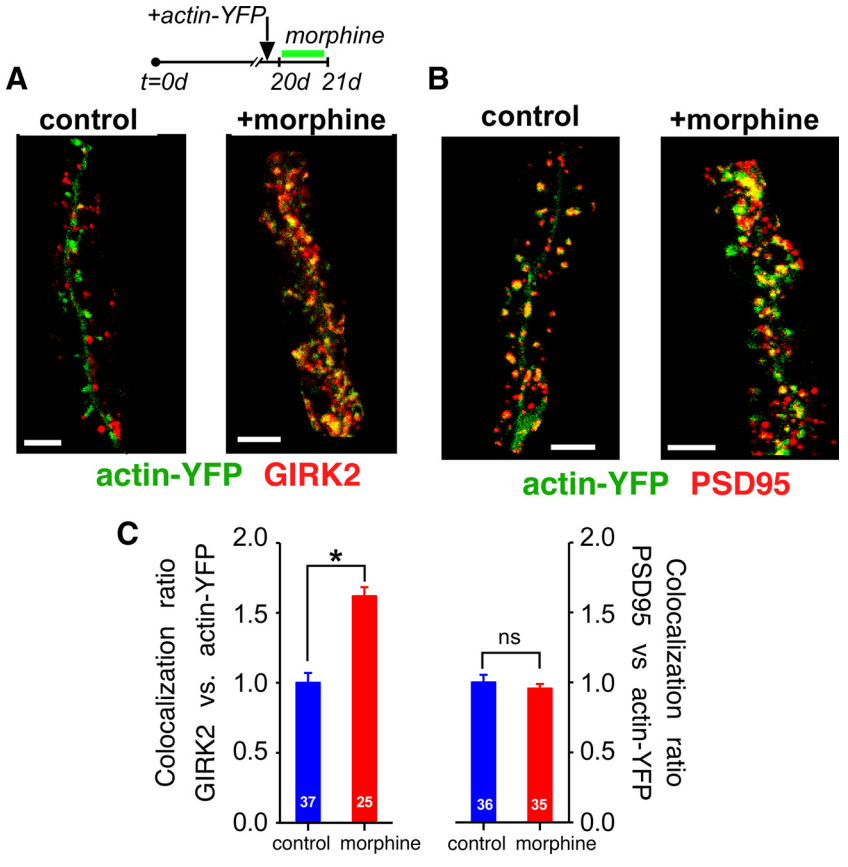

Figure 3. Morphine increases colocalization of GIRK2 but not PSD95 in actin-filled dendritic spines. Dissociated hippocampal cultures were infected with Sindbis actin-YFP virus at 20 DIV and were either untreated (control) or exposed to morphine (100 μM) for ~20 h. ***A***, ***B***, Examples of actin-YFP-infected dendrites immunostained for GIRK2 or PSD95 in control and morphine-treated cultures at 21 DIV. Morphine significantly increased colocalization of GIRK2 with actin-YFP but did not appear to alter colocalization of PSD95 with actin-YFP. Scale bar, 5 μm. ***C***, Bar graphs show average colocalization for GIRK2/actin-YFP and PSD95/actin-YFP in untreated (control) or morphine-treated neurons. *, $p < 0.05$, Student's *t* test. Numbers in bars represent number of dendritic fields analyzed. ns, Not significant.

Involvement of CaMKII and Ca^{2+} in morphine-dependent regulation of GIRK channels

Morphine has been shown to increase the activity of CaMKII in neurons (Wang and Wang, 2006). Furthermore, morphine stimulation of receptors that couple to PTX-insensitive G-proteins could lead to activation of CaMKII via changes in intracellular Ca^{2+}. We first examined the effect of CaMKII inhibitors on the morphine-dependent increase in colocalization between GIRK2 and PSD95 (Fig. 6). Hippocampal neurons treated with a membrane permeant inhibitor of CaMKII, KN-93 (10 μM) (Sumi et al., 1991), for the last 2 h of the ~20 h morphine treatment, prevented the morphine-dependent increase in colocalization of GIRK2 and

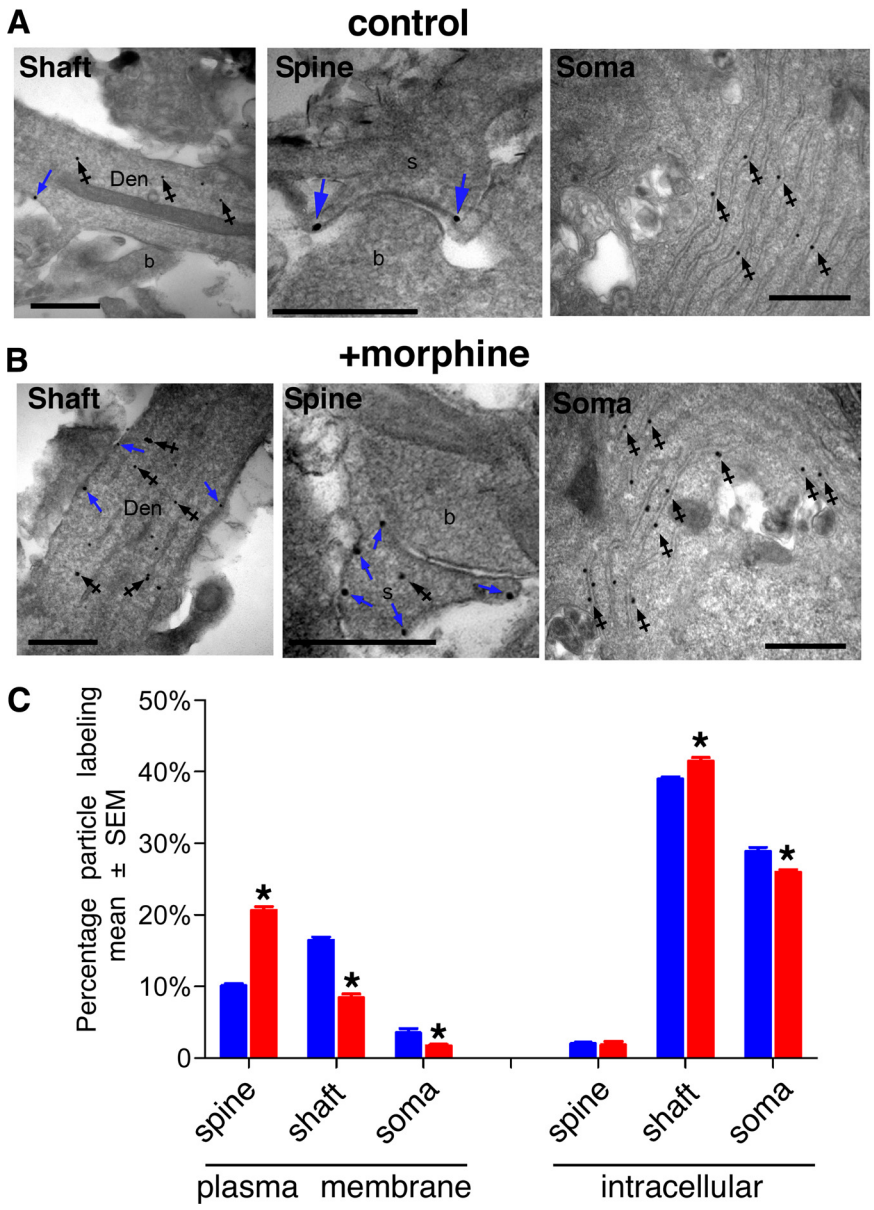

Figure 4. Ultrastructural analyses reveal that morphine increases expression of GIRK2 in the dendritic spines of hippocampal neurons. The effect of morphine on the expression of GIRK2 was studied at the EM level using pre-embedding immunogold method. ***A***, ***B***, EM micrographs show immunoreactivity for GIRK2 in control-cultured hippocampal neurons (***A***) and after 20 h treatment with morphine (***B***). In control neurons (***A***), immunogold particles for GIRK2 were detected in dendritic shafts (Den) of cultured cells, both along the plasma membrane (blue arrows) and at intracellular sites (crossed arrows), and especially along the plasma membrane of dendritic spines (s, blue arrows), establishing asymmetrical synapses with axon terminal boutons (b). ***B***, After 20 h treatment with morphine, immunoreactivity for GIRK2 increased along the plasma membrane of dendritic shafts (blue arrows), dendritic spines (blue arrows), and endoplasmic reticulum in soma, as well as at intracellular sites (crossed arrows). Scale bars, 0.5 μm. ***C***, Bar graph shows the mean relative percentage of GIRK2 immunoreactivity detected in the soma, shaft, and spines of intracellular and plasma membrane compartments for control and morphine ($n = 4$ coverslips, Student's t test, $p < 0.05$). Overall, the total number of immunogold particles for GIRK2 increased from 1578 to 2596 in morphine-treated cultures.

PSD95 (Fig. 6*B*,*D*). By contrast, the inactive peptide, KN-92 (10 μM), did not inhibit the morphine-dependent increase in GIRK2/PSD95 colocalization (Fig. 6*C*,*D*). These results suggest that activation of CaMKII is necessary for producing the morphine-dependent increase in colocalization between GIRK2 and PSD95 in dendritic spines.

To examine the role of CaMKII directly and bypass the opioid receptor, hippocampal neurons were infected with a Sindbis virus encoding the constitutively active form of CaMKII fused to GFP (ΔCaMKII-GFP), which was sufficient to produce LTP and insertion of GluR1 receptors into the dendritic spine (Hayashi et al., 2000). Similar to the effect of morphine, expression of ΔCaMKII-GFP significantly increased the colocalization of GIRK2 and PSD95 compared with uninfected dendrites (uninfected, 1.00 ± .035, $n = 49$ vs GFP-positive, 1.477 ± 0.015, $n = 101$ fields) (Fig. 7*A*,*B*). Thus, either stimulating with morphine or expressing activated CaMKII in hippocampal neurons upregulates GIRK2 channels in the dendritic spines. To determine the functional consequence of activated CaMKII in cultured hippocampal neurons, we recorded macroscopic GIRK currents from neurons infected with ΔCaMKII-GFP virus. Similar to the effect of ~20 h morphine treatment on GIRK currents (Fig. 5*F*), coexpressing ΔCaMKII-GFP increased the amplitude of the Ba^{2+}-sensitive basal potassium currents (Fig. 7*C*,*D*). Furthermore, $GABA_B$ receptor-activated currents were >50% smaller in neurons coexpressing ΔCaMKII-GFP (Fig. 7*D*). Together, these results suggest that activation of CaMKII is not only necessary but is sufficient for the morphine-dependent increase in colocalization of GIRK2 and PSD95 in dendritic spines and the reduction of $GABA_B$ receptor-activated currents.

Previous studies indicated that elevations in intracellular Ca^{2+} were required for mediating the morphine-dependent activation of CaMKII (Wang and Wang, 2006). To explore this further, we tested the effect of a membrane permeant Ca^{2+} chelator, BAPTA-AM. Coapplication of 50 μM BAPTA-AM prevented the morphine-induced increase in colocalization of GIRK2 and PSD95 (Fig. 8*A*). Therefore, a plausible pathway for upregulating GIRK channels involves activation of Gq G-proteins, increases in intracellular Ca^{2+}, and activation of CaMKII. If activation of Gq G-proteins was sufficient to trigger the downstream signaling pathways to upregulate GIRK2 channels, we hypothesized that activation of a different GPCR that couples to Gq G-proteins might produce the same effects as morphine. To test this, we investigated the effect of activating metabotropic glutamate receptors, implicated previously in producing a form of long-term depression in the hippocampus (Oliet et al., 1997). Like morphine, ~20 h treatment with (±)-1-aminocyclopentane-*trans*-1, 3-dicarboxylic acid (ACPD; 100 μM) increased the colocalization of GIRK2 and PSD95 (Fig. 8*A*). Together, these data show that multiple pathways exist for upregulating GIRK2 channels in hippocampal neurons (Fig. 8*B*).

Discussion

Here, we describe a novel morphine- and CaMKII-dependent pathway for enhancing GIRK signaling in hippocampal neurons. Morphine stimulation of hippocampal neurons for ~20 h leads to an

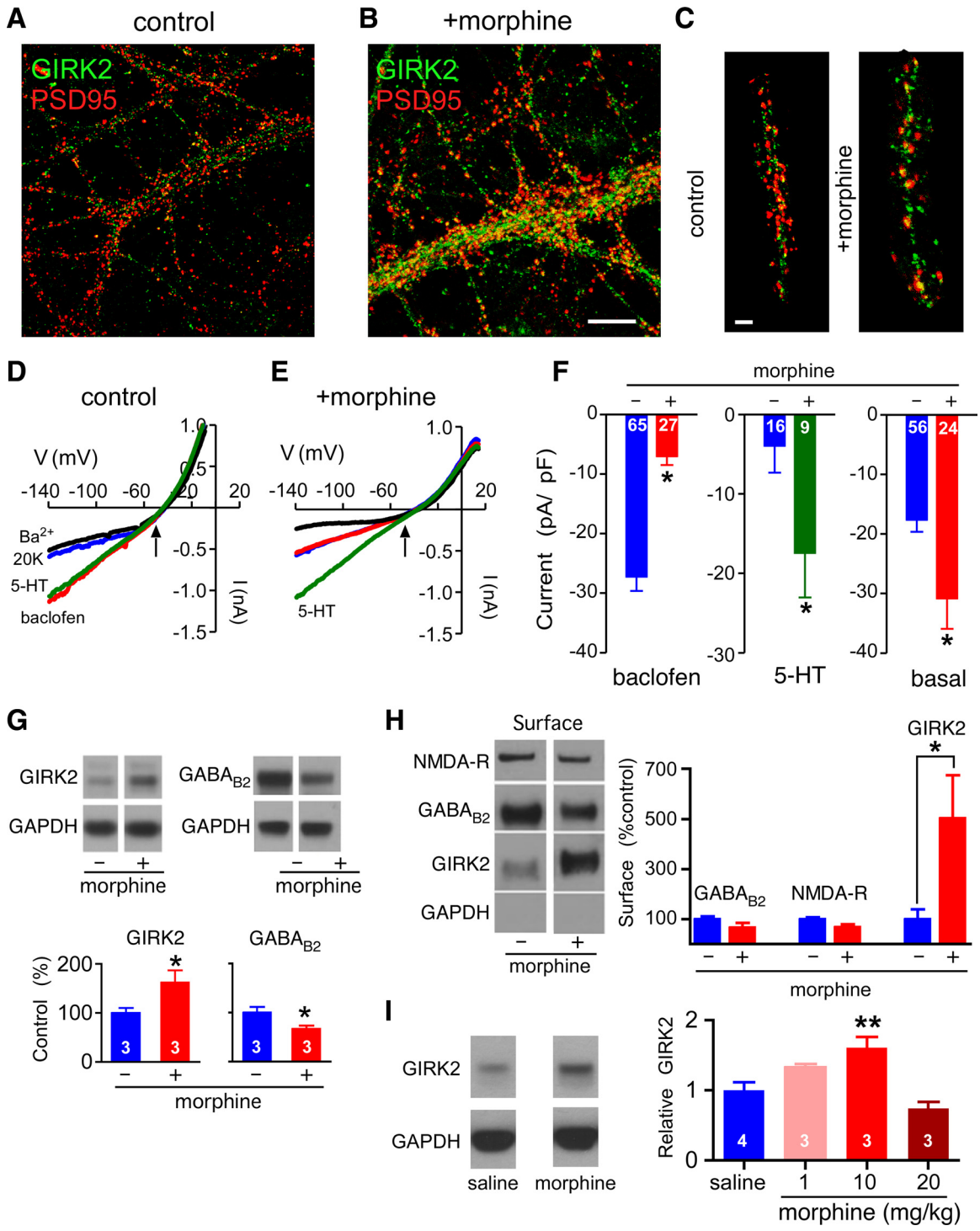

Figure 5. Morphine treatment increases 5-HT-induced GIRK currents and Ba^{2+}-sensitive basal currents but reduces $GABA_B$ receptor-activated GIRK currents. Immunostaining of GIRK2 and PSD95 in untreated hippocampal neurons (10–14 DIV) or after ~20 h morphine treatment (100 μM). ***A–C***, Colocalization between GIRK2 and PSD95 increased with morphine, demonstrating a response in 11–14 DIV that was similar to 21 DIV neurons. ***C***, Zoom of dendrites from control and morphine-treated neurons. Scale bars: ***B***, 10 μm; ***C***, 2 μm. ***D–F***, Whole-cell patch-clamp recordings show changes in GIRK currents with morphine treatment. The 5-HT-induced GIRK currents and the Ba^{2+}-sensitive basal currents increased following 20 h morphine treatment. The $GABA_B$-activated GIRK current decreased in morphine-treated neurons. Representative current–voltage (V) plots show the macroscopic currents recorded in 20K, 20K + 1 mM Ba^{2+}, 20K + 1 μM 5-HT, or 20K + 100 μM baclofen for control (***D***) and morphine-treated (***E***) neurons. Arrow marks the equilibrium potential for potassium. ***F***, Bar graphs showing mean current density (±SEM) for baclofen, 5-HT, and Ba^{2+}-sensitive basal K^+ currents. *, $p < 0.05$, Student's *t* test for significance. Numbers in bars represent the number of recordings. ***G***, Western staining shows increase in total protein for GIRK2 and decrease in $GABA_{B2}$ from hippocampal neurons exposed to morphine for ~20 h. Representative immunoblots for GIRK2, $GABA_{B2}$, and GAPDH (loading control) are shown. Bar graph shows quantification of optical density for indicated protein (see Materials and Methods). ***H***, Surface biotinylation experiment shows an increase in surface expression of GIRK2 in neurons exposed to morphine. Representative immunoblots for NMDA receptor, $GABA_{B2}$, GIRK2, and GAPDH (lack of GAPDH confirms isolation of plasma membrane fraction). Bar graph shows quantification of surface expression and increase in GIRK2 ($n = 3$ separate experiments). *, $p < 0.05$, Student's *t* test for significance. ***I***, Systemic morphine increases GIRK2 protein levels *in vivo*. Western blot shows increase in total protein for GIRK2 in rat hippocampi isolated 5 h following a single morphine injection (10 mg/kg). Bar graph shows relative change in GIRK2 (mean optical density normalized to GAPDH) for three different morphine concentrations ($n = 3$) versus saline injection ($n = 4$). **, $p < 0.05$, one-way ANOVA followed by Bonferroni's multiple-comparison test for significance.

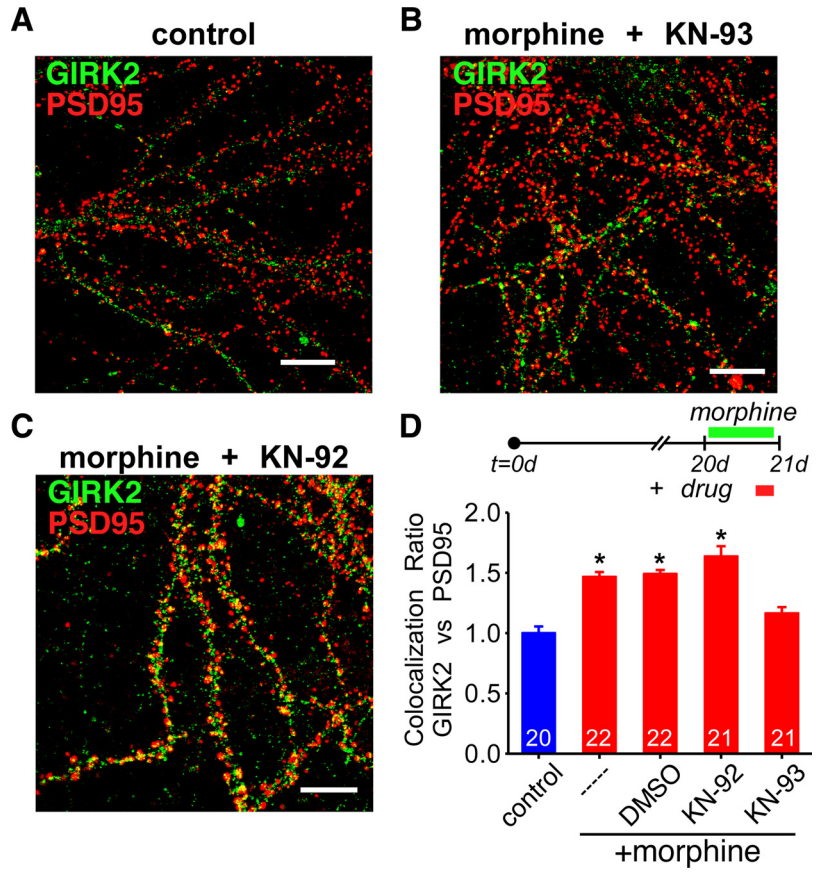

Figure 6. Morphine-dependent increase in colocalization of GIRK2 and PSD95 requires activated CaMKII. Hippocampal neurons (20 DIV) were exposed to 100 μM morphine for ~18 h and during the last 2 h were either treated with the selective CaMKII inhibitor KN-93 (10 μM) and morphine or with the inactive peptide KN-92 (10 μM) and morphine. ***A–C***, Representative images show immunostaining for GIRK2 and PSD95. KN-93 but not KN-92 or the vehicle DMSO prevents the morphine-dependent increase in GIRK2 colocalization with PSD95. Scale bar, 10 μm. ***D***, Bar graph shows the mean colocalization ratio (±SEM) with the numbers in the bars indicating the number of dendritic regions analyzed. *, $p < 0.05$, one-way ANOVA followed by Bonferroni *post hoc* test for significance.

upregulation of GIRK2 channel protein and expression on the plasma membrane, with a significant fraction expressed in the dendritic spines. The increase in GIRK2 expression occurs in parallel with the enhancement of 5-HT-induced GIRK currents and Ba^{2+}-sensitive basal currents, and requires an elevation in intracellular Ca^{2+} (BAPTA-AM-sensitive) and activated CaMKII (KN93-sensitive). We discuss the mechanisms and implications of this form of CaMKII-dependent plasticity in GIRK signaling in the hippocampus.

Morphine treatment enhanced both 5-HT-induced GIRK currents and Ba^{2+}-sensitive basal currents but reduced baclofen-induced GIRK currents. Recently, a similar segregation of signaling was reported for adenosine and $GABA_B$ receptors (Chung et al., 2009). NMDA receptor stimulation selectively upregulated adenosine-induced GIRK currents without affecting $GABA_B$-GIRK currents. Together, these studies indicate there are distinct GPCR/GIRK signaling pathways in hippocampal neurons, perhaps one for $GABA_B$ and one for 5-HT or adenosine. Our biochemical studies revealed increases in *Girk2* mRNA, total GIRK2 protein, and surface expression of GIRK2 channels, which suggest that transcriptional regulation and specific protein targeting pathways underlie the enhancement of GIRK currents. A similar pathway for regulating GIRK2 expression may also exist *in vivo*; systemic injection of morphine significantly increased levels of total GIRK2 protein in the hippocampus. Recently, Ingram et al. (2008) also found that a single morphine injection enhances GIRK currents in the periaqueductal gray, suggesting changes in GIRK signaling may be a common response to morphine. Systemic opioids have also been found to enhance glutamatergic signaling in the ventral tegmental area (VTA), increasing expression of GluR1 (Fitzgerald et al., 1996) and the ratio of AMPA/NMDA currents (Saal et al., 2003). The morphine-dependent increase in 5-HT-induced GIRK currents could provide a serotoninergic pathway for dampening pyramidal neuron excitability. In fact, acute administration of morphine rapidly increases the synthesis of 5-HT (Ahtee and Carlsson, 1979), which may parallel the increase in 5-HT-induced currents.

Interestingly, morphine selectively reduced the amplitude of $GABA_B$ receptor-activated GIRK currents. In parallel with this, the levels of total $GABA_{B2}$ receptors were reduced; however there was no statistical decrease in surface expression of $GABA_{B2}$. Thus, it is unclear whether surface levels of $GABA_B$ receptors are altered by morphine. $GABA_B$ receptor turnover is quite complex. Depending on the cell type, $GABA_B$ receptors have been shown to undergo either rapid constitutive endocytosis, no endocytosis, or agonist-induced endocytosis (Gonzalez-Maeso et al., 2003; Fairfax et al., 2004; Grampp et al., 2007). It is possible that morphine induces heterologous desensitization, which leads to uncoupling of $GABA_B$ receptors with G-proteins and reduces the baclofen-activated currents without changing the levels of receptor. For example, prolonged treatment with delta9-tetrahydrocannabinol leads to downregulation and desensitization of CB1 receptors as well as heterologous reduction of $GABA_B$ and A1 receptor-mediated inhibition of adenylyl cyclase (Selley et al., 2004). Future studies are needed to clarify the changes in $GABA_B$ receptors that occur with morphine.

Opioids have been shown to modulate synaptic transmission by altering glutamatergic transmission, long-term potentiation, and dendritic stability in the hippocampus (Salmanzadeh et al., 2003; Guo et al., 2005; Liao et al., 2005; Bao et al., 2007). Although morphine is a strong agonist of the μ-opioid receptor, it is also an agonist for δ- and κ-opioid receptors. The mRNA for all three has been detected in the hippocampus (Mansour et al., 1987) and a majority of opioid receptors are expressed in GABA interneurons in the hippocampus (Wimpey and Chavkin, 1991; Stumm et al., 2004; McQuiston, 2007). μ-Opioid receptors, in particular, regulate presynaptic release of GABA (Drake and Milner, 1999). Opioid receptors may also be expressed postsynaptically in glutamatergic hippocampal neurons. There is low-level expression for μ- and δ-opioid receptor mRNA in principal neurons of hippocampus (Stumm et al., 2004). Furthermore, immunohistochemistry reveals the localization of both μ- and δ-opioid receptors in principal neurons of hippocampus (this study and Arvidsson et al., 1995; Bausch et al., 1995; Ding et al., 1996; Drake

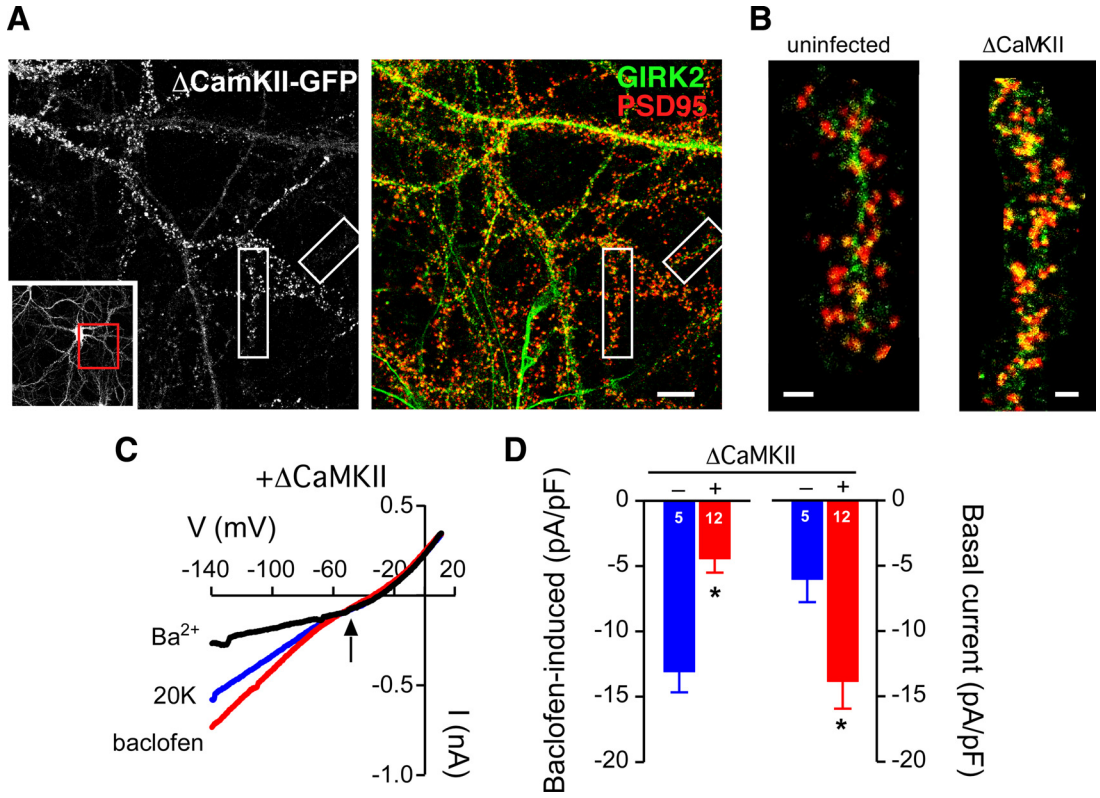

Figure 7. Constitutively active CaMKII mimics the effects of morphine on GIRK signaling. Hippocampal neurons (20 DIV) were infected with a virus expressing constitutively active CaMKII (ΔCaMKII-GFP). ***A***, ***B***, Representative images of dendrites 1 d after infection. Low-magnification images show the location of infected and uninfected dendrites immunostained for GIRK2 and PSD95 (***A***). Grayscale image shows GFP-channel. ***B***, Zoom of dendrites immunostained for GIRK2 and PSD95 from infected and uninfected neurons. Scale bars: ***A***, 10 μm; ***B***, 2 μm. ***C***, Whole-cell patch-clamp recording from a representative neuron infected with ΔCaMKII-GFP (11–14 DIV). The response to baclofen and Ba^{2+} are shown. Arrow indicates potassium. V, Voltage. ***D***, Bar graphs show mean (±SEM) amplitude for baclofen-induced (left) and Ba^{2+}-sensitive basal K^+ (right) currents for uninfected and infected neurons. *, $p < 0.05$, Student's *t* test for significance.

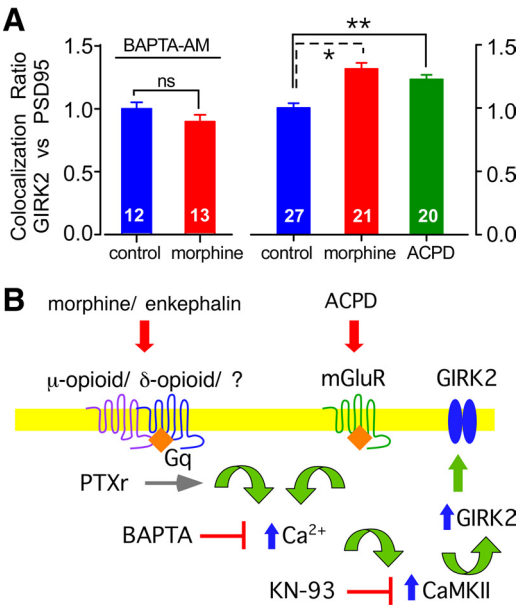

Figure 8. Proposed pathway for morphine-induced enhancement of GIRK signaling in hippocampal neurons. ***A***, Inhibition of intracellular Ca^{2+} with 50 μM BAPTA-AM prevents the morphine-induced colocalization of GIRK2 and PSD95 in 20 DIV hippocampal neurons (left). Exposure to the metabotropic glutamate receptor agonist ACPD (100 μM) for 20 h increases the colocalization ratio for GIRK2 and PSD95 in 20 DIV hippocampal neurons (right). ***B***, Proposed signaling pathways for morphine and ACPD-dependent upregulation of GIRK2 in hippocampal neurons.

and Milner, 1999) and postsynaptic μ-opioid receptors have been implicated in changes of spine morphology and modulation of excitatory synaptic activity in cultured hippocampal neurons (Liao et al., 2005, 2007). Due to this complex network of neuronal connections and expression of opioid receptors, morphine could produce different effects depending whether it targets presynaptic or postsynaptic receptors. For example, opioid stimulation of presynaptic μ-opioid receptors on interneurons would inhibit release of GABA (Wimpey and Chavkin, 1991; Stumm et al., 2004; McQuiston, 2007) and disinhibit pyramidal glutamate neurons, resulting in enhanced neuronal activity and glutamate release. AMPA and NMDA glutamate receptor antagonists did not inhibit the morphine-induced change in GIRK2 localization, however, suggesting the activation of these ionotropic glutamate receptors is not required. Similarly, neither activation of $GABA_A$ receptors nor electrical activity is required for the morphine-dependent change (Table 1). Constitutively active CaMKII expressed directly in principal hippocampal neurons, on the other hand, mimics many of the changes produced by morphine. Together, these results support the interpretation that postsynaptic opioid receptors mediate the morphine-induced increase in GIRK2 spine expression, though additional experiments are needed to assess the contribution of other pathways, such as transsynaptic messengers and/or relocation of GIRK2 from other compartments.

The naloxone-inhibition of the morphine-dependent increase in colocalization of GIRK2 and PSD95 confirms the involvement of opioid receptors. The selective μ-opioid receptor agonist DAMGO, however, had no effect on GIRK2 channel expression. Morphine

but not DAMGO induces little μ-opioid receptor desensitization (Whistler et al., 1999). Moreover, morphine can inhibit GIRK channels through a direct action on the channel without triggering internalization (Ulens et al., 1999; Blanchet et al., 2003). The inability of DAMGO treatment (~20 h) to alter GIRK2 expression could therefore be due to DAMGO-dependent internalization. However, met-enkephalin, an agonist for both μ- and δ-opioid receptors (Drake et al., 2007a), produced a similar increase in the colocalization ratio of GIRK2/PSD95, whereas the δ-opioid receptor agonist DPDPE had no effect. This pharmacological profile for the morphine-dependent change in GIRK signaling indicates it is unlikely that μ-opioid receptors are exclusively involved. In addition to this pharmacological profile, the PTX-insensitivity, the requirement for high concentration of morphine, Ca^{2+} dependence, and CaMKII dependence all implicate an alternative opioid signaling pathway. One possibility is that morphine stimulates μ/δ-opioid receptor heterodimers, which have been shown to generate novel ligand binding properties, including a reduction in agonist affinity and insensitivity to pertussis toxin (Levac et al., 2002). Recently, μ/δ-opioid receptors were found to coexpress in nociceptive DRG neurons (Wang et al., 2010). μ-Opioid receptors have also been shown to dimerize with non-opioid receptors (Vilardaga et al., 2008), which could provide additional signaling pathways. Other G-protein signaling pathways are also possible, such as μ-opioid receptor stimulation of PTX-insensitive Gz subunits, which have been shown to regulate Src and increase NMDAR expression and CaMKII activity (Sánchez-Blázquez et al., 2009). Stimulation of Gq but not Gz pathway, however, would be expected to elevate intracellular Ca^{2+} and the chelating Ca^{2+} with BAPTA-AM prevented the morphine-dependent change in GIRK2 expression. Finally, if morphine stimulates an opioid receptor that couples to PTX-insensitive Gq G-proteins, we predicted that stimulation of a different GPCR that also couples to Gq should mimic the effects of morphine. Indeed, the metabotropic glutamate receptor agonist ACPD (~20 h) significantly increased GIRK2 colocalization with PSD95 in hippocampal neurons.

Activation of postsynaptic opioid receptors that couple to Gq G-proteins could increase intracellular Ca^{2+} and provide a pathway for activating CaMKII (Lou et al., 1999; Wang and Wang, 2006). In support of this theory, the CaMKII antagonist KN93 inhibited the effects of morphine and, importantly, the constitutively activated form of CaMKII (ΔCaMKII) mimicked the morphine-dependent increase in GIRK2 expression. Both chronic and acute morphine treatments have been shown to significantly increase CaMKII activity in the hippocampus of rats (Wang and Wang, 2006) and CaMKII is highly enriched in postsynaptic densities of hippocampus (Kennedy, 2000). Also, inhibition of CaMKII *in vivo* can attenuate morphine tolerance, dependence, and morphine-dependent place preference (Wang and Wang, 2006). Could GIRK channels be a direct target of CaMKII-dependent phosphorylation? There are six putative CaMKII sites on GIRK2; one is located in the *N*-terminal domain and was recently implicated in regulating the stability of channels on the membrane (Chung et al., 2009). We suggest that morphine stimulates a postsynaptic heteromeric, opioid receptor-containing complex that couples to Gq G-proteins, which in turn leads to elevations in intracellular Ca^{2+}, activation of CaMKII, and upregulation of GIRK2 channels in dendritic spines (Fig. 8*B*). Interestingly, long-term potentiation of glutamatergic signaling has been shown to potentiate the GIRK-dependent slow IPSC via CaMKII (Huang et al., 2005). Future studies will need to elucidate the cellular mechanism for selectively upregulating and targeting GIRK channels to the dendritic spine. Morphine has been shown to alter the levels of a kinesin light chain 1, which acts as the primary regulator of kinesin action, and colocalizes with CaMKII in hippocampal neurons (Bilecki et al., 2009). The enhanced activity of kinesin might facilitate the transport of GIRK channels into the spines.

In summary, morphine treatment for ~1 d increases GIRK2 channel expression in the dendritic spines of hippocampal neurons and enhances the 5-HT-receptor-activated and basal K^+ currents through a CaMKII-dependent mechanism. The involvement of CaMKII in attenuating morphine tolerance and dependence *in vivo* (Wang and Wang, 2006) raises the possibility that enhancement of GIRK signaling may be a pathway involved in the abusive effects of morphine.

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