

Special Section on 50 Years of Opioid Research

Chronic Treatment with Morphine Disrupts Acute Kinase-Dependent Desensitization of GPCRs[§]

Emily R. Leff, Seksiri Arttamangkul, and John T. Williams

Vollum Institute, Oregon Health and Science University, Portland, Oregon

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ABSTRACT

Based on studies using mutations of the μ -opioid receptor (MOR), phosphorylation of multiple sites on the C-terminus has been recognized as a critical step underlying acute desensitization and the development of cellular tolerance. The aim of this study is to explore which kinases mediate desensitization of MOR in brain slices from drug-naïve and morphine-treated animals. Whole-cell recordings from locus coeruleus neurons were made, and the agonist-induced increase in potassium conductance was measured. In slices from naïve animals, pharmacological inhibition of G-protein receptor kinase (GRK2/3) with compound 101 blocked acute desensitization. Following chronic treatment with morphine, compound 101 was less effective at blocking acute desensitization. Compound 101 blocked receptor internalization in tissue from both naïve and morphine-treated animals, suggesting that GRK2/3 remained active. Kinase inhibitors aimed at blocking protein kinase C and c-Jun N-terminal kinase had no effect on desensitization in tissue taken from naïve animals. However, in slices taken from morphine-treated animals, the combination of these blockers along with compound 101 was required to block acute desensitization. Acute desensitization of the potassium

conductance induced by the somatostatin receptor was also blocked by compound 101 in slices from naïve but not morphine-treated animals. As was observed with MOR, it was necessary to use the combination of kinase inhibitors to block desensitization of the somatostatin receptor in slices from morphine-treated animals. The results show that chronic treatment with morphine results in a surprising and heterologous adaptation in kinase-dependent desensitization.

SIGNIFICANCE STATEMENT

The results show that chronic treatment with morphine induced heterologous adaptations in kinase regulation of G protein coupled receptor (GPCR) desensitization. Although the canonical mechanism for acute desensitization through phosphorylation by G protein-coupled receptor kinase is supported in tissue taken from naïve animals, following chronic treatment with morphine, the acute kinase-dependent desensitization of GPCRs is disrupted such that additional kinases, including protein kinase C and c-Jun N-terminal kinase, contribute to desensitization.

Introduction

Acute desensitization of many GPCRs is initiated by G protein-coupled receptor kinase (GRK)-dependent phosphorylation (Gurevich and Gurevich, 2019). Most work to date on the μ -opioid receptor (MOR) has focused on phosphospecific antibodies and mutant receptors where phosphorylation sites on the C-terminus have been substituted with alanine (Williams et al., 2013). Two clusters of amino acid residues, 354–357 (TSST) and 375–379 (STANT), are phosphorylated following application of high-efficacy agonists (Wang et al.,

2002; Doll et al., 2011; Lau et al., 2011; Chen et al., 2013; Just et al., 2013; Miess et al., 2018). Four additional phosphorylation sites, constituting 11 total phosphorylation sites on the C-terminal tail of MOR, are either phosphorylated constitutively or by activity-dependent kinases, such as PKC (Williams et al., 2013). All 11 sites contribute to acute desensitization and long-term tolerance in varying degrees. Partially phosphorylation-deficient mutant MORs virally expressed in the rat locus coeruleus (LC) blocked internalization and reduced acute desensitization (Birdsong et al., 2015; Yousuf et al., 2015; Arttamangkul et al., 2019). In addition, total phosphorylation-deficient (TPD) MORs, in which all 11 phosphorylation sites are mutated to alanine, nearly abolished acute desensitization in the LC of both naïve and morphine-treated animals (Arttamangkul et al., 2018; Kliever

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ABBREVIATIONS: AAV2, adeno-associated virus type 2; ACSF, artificial cerebrospinal fluid; β -CNA, β -chlornaltrexamine; CMP101, compound 101; exWT, virally expressed wild-type; GPCR, G protein coupled receptor; GRK, G protein-coupled receptor kinase; JNK, c-Jun N-terminal kinase; LC, locus coeruleus; ME, [Met⁵]enkephalin; MOR, μ -opioid receptor; MTA, morphine-treated animal; PKC, protein kinase C; SST, somatostatin; TPD, total phosphorylation-deficient; WT, wild type.

et al., 2019), indicating that C-terminal phosphorylation is critical for MOR desensitization.

Acute desensitization in LC neurons is augmented and the recovery from desensitization is prolonged in slices taken from morphine-treated animals (Dang and Williams, 2004; Quillinan et al., 2011; Levitt and Williams, 2012; Arttamangkul et al., 2018). The mechanism that underlies this augmentation is not understood. In slices from naïve animals, inhibition of GRK2/3 with compound 101 (CMP101) blocked one measure of acute desensitization in LC neurons (Lowe et al., 2015). Following chronic morphine treatment, a second component of desensitization that was dependent on PKC was observed (Bailey et al., 2009; Levitt and Williams, 2012). In addition, spinally mediated acute analgesic tolerance induced by morphine administration involved JNK2 (Melief et al., 2010). Morphine-induced acute MOR desensitization in the dorsal root ganglion was also mediated by JNK (Mittal et al., 2012). Taken together, these results suggest that chronic treatment with morphine induced the involvement of additional kinases that augment desensitization.

In the present study, the activation of potassium conductance in rat brain slices induced by opioids and somatostatin on LC neurons was used to examine how kinase inhibitors affect acute desensitization before and following chronic treatment with morphine. Although the GRK2/3 inhibitor, CMP101, blocked acute desensitization of MORs and somatostatin receptors in slices from untreated animals, CMP101 did not block desensitization of either receptors in slices from morphine-treated animals. Though inhibitors of PKC or JNK (Go6976 and SP600125, respectively) did not block acute desensitization in naïve animals, these inhibitors in combination with CMP101 nearly abolished acute desensitization in chronically treated animals. The results indicate that kinase regulation of GPCR desensitization fundamentally changed following chronic morphine treatment.

Materials and Methods

Drugs. Morphine sulfate was obtained from the National Institute on Drug Abuse, Neuroscience Center (Bethesda, MD). [Met]⁵enkephalin (ME), idazoxan, and β -chloralnaloxamine (β -CNA) were from Sigma-Aldrich (St. Louis, MO). Somatostatin was from ProSpec (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel). MK-801 ((5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine maleate) and compound 101 (CMP101, 3-[(4-methyl-5-pyridin-4-yl-1,2,4-triazol-3-yl)methylamino]-N-[[2-(trifluoromethyl)phenyl]methyl]benzamide hydrochloride) were purchased from Hello Bio (Princeton, NJ); Go6976 (3-(13-methyl-5-oxo-6,7-dihydro-5H-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazol-12(13H)-yl)propanenitrile), SP600125 (1,9-Pyrazoloanthrone), and UK14304 tartate (5-Bromo-6-(2-imidazolin-2-ylamino)quinoxaline) from Tocris (Bio-Techne Corp., Minneapolis, MN); and staurosporine was from LC Laboratories (Woburn, MA). Potassium methanesulfonate was acquired from Alfa Aesar (Ward Hill, MA).

Somatostatin and ME (10 mM) were dissolved in water, diluted to the appropriate concentration in ACSF, and applied by superfusion. Go6976, SP600125, and staurosporine (all 10 mM) were dissolved in DMSO. CMP101 was first dissolved in a small amount of DMSO (10% of final volume), sonicated, and then brought to its final volume with 20% (2-Hydroxypropyl)- β -cyclo-dextrin (Sigma-Aldrich) and sonicated again to create a 10-mM solution. Slices were incubated in inhibitors diluted in ACSF for at least 1 hour prior to recording, and inhibitors were included in the bath and drug solutions at lower

concentrations. β -CNA (10 mM) was dissolved in methanol and used at 30–100 nM in ACSF.

Animals. Adult rats of both sexes were used with ages between 5 and 8 weeks. Wild-type Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). MOR-knockout Sprague-Dawley rats were also used as described in Arttamangkul et al. (2019). All animal experiments were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee of the Oregon Health & Science University (Portland, OR).

Microinjections. Animals (P24–30) were anesthetized with isoflurane and placed in a stereotaxic frame for microinjection of adeno-associated virus type 2 (AAV2) encoding either virally expressed wild-type MORs (exWT, AAV2-CAG-SS-GFP-MOR-WT-WPRE-SV40pA) or total phosphorylation-deficient MORs (TPD, AAV2-CAG-SS-GFP-MOR-TPD-WPRE-SV40pA) in the LC of MOR knockout rats. A total of 200 nl of virus was injected at 0.1 μ l/min bilaterally in the LC (anteroposterior: -9.72 mm, mediolateral: ± 1.25 mm, dorsoventral: -6.95 mm, from bregma) using a computer-controlled stereotaxic frame (Neurostar, Tübingen, Germany). Both viruses were obtained from Virovek (Hayward, CA). Electrophysiology experiments were carried out 2–4 weeks following injection.

Chronic Opioid Treatment. Rats (5 to 6 weeks) were treated with morphine sulfate continuously released from osmotic pumps as described in Quillinan et al. (2011). Osmotic pumps (2ML1; Alzet, Cupertino, CA) were filled with the required concentration of morphine sulfate in water to deliver 80 mg/kg per day. Osmotic pumps were implanted subcutaneously in the mid-scapular region of rats maintained on isoflurane anesthesia and remained in the animals until they were used for experiments 6 or 7 days later.

Tissue Preparation. Horizontal slices (260 μ m) containing LC neurons were prepared as previously described (Williams and North, 1984). Rats were deeply anesthetized and euthanized by cardiac percussion. Brains were removed, blocked, and placed in warm (34°C) ACSF containing (in millimolars) 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 2.6 CaCl₂, 1.2 NaH₂PO₄, 21.4 NaHCO₃, and 11 D-glucose with +MK-801 (10 μ M) and cut horizontally (260 μ m) by using a vibratome (VT 1200S; Leica, Nussloch, Germany). Slices were allowed to recover in warm ACSF containing +MK-801 (10 μ M) for at least 30 minutes and then stored in glass vials with warm (34°C) oxygenated (95% O₂/5% CO₂) ACSF until used.

Electrophysiology. Slices were hemisected and then transferred to the recording chamber, which was continuously superfused with 34°C carbogenated ACSF at 1.5–2 ml/min. Whole-cell recordings from LC neurons were obtained with an Axopatch 200B amplifier (Axon Instruments) in voltage-clamp mode holding potential (V_{hold} = -60 mV). Recording pipettes (World Precision Instruments, Sarasota, FL) with a resistance of 1.5–2 M Ω were filled with an internal solution of (in millimolars) 115 potassium methanesulfonate or potassium methyl sulfate, 20 KCl, 1.5 MgCl₂, 5 HEPES(K), 10 BAPTA, 2 Mg-ATP, 0.2 Na-GTP, pH 7.4, and 275–280 mOsm. Only recordings in which the series resistance remained <15 M Ω were included. Data were collected at 400 Hz with PowerLab (Chart Version 8.1.8; AD Instruments, Colorado Springs, CO).

Internalization and Imaging. Trafficking of virally expressed wild-type MORs was visualized as previously described (Arttamangkul et al., 2019). Briefly, acute brain slices (260 μ m) were prepared and then incubated with an anti-GFP nanobody conjugated to Alexa594 (Nb-A594, 10 mg/ml, 30–45 minutes). Images were captured before and after application of a saturating concentration of ME (30 μ M, 10 minutes) by using a two-photon microscope. A z-series of 10 sections was acquired at 1- μ m intervals so that the whole neuron could be qualitatively compared.

Data Analysis. For all conditions, animals were used to obtain at least six technical replicates per group; if more than six could be analyzed, all were included. Analysis was performed by using GraphPad Prism 6 (GraphPad Software, version 6.0d; San Diego, CA) based on number of technical replicates (number of slices). Values are

presented as average \pm S.D. Statistical comparisons were made using one-way or two-way ANOVA as well as multiple comparison adjusted Tukey's post hoc tests, as appropriate. For all experiments, $P < 0.05$ was used to describe significance.

Results

Naïve Animals

GRK2/3 Is Necessary for Acute Desensitization of MOR in Naïve Animals. The selective, membrane-permeable, small-molecule GRK2/3 inhibitor, CMP101 (Ikeda et al., 2007; Thal et al., 2011), was used to test the canonical mechanism of MOR desensitization by GRKs. Slices were incubated in CMP101 (30 μ M, 1 hour) and perfused throughout the recording at a lower concentration (CMP101, 1 μ M), and CMP101 (10 μ M) was included in drug-containing solutions. Recordings were made in brain slices containing the LC to measure the outward current induced by activation of G protein-coupled inwardly rectifying potassium channels by [Met]⁵enkephalin (ME).

Two measures of desensitization that have been examined in the past were also used in the present study. First, the current induced by an EC₅₀ concentration of ME (0.3 μ M) was measured before and after application of a saturating concentration of ME (30 μ M, 10 minutes). The degree of desensitization was taken as the relative current amplitude induced by ME (0.3 μ M) 5 minutes after washing the saturating concentration of ME (30 μ M) compared with the initial current amplitude induced by ME (0.3 μ M). Recovery from desensitization was measured with repeated applications of ME (0.3 μ M) for 20–40 minutes following the washout of the saturating concentration of ME (30 μ M, 10 minutes). The decline in the peak current during the application of the saturating concentration of ME (30 μ M, 10 minutes) was used as a second measure of desensitization, henceforth referred to as acute decline.

ME induced desensitization (30 μ M, 10 minutes) as measured by both acute decline (58.8% \pm 6.8% of peak, $n = 15$, Fig. 1, A and E) and the decrease in the current induced by the EC₅₀ concentration of ME (0.3 μ M) 5 minutes after washing ME (30 μ M, 31.4% \pm 9.1%, $n = 12$, Fig. 1, A and D). As reported previously, recovery from desensitization occurred over a period of 20–35 minutes. However, in the presence of CMP101 (10 μ M), both measures of acute desensitization were substantially reduced (Fig. 1, C–E). The inhibition of desensitization induced by CMP101 in WT rats, measured by both acute decline and recovery from desensitization, was indistinguishable from that found using mutant receptors having alanine mutations in all 11 C-terminus phosphorylation sites (total phosphorylation-deficient MORs, TPD MORs) expressed in MOR knockout rats (decline: WT CMP101: 83.5% \pm 2.4% of peak, $n = 6$; TPD: 80.2% \pm 6.0% of peak, $n = 7$; $P = 0.591$; relative current at 5 minute: WT CMP101: 78.8% \pm 12.1% of initial, $n = 6$; TPD: 76.9% \pm 9.5% of initial, $n = 7$; $P = 0.931$; Fig. 1, B–E; Arttamangkul et al., 2018). This block was not due to an increased sensitivity to agonist induced by CMP101 as the concentration-response curve for ME was not changed in the presence of CMP101 (Supplemental Fig. 1, A and B). These results indicate that GRK2/3 is necessary for acute desensitization in naïve rats. To test whether GRK primarily acted on the phosphorylation sites on the C-terminal tail of MOR, the effect of CMP101 on MOR desensitization in rats expressing TPD MORs was measured. Notably, there was no additional

effect of CMP101 on desensitization in slices from rats expressing TPD MORs (Supplemental Fig. 2, A–C). Thus, the 11 phosphorylation sites on the C-terminus are the main sites involved in GRK2/3-mediated acute MOR desensitization.

GRK2/3 Is Necessary for MOR Internalization. The internalization of MORs induced by efficacious agonists is also known to be dependent on phosphorylation of the C-terminus. Wildtype MORs (exWT MORs), but not TPD MORs, that were virally expressed in the LC of MOR knockout rats were efficiently internalized during application of a saturating concentration of ME (30 μ M, 10 minute, Arttamangkul et al., 2018). The internalization of virally expressed wildtype MORs was examined in slices from naïve animals in the absence and presence of CMP101. Expressed receptors had an N-terminus GFP-tag such that plasma membrane-associated receptors were immuno-labeled with an anti-GFP nanobody conjugated to Alexa594 and imaged using two-photon microscopy (Fig. 2). Labeled receptors were visualized before and after application of a saturating concentration of ME (30 μ M, 10 minute). As in previous studies, MORs were internalized following the application of ME (Fig. 2A, Arttamangkul et al., 2018). When slices were incubated in CMP101 (30 μ M, 1 hour) along with the nanobody, internalization of exWT receptors was blocked (Fig. 2B). Thus, phosphorylation of MOR by GRK2/3 is a critical step in the process of internalization as well as desensitization.

Other Kinase Inhibitors. In addition to GRK2/3, other kinases have been shown to play a role in acute MOR desensitization that include PKC and JNK. The acute decline, the decrease in the EC₅₀-induced current, and the recovery from ME-induced desensitization were measured in the presence of selective kinase inhibitors for PKC and JNK (Go6976 and SP600125, respectively). Brain slices from wild-type rats were incubated in Go6976 (1 μ M) or SP600125 (20 μ M) for at least 1 hour prior to the experiment. Acute desensitization, as measured by acute decline and recovery from desensitization, in the presence of Go6976 did not differ from that in control slices (Fig. 3, B–D). Likewise, the JNK inhibitor, SP600125, had no effect on acute desensitization; there was, however, an unexpected decrease in the recovery from desensitization at 10 minute (Fig. 3, A, C, and D). Experiments were also performed in the presence of the non-selective kinase inhibitor, staurosporine, that does not act on GRKs. Slices were incubated in staurosporine (1 μ M) for at least an hour and staurosporine (100 nM) was included in the superfusion solution. As in experiments containing PKC or JNK inhibitors, staurosporine had no effect on acute MOR desensitization or acute decline in naïve animals (relative current at 5 minute: 31.6% \pm 5.4% of initial, $n = 6$, $P > 0.999$; decline: 59.3% \pm 8.3% of peak, $n = 6$, $P = 0.999$). Thus, GRK2/3 is the major kinase(s) involved in acute MOR desensitization in the LC in slices from naïve animals and PKC or JNK alone are not sufficient to mediate acute desensitization of MOR under naïve conditions.

Morphine Treated Animals. In experiments with virally expressed TPD MORs, acute desensitization and signs of tolerance were blocked in morphine treated animals, indicating that phosphorylation of the receptor is necessary for the development of cellular tolerance (Arttamangkul et al., 2018). Previous work indicated that chronic morphine treatment induced a component of desensitization that was PKC-dependent in LC neurons, suggesting chronic morphine treatment altered

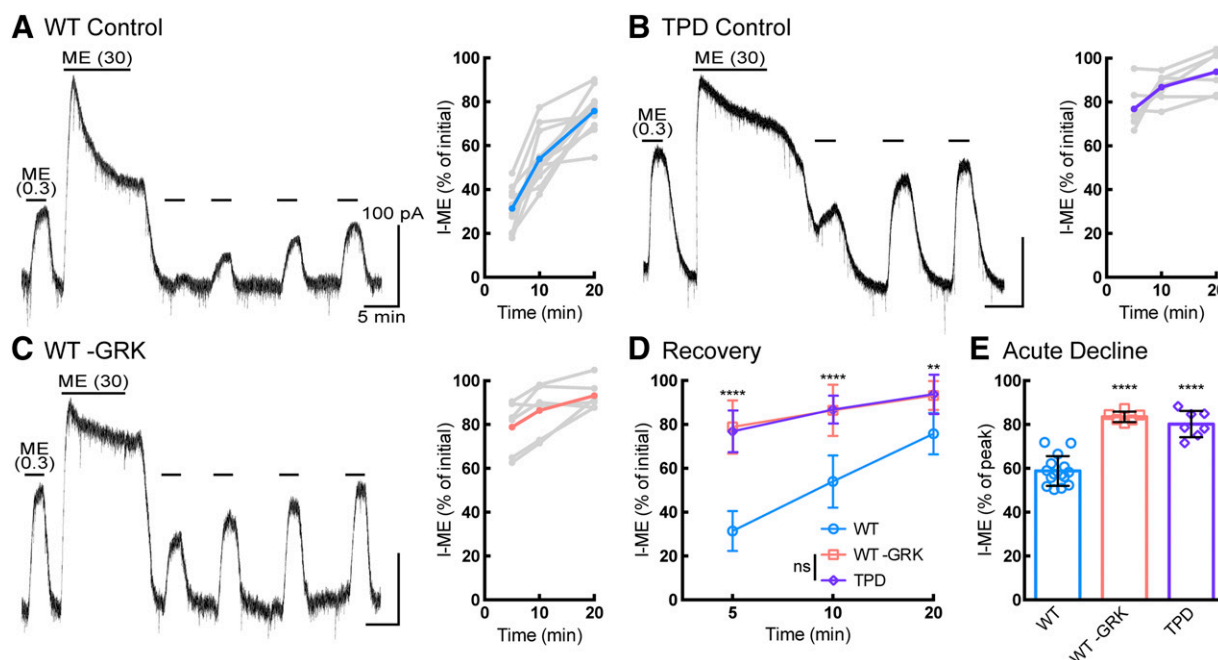


Fig. 1. GRK2/3 is necessary for acute desensitization of MOR in naïve animals. Example trace showing the G protein-coupled inwardly rectifying potassium channel currents induced by [Met]⁵enkephalin (ME, 0.3 μM) before and following application of ME (30 μM, 10 minutes) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes postdesensitization in locus coeruleus (LC) slices from (A) wild-type (WT) controls ($n = 12$ slices, eight animals), (B) MOR knockout animals virally expressing total phosphorylation deficient (TPD) mutant MORs in the LC ($n = 7$ slices, seven animals), and (C) WT incubated in the GRK2/3 inhibitor, compound 101 (CMP101, 30 μM, 1 hour, $n = 6$ slices, four animals). CMP101 was also included in the bath (1 μM) and drug solutions (10 μM). Gray lines indicate individual experiments, and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (D) recovery from desensitization (two-way ANOVA, Tukey's post hoc) and (E) acute decline (one-way ANOVA, Tukey's post hoc) for WT controls (light-blue open circles), TPD controls (purple open diamonds), and WT incubated in CMP101 (salmon open squares). Data presented as average \pm S.D., ** $P < 0.01$, **** $P < 0.0001$.

the kinase regulation of MOR (Levitt and Williams, 2012). In the present study, the change in kinase-dependent modulation of MOR signaling following chronic morphine treatment was examined using kinase inhibitors. Wildtype rats were treated with morphine (80 mg/kg per day) for 6 to 7 days with osmotic mini pumps. Brain slices were maintained in morphine-free solutions such that they were in a state of acute withdrawal. Acute desensitization was examined in slices from morphine treated animals in the absence and presence of the GRK2/3 inhibitor, CMP101. In slices not treated with CMP101, the recovery from desensitization was slowed as previously reported (Fig. 4, A and C; Dang and Williams, 2004; Quillinan et al., 2011; Arttamangkul et al., 2018, 2019). In slices that were incubated with CMP101, the acute decline in the peak ME current was

blocked, but CMP101 had no effect on the decrease in the current induced by the EC₅₀ concentration (ME, 0.3 μM) and the incomplete recovery from desensitization (Fig. 4, B–D). Finally, the relative current induced by an EC₅₀ concentration of ME (0.3 μM) compared with that induced by a saturating concentration of ME (30 μM) was the same in the absence and presence of CMP101, suggesting that CMP101 did not change the sensitivity to ME (Fig. 4E). Thus, although CMP101 blocked two measures of desensitization in slices from naïve animals, following chronic treatment with morphine it had differing effects on the two forms of desensitization.

This observation could result from a difference in sensitivity of the two measures. A rightward shift in the concentration response curve could decrease the current induced by an EC₅₀

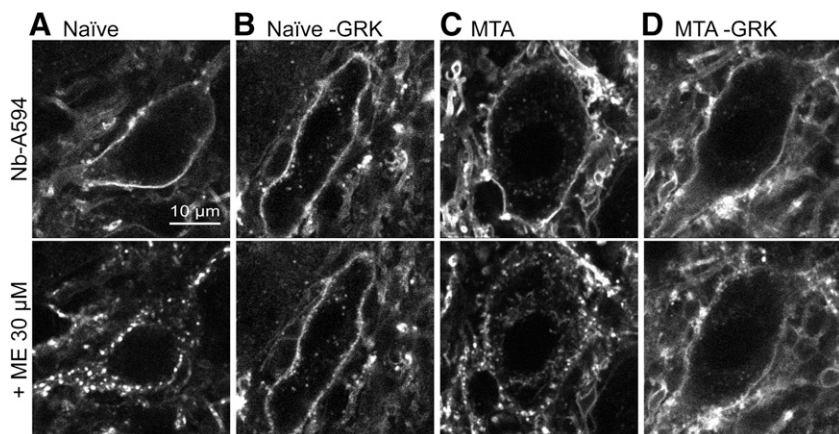


Fig. 2. GRK2/3 inhibitor blocks internalization in both naïve and morphine-treated animals. An anti-GFP nanobody conjugated to alexa594 was used to image expressed WT (exWT) receptors in the LC before (top) and following application of ME (30 μM, 10 minutes, bottom). Receptor distribution was imaged in both (A and B) naïve and (C and D) MTAs both in the (A and C) absence and (B and D) presence of CMP101 (1 μM). Experiments containing CMP101 were incubated in CMP101 (30 μM, 1 hour) prior to imaging. The exWT receptors internalized and became punctate in slices from (A, bottom) naïve ($n = 4$ slices, four animals) and (C, bottom) MTAs ($n = 3$ slices, four animals) in the absence of CMP101. Receptor trafficking was inhibited in slices both (B, bottom) naïve ($n = 6$ slices, four animals) and (D, bottom) MTAs ($n = 6$ slices, four animals) in the presence of CMP101. Scale bar, 10 μm.

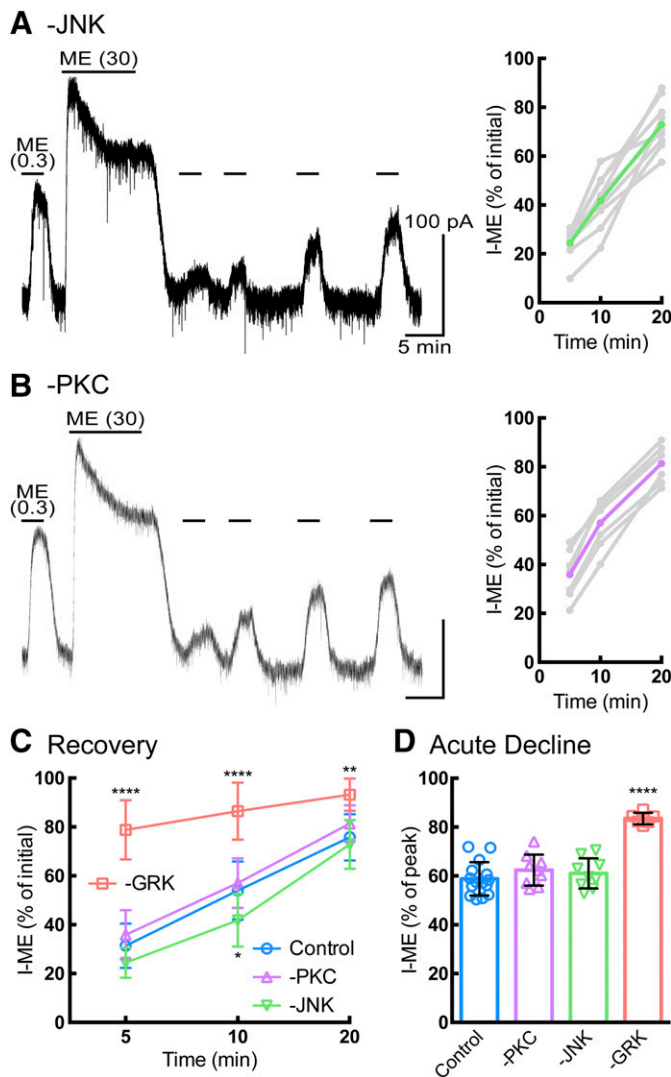


Fig. 3. PKC and JNK kinases not sufficient for acute MOR desensitization in naïve animals. Example trace showing the currents induced by ME (0.3 μ M) before and following application of ME (30 μ M, 10 minutes) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes postdesensitization in slices from (A) WT animals incubated in a JNK inhibitor (SP600125, 20 μ M, 1 hour, $n = 9$ slices, five animals) or (B) a PKC inhibitor (Go6976, 1 μ M, 1 hour, $n = 7$ slices, five animals). Inhibitors were also included in the bath (1 μ M, 100 nM, respectively) and drug solutions (10, 1 μ M, respectively). Gray lines indicate individual experiments, and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (C) recovery from desensitization (two-way ANOVA, Tukey's post hoc) and (D) acute decline (one-way ANOVA, Tukey's post hoc) for WT controls (light-blue open circles), WT incubated in SP600125 (light-green open upside-down triangles), WT incubated in Go6976 (light-purple open triangles), and WT incubated in CMP101 (salmon open squares). Data presented as average \pm S.D.; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

but not affect the peak outward current in slices from morphine treated animals. This possibility was examined by measuring the acute decline after partial irreversible block of receptors with the irreversible opioid receptor antagonist, β -chloralntrexamine (β -CNA, 30–100 nM, 5 minute) to reduce receptor reserve. Slices were incubated in β -CNA (100 nM, 5 minute) after treatment with CMP101 (30 μ M, 1 hour). The block of MORs was normalized to the current induced by the α 2-adrenergic receptor agonist, UK14304 (3 μ M). The peak current induced by ME (30 μ M) decreased from 124.1% \pm 10.9% in

control ($n = 4$) to 48.6% \pm 16.2% after treatment with β -CNA ($n = 12$). There was no correlation between the ratio of the peak current induced by ME (30 μ M) relative to the peak current induced by UK14304 (3 μ M) and the extent of acute decline in the presence of β -CNA (Fig. 5F). In slices from naïve animals treated with β -CNA, CMP101 still blocked acute decline (Fig. 5, B and E). However, in slices from morphine treated animals treated with β -CNA, CMP101 no longer blocked the acute decline (Fig. 5, D and E). Although CMP101 blocked acute decline in slices from morphine treated animals, it was no longer effective following partial irreversible removal of receptors with β -CNA. Thus, inhibition of GRK2/3 no longer blocked this measure of desensitization.

The internalization of MOR induced by ME was also examined in slices from morphine treated animals incubated with and without CMP101. Slices from MOR knockout animals that expressed wildtype N-terminus linked GFP-MORs (exWT MORs) were incubated in a solution containing anti-GFP nanobodies conjugated with alexa594 (Arttamangkul et al., 2018). Receptor trafficking was visualized with two-photon microscopy in slices taken from morphine treated animals. Treatment with ME (30 μ M, 10 minute) induced internalization of the exWT MORs in slices from MTAs but internalization was blocked in slices that were incubated with CMP101 (Fig. 2, C and D). Thus, GRK2/3 is necessary for internalization of MOR in both naïve and MTAs. The observation that internalization was blocked by CMP101 but desensitization measured by the recovery from desensitization was not affected further suggests that desensitization and internalization are separate processes (Arttamangkul et al., 2006). The results also indicate that GRK2/3 activity was not eliminated following chronic morphine treatment, suggesting that something other than, or in addition to, GRK2/3 must mediate desensitization in MTAs.

PKC and JNK Contribute to Acute Desensitization after Chronic Morphine Treatment. Given that the decrease in the recovery from desensitization following chronic morphine treatment was insensitive to CMP101, the action of PKC and JNK inhibitors on this measure was examined in slices from morphine treated animals. There was no change in the extent or rate of recovery of the current induced by ME (0.3 μ M) in slices that were incubated with the JNK inhibitor alone (Fig. 6, A and E). In contrast, this measure of desensitization was significantly attenuated when slices were incubated with both the JNK inhibitor and CMP101 (Fig. 6, B and E). When the combination of CMP101 and the PKC inhibitor, Go6976, were examined, there was a small reduction in acute desensitization following chronic morphine treatment (Fig. 6, C and E). Finally, when all three kinase inhibitors were applied, a near complete inhibition of desensitization was observed (Fig. 6, D and E). In these experiments, the desensitization was the same as that observed in experiments using expression of the GFP-TPD MORs in MOR knockout animals (Arttamangkul et al., 2018). It is interesting to note that neither the PKC nor JNK inhibitor had an additional effect on the acute decline of the current induced by a saturating concentration of ME (30 μ M, Fig. 6F).

The non-selective kinase blocker, staurosporine, was used to determine if additional kinase activity contributed to the induction of desensitization in slices from morphine treated animals. Staurosporine alone had no effect on acute desensitization measured by the acute decline (decline: 58.7% \pm 5.7% of

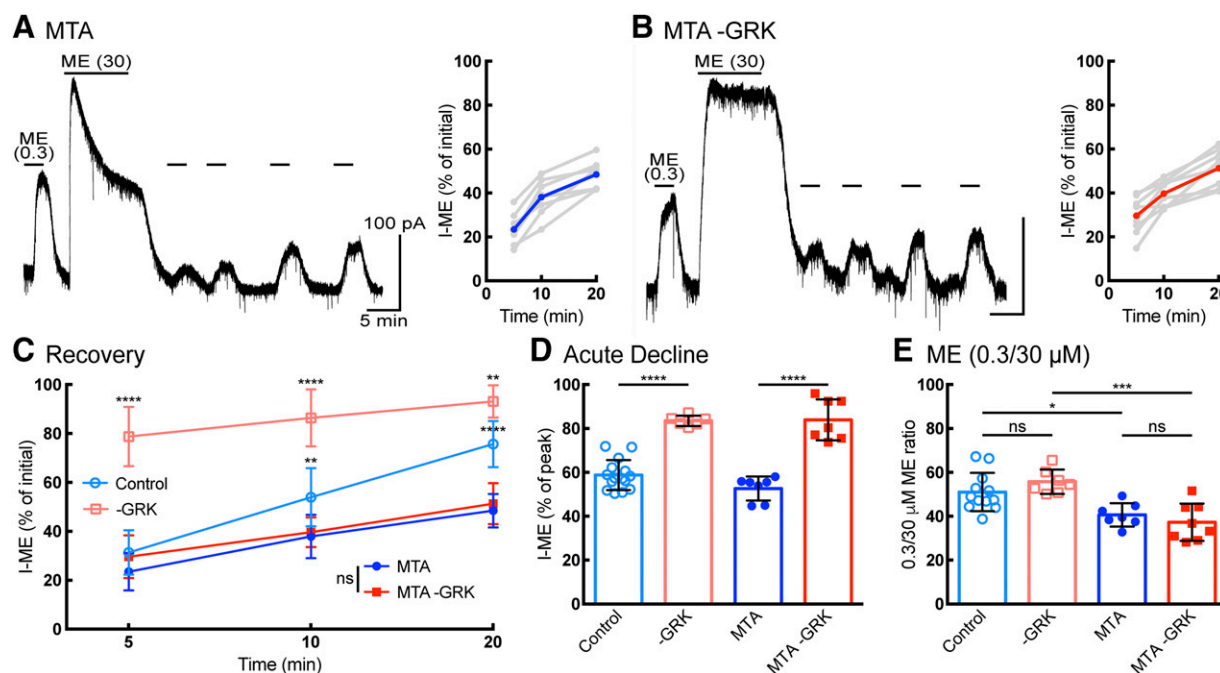


Fig. 4. GRK2/3 inhibitor blocks acute desensitization in naïve but not morphine-treated animals. Experiments were in slices from morphine-treated animals (MTAs). Example trace showing the currents induced by ME (0.3 μ M) before and following application of ME (30 μ M, 10 minutes) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes postdesensitization in slices from (A) WT MTA ($n = 7$ slices, six animals) or (B) WT MTA incubated in CMP101 (30 μ M, 1 hour, $n = 8$ slices, five animals). CMP101 was also included in the bath (1 μ M) and drug solutions (10 μ M). Gray lines indicate individual experiments, and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (C) recovery from desensitization (two-way ANOVA, Tukey's post hoc), (D) acute decline (one-way ANOVA, Tukey's post hoc), and (E) ME 0.3/30 μ M ratio (one-way ANOVA, Tukey's post hoc) for WT controls (light-blue open circles), WT MTA (dark-blue closed circles), WT incubated in CMP101 (salmon open squares), and WT MTA incubated in CMP101 (red closed squares). Data presented as average \pm S.D.; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant ($P > 0.05$).

peak, $n = 7$, $P = 0.264$, Fig. 7, A and D) or the recovery from desensitization at 5 minute (relative current at 5 minute: $31.1\% \pm 5.6\%$ of initial, $n = 7$, $P = 0.173$, Fig. 7, A and C). Experiments with staurosporine in combination with CMP101 were not significantly different from those using all three specific inhibitors (CMP101, Go6976, and SP600125; relative current at 5 minute: $75.2\% \pm 9.0\%$ of initial, $n = 6$; $P = 0.502$; Fig. 6, D and E; Fig. 7, B–D). This further supports the idea that while kinases other than GRK2/3 are upregulated following chronic treatment, GRK2/3 activity is not downregulated as the block of desensitization still required treatment with CMP101. In addition, although CMP101 no longer blocked the acute decline in slices from morphine treated animals after treatment with β -CNA, the acute decline was blocked in slices from morphine treated animals treated with β -CNA in the presence of CMP101 and staurosporine (30, 1 μ M respectively; MTA β CNA CMP101: $50.9\% \pm 8.7\%$ of peak, $n = 8$; MTA β CNA CMP101 staurosporine: $76.4\% \pm 6.2\%$ of peak, $n = 8$, $P < 0.0001$). Taken together, the results indicate that chronic morphine treatment induced an adaptive response in the kinase regulation of MORs that involves GRK2/3, JNK, and PKC.

Chronic Morphine Treatment and Heterologous Desensitization of Somatostatin Receptors. Somatostatin activates the same potassium conductance as opioids as determined by occlusion experiments (Fiorillo and Williams, 1996) and is known to be phosphorylated by GRK2/3 (Gunther et al., 2018). Desensitization of the somatostatin receptor was induced by somatostatin (SST, 20 μ M, 10 minute) and the decline in the peak current during the application was

measured. The recovery from desensitization could not be tested because of the extended time it took to wash from the slice preparation. To obtain a baseline at the end of the application of SST, BaCl₂ (100 μ M) was used to reverse current by blocking the potassium conductance.

The inhibition of desensitization induced by the kinase inhibitors was tested in slices from naïve and morphine treated animals. Treatment with SST (20 μ M, 10 minute) resulted in robust desensitization that was significantly reduced in slices incubated with CMP101 (Fig. 8, A, B, and F). The desensitization induced by SST was larger in slices taken from morphine treated animals (Fig. 8, C and F). The acute decline in the current induced by somatostatin measured in slices from morphine treated animals was insensitive to CMP101 (Fig. 8, D and F). The insensitivity to CMP101 was similar to that found with MORs with measures of recovery from desensitization and the acute decline (following treatment with β -CNA). When the desensitization of the somatostatin receptor was examined in the presence of the kinase inhibitors, CMP101, Go6976, and SP600125, desensitization was significantly reduced (Fig. 8, E and F). Thus, chronic morphine treatment induced a heterologous adaptive response on the kinase regulation of both MOR and the somatostatin receptor.

Discussion

The present study examined kinase regulation of MORs and somatostatin (SST) receptors in LC neurons before and following chronic treatment with morphine. Inhibition of

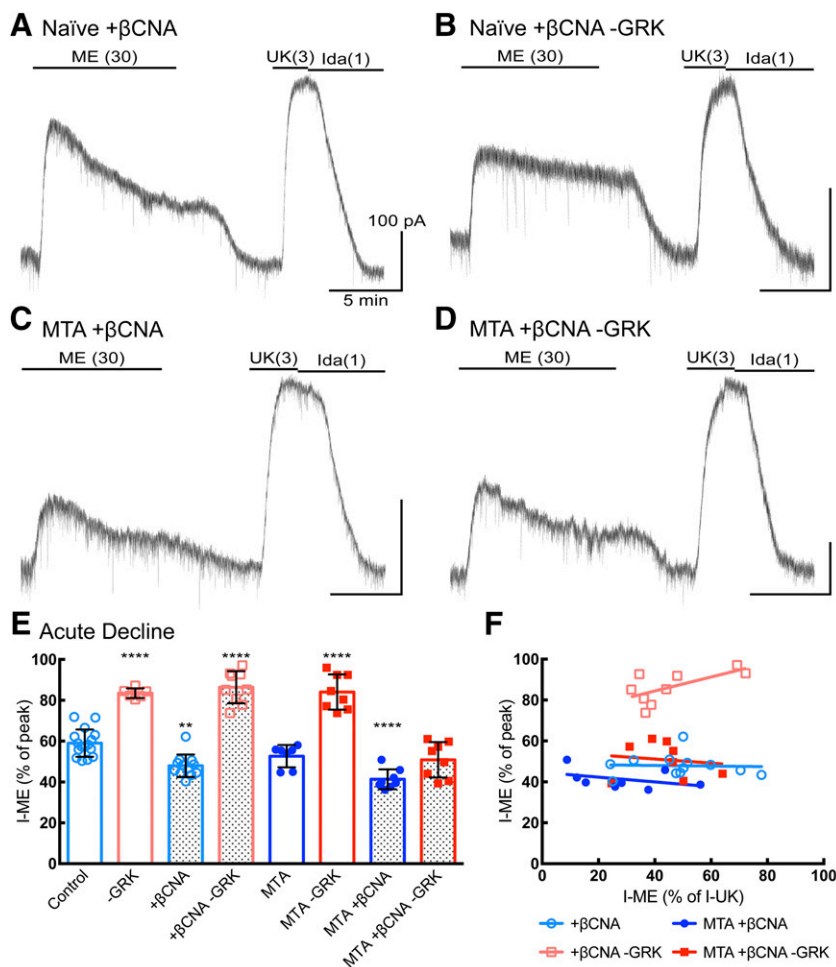


Fig. 5. GRK2/3 inhibitor does not block acute decline in morphine-treated animals after partial irreversible block of MORs. Experiments were performed in slices from both naïve and MTAs. Example trace showing the decline in the peak current during application of ME (30 μ M, 10 minutes) followed by the current induced by UK14304 (UK, 3 μ M) and reversed with idazoxan (1 μ M) in slices from (A) WT naïve incubated in the irreversible MOR antagonist, β -CNA (30–100 nM, 5 minutes, n = 12 slices, four animals), (B) WT naïve incubated in CMP101 (30 μ M, 1 hour) and then β -CNA (30–100 nM, 5 minutes, n = 9 slices, six animals), (C) WT MTA incubated in β -CNA (30–100 nM, 5 minutes, n = 8 slices, four animals), and (D) WT MTA incubated in CMP101 (30 μ M, 1 hour) and then β -CNA (30–100 nM, 5 minutes, n = 8 slices, four animals). CMP101 was also included in the bath (1 μ M) and drug solutions (10 μ M). Summary graphs showing (E) acute decline (one-way ANOVA, Tukey's post hoc) and (F) the correlation between the ratio of the peak current induced by ME (30 μ M) relative to the peak current induced by UK (3 μ M) and the extent of acute decline in the presence of β -CNA (linear regression) for WT naïve incubated in β -CNA (light-blue open circles, shaded box), WT naïve incubated in CMP101 and then β -CNA (salmon open squares, shaded box), WT MTA incubated in β -CNA (dark-blue closed circles, shaded box), and WT MTA incubated in CMP101 and then β -CNA (red closed squares, shaded box). Data presented as average \pm S.D., *** P < 0.01; **** P < 0.0001.

GRK2/3 with the selective inhibitor, CMP101, blocked acute MOR and SST desensitization in slices from naïve animals. Following chronic treatment with morphine, the kinase regulation of both MORs and SST receptors changed. Internalization of MORs induced by ME remained sensitive to CMP101, however the slowed recovery from desensitization was insensitive to CMP101. In addition, following the partial irreversible block of MORs in slices from morphine treated animals, CMP101 no longer blocked the acute decline. Thus, measures of acute desensitization and cellular tolerance to ME were insensitive to inhibition of GRK2/3. Although inhibitors of PKC and JNK had no effect on acute MOR desensitization in slices from naïve animals, in combination with CMP101, these inhibitors resulted in a near complete block of desensitization in slices from morphine treated animals. Taken together, the results show that chronic morphine treatment induced a heterologous adaptation in the kinase regulation of two GPCRs.

Naïve Animals. Canonically, phosphorylation by GRKs initiates acute homologous desensitization of GPCRs (Gurevich & Gurevich, 2019). As previously reported, inhibition of GRK2/3 with CMP101 blocked both measures of desensitization in slices taken from naïve animals (Lowe et al., 2015). Multiple sites on the C-terminus of MOR are directly phosphorylated by GRK2/3, suggesting that GRK2/3-dependent desensitization is likely through direct phosphorylation of MOR (Doll et al., 2011; Lau

et al., 2011; Chen et al., 2013; Just et al., 2013). The effect of CMP101 on MOR desensitization in WT rats, as measured by both acute decline and recovery from desensitization, was indistinguishable from that of desensitization of expressed TPD MORs, where all 11 phosphorylation sites on the C-terminus of MOR were mutated to alanine. In addition, CMP101 did not alter the small amount of desensitization in slices from rats expressing TPD MORs. The results suggest that the 11 phosphorylation sites on the C terminus underlie GRK2/3-dependent desensitization with little role of additional phosphorylation sites on the intracellular loops.

Morphine-Treated Animals. Although CMP101 blocked two measures of acute MOR desensitization and internalization in slices from naïve animals, it had mixed actions in slices taken from morphine-treated animals. The acute decline and internalization of MORs remained sensitive to CMP101 in slices from morphine-treated animals. However, the slowed recovery from desensitization measured using the EC₅₀ concentration was no longer sensitive to CMP101. It is possible that the two different measures of desensitization are dependent on separate processes (Arttamangkul et al., 2015; Birdsong et al., 2015), although following partial irreversible block of receptors with β -CNA, the acute decline was also insensitive to CMP101. Therefore, although inhibiting the activity of GRK2/3 blocked desensitization in naïve animals, it was not sufficient to block desensitization following

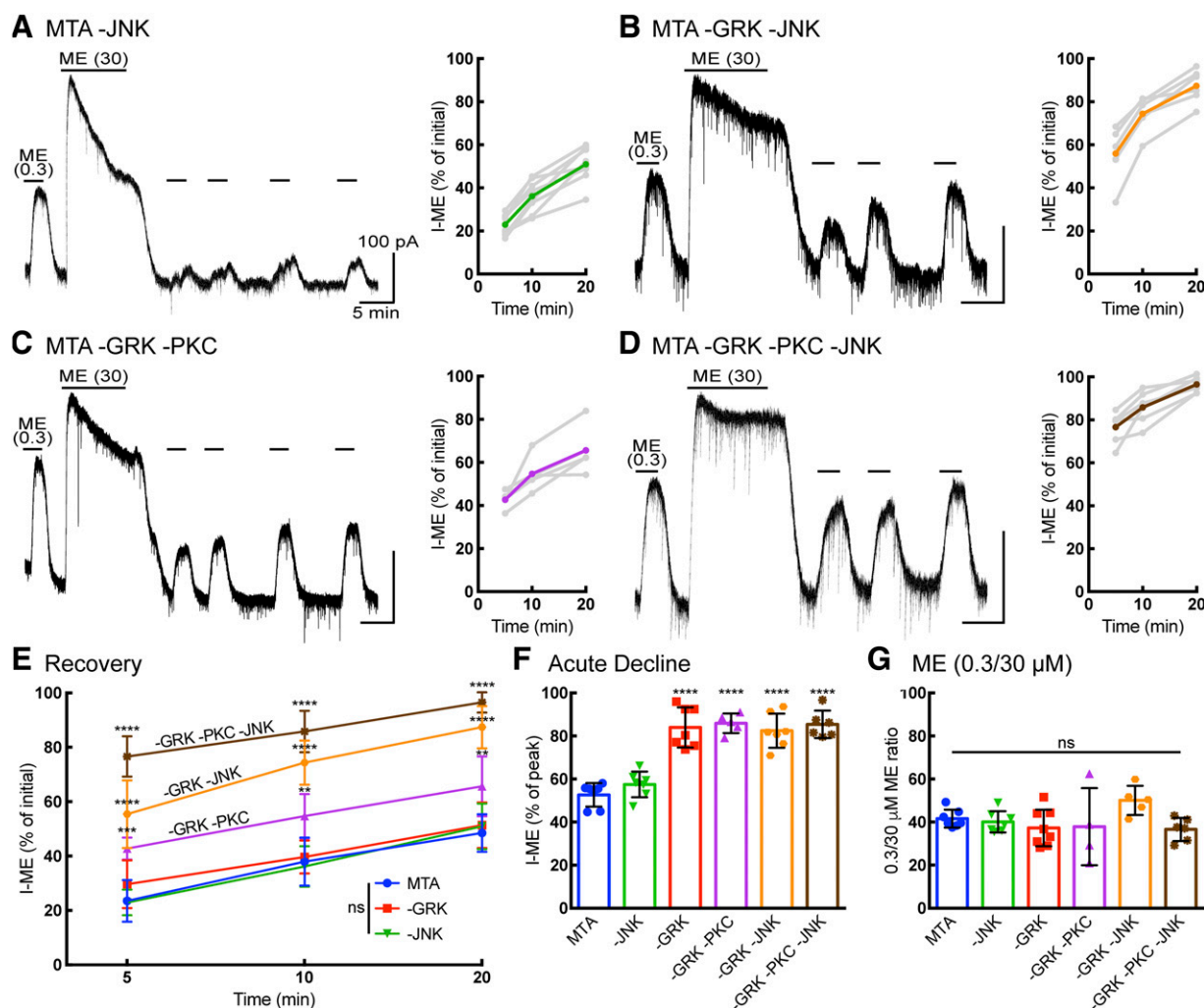


Fig. 6. PKC and JNK are sufficient to cause acute desensitization in morphine-treated animals. Experiments were conducted in slices from MTAs. Example trace showing the currents induced by ME (0.3 μ M) before and following application of ME (30 μ M, 10 minutes) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes postdesensitization in slices from (A) WT MTA incubated in CMP101 and Go6976 (30 and 1 μ M, 1 hour, $n = 5$ slices, three animals), (B) WT MTA incubated in CMP101 and SP600125 (30 and 20 μ M, 1 hour, $n = 6$ slices, four animals), (C) WT MTA incubated in SP600125 (20 μ M, 1 hour, $n = 8$ slices, six animals), and (D) WT MTA incubated in all three kinase inhibitors, CMP101, Go6976, and SP600125 (30, 1, and 20 μ M, 1 hour, $n = 6$ slices, four animals). Inhibitors were also included in the bath and drug solutions at lower concentrations. Gray lines indicate individual experiments, and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (E) recovery from desensitization (two-way ANOVA, Tukey's post hoc), (F) acute decline (one-way ANOVA, Tukey's post hoc), and (G) ME 0.3/30 μ M ratio (one-way ANOVA, Tukey's post hoc) for WT MTA controls (dark-blue circles), WT MTA incubated in CMP101 (red squares), WT MTA incubated in SP600125 (green upside-down triangles), WT MTA incubated in CMP101 and Go6976 (purple triangles), WT MTA incubated in CMP101 and SP600125 (orange hexagons), and WT MTA incubated in all three inhibitors (brown asterisks). Data presented as average \pm S.D.; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant ($P > 0.05$).

chronic morphine treatment. Given CMP101 blocked internalization and the acute decline of the current induced by ME (without preincubation with β -CNA) in morphine-treated animals, this suggests GRK2/3 remained active following chronic morphine treatment. There was also no indication that the sensitivity to ME was changed by CMP101 given that there was no change in the ratio of the current induced by ME (0.3/30 μ M) in slices taken from both naïve and morphine-treated animals.

The results support the idea that following chronic morphine treatment, additional kinases contribute to desensitization. One component of acute desensitization was dependent on PKC following chronic morphine treatment (Bailey et al., 2009; Levitt and Williams, 2012). In addition, morphine-induced acute MOR desensitization in the dorsal root ganglion (Mittal et al., 2012), spinally mediated acute analgesic tolerance

induced by morphine administration (Melief et al., 2010), and centrally mediated tolerance to morphine all involved JNK (Kuhar et al., 2015). In the present study, when the combination of staurosporine and CMP101 was used, the results were indistinguishable from those using the combination of selective inhibitors of GRK2/3, PKC, and JNK. Thus, GRK2/3, PKC, and JNK all contribute to desensitization following chronic treatment with morphine.

Though inhibitors of GRK2/3, PKC, and JNK blocked the majority of acute desensitization ($\sim 80\%$ of initial), a small amount of desensitization remained. This incomplete block could be either incomplete inhibition of kinases because of incomplete penetration or an as-yet unknown phosphorylation-independent mechanism of acute desensitization. It is also possible that phosphorylation by kinases not affected by the inhibitors at sites other than the C-terminal tail could be responsible.

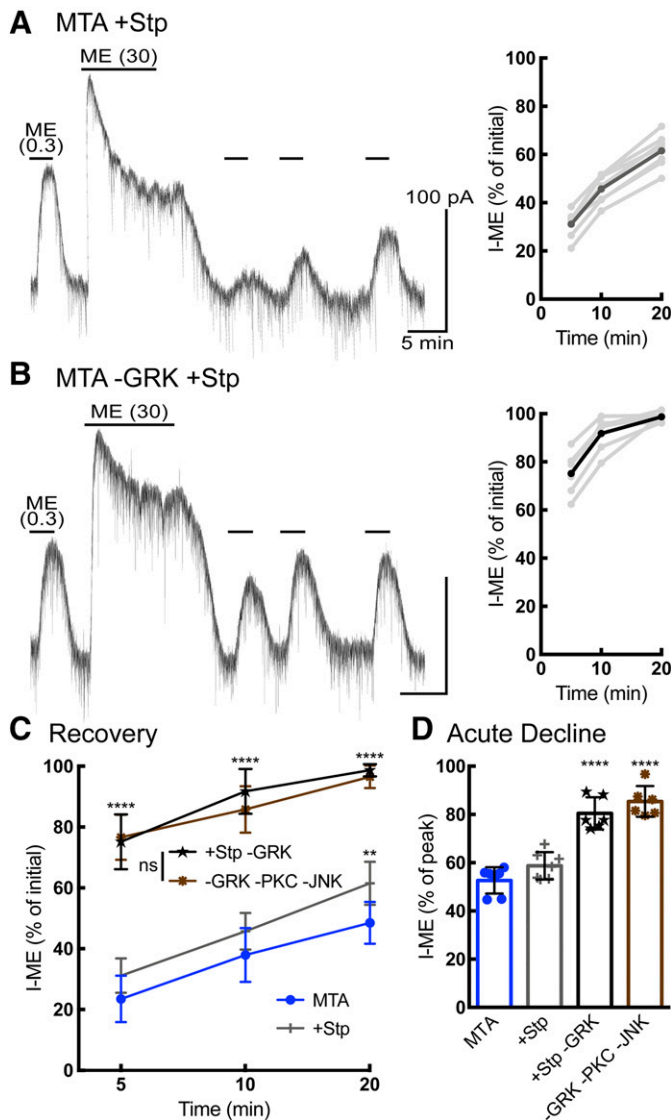


Fig. 7. GRK2/3, PKC, and JNK are the three main kinases involved in acute desensitization in morphine-treated animals. Experiments were conducted in slices taken from MTAs. Example trace showing the currents induced by ME (0.3 μ M) before and following application of ME (30 μ M, 10 minutes) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes postdesensitization in slices from (A) WT MTA incubated in the nonspecific kinase inhibitor, staurosporine (Stp, 1 μ M, 1 hour, $n = 7$ slices, four animals) or (B) WT MTA incubated in Stp and CMP101 (1 and 30 μ M, 1 hour, $n = 6$ slices, four animals). Gray lines indicate individual experiments, and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (C) recovery from desensitization (two-way ANOVA, Tukey's post hoc) and (D) acute decline (one-way ANOVA, Tukey's post hoc) for WT MTA controls (dark-blue circles), WT incubated in Stp (dark gray plusses), WT incubated in Stp and CMP101 (black stars), and WT incubated in the three inhibitors: CMP101, Go6976, and SP600125 (brown asterisks). Data presented as average \pm S.D.; ** $P < 0.01$; **** $P < 0.0001$.

It is unclear how PKC and JNK are contributing to desensitization in slices from morphine-treated animals. Although GRK2/3 has been shown to directly phosphorylate MOR, there is no evidence for direct phosphorylation of MOR by JNK. Arrestin can act as a scaffold for JNK (Kook et al., 2013; Zhan et al., 2013); however, morphine-induced JNK activation was arrestin-independent and PKC- and Proto-oncogene tyrosine-protein kinase Src-dependent (Kuhar et al.,

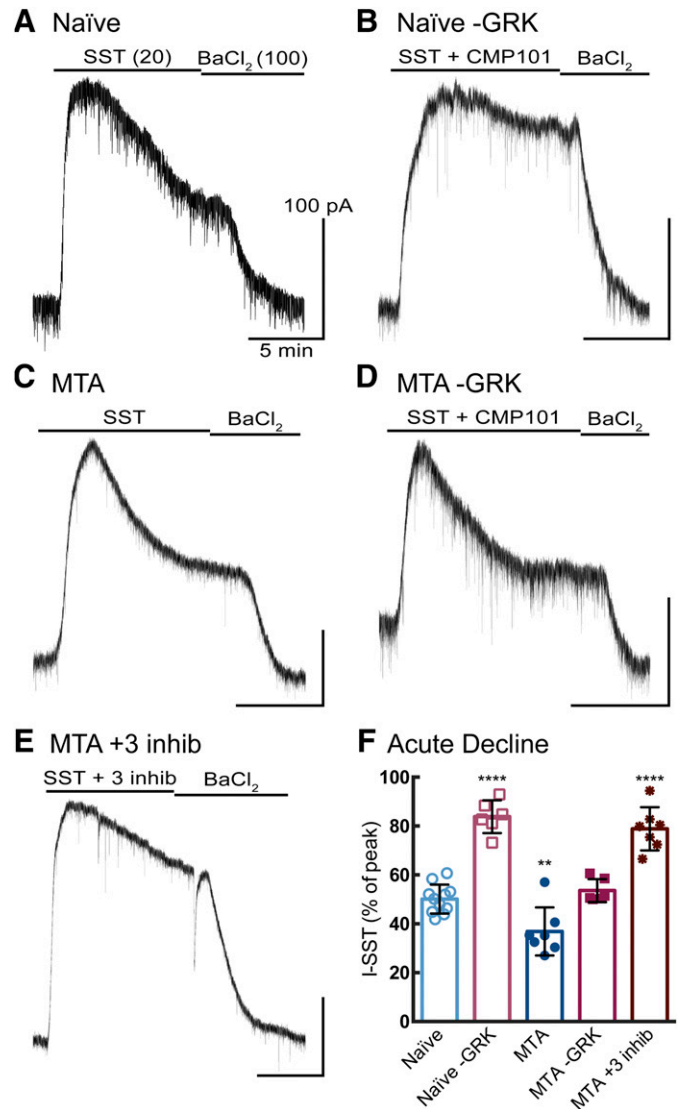


Fig. 8. Chronic treatment with morphine induces a heterologous adaptation in the kinase regulation of acute desensitization. Example trace showing the decline in current induced by somatostatin (SST, 20 μ M, 10 minutes) in slices from (A) WT naïve animals ($n = 11$ slices, six animals), (B) WT naïve incubated in CMP101 (30 μ M, 1 hour, $n = 6$ slices, five animals), (C) WT MTA ($n = 7$ slices, six animals), (D) WT MTA incubated in CMP101 (30 μ M, 1 hour, $n = 6$ slices, five animals), and (E) WT MTA incubated in all three inhibitors: CMP101, Go6976, and SP600125 (30, 1, and 20 μ M, 1 hour, $n = 7$ slices, five animals). CMP101 was also included in the bath (1 μ M) and drug solutions (10 μ M) as were CMP101, Go6976, and SP600125 when all three were used. (F) Summary graph showing acute decline (one-way ANOVA, Tukey's post hoc) for WT naïve (light-blue open circles), WT naïve incubated in CMP101 (light-pink open squares), WT MTA (dark-blue circles), WT MTA incubated in CMP101 (dark-pink squares), and WT MTA incubated in all three inhibitors (brown asterisks). Data presented as average \pm S.D.; ** $P < 0.01$; **** $P < 0.0001$.

2015). Phosphorylation of MOR by PKC is known, but the mechanism that underlies the morphine-induced activation of PKC has not been characterized (Wang et al., 2002; Doll et al., 2011; Feng et al., 2011; Chen et al., 2013; Illing et al., 2014). The activation of PKC induced by muscarine enhanced desensitization of wild-type and also TPD MORs, indicating that PKC could increase desensitization through a mechanism that is independent of phosphorylation of the C-terminal tail (Arttamangkul et al., 2018). Thus, PKC and JNK could contribute to

desensitization indirectly through phosphorylation of a component of the receptor signaling complex, preventing G-protein activation through an alternative process to GRK2/3-mediated desensitization. Heterologous desensitization in HEK293 cells through PKC-dependent phosphorylation of $G\alpha_{i2}$ has been reported (Chu et al., 2010). An alternative possibility is that PKC and/or JNK may act to mediate desensitization through phosphorylation of Regulator of G protein Signaling (RGS) proteins to turn off signaling, as has been reported in some cell types (Ogier-Denis et al., 2000; Garzon et al., 2005). Lastly, JNK-dependent desensitization resulted from JNK-dependent activation of peroxiredoxin 6 to generate reactive oxygen species via NADPH oxidase that reduced the palmitoylation of receptor $G\alpha_i$ subunits that impacted MOR signaling (Schattauer et al., 2017).

Heterologous Kinase-Dependent Signaling following Morphine Treatment. Chronic morphine treatment resulted in a heterologous modulation of GRK2/3-induced desensitization of the somatostatin receptor. Desensitization of somatostatin receptors was augmented in tissue taken from morphine treated animals, and the inhibition of GRK2/3 by CMP101 was less effective. The coapplication of PKC and JNK inhibitors along with CMP101 was required to substantially block somatostatin-induced desensitization in slices taken from morphine-treated animals, indicating a heterologous adaptation of kinase regulation following chronic morphine treatment. The somatostatin receptor contains phosphorylation sites on the C-terminus that are phosphorylated by GRK2/3 and PKC but not JNK (Gunther et al., 2018). There is also evidence that acute desensitization of MOR in slices from naïve animals results in heterologous desensitization of the somatostatin receptor (Fiorillo and Williams, 1996). The present results suggest that this heterologous action is the result of the recruitment of GRK2/3 to the plasma membrane to affect not only MORs but also SST receptors.

That the desensitization induced by SST was not sensitive to the inhibition induced by CMP101 following chronic morphine treatment is unlike what was observed for the same measure of MOR desensitization. The acute decline in the current induced by ME (30 μ M) remained sensitive to inhibition by CMP101. Thus, that measure of desensitization was dependent on GRK2/3. However, following the partial irreversible block of MORs with β -CNA, the acute decline in the current was no longer sensitive to CMP101. Given that a decrease in receptor reserve in LC neurons is induced following chronic morphine (Christie et al., 1987), it seems unlikely that a decrease in receptor reserve alone is responsible for the change in sensitivity to CMP101 following treatment with β -CNA. It is clear, however, that as was observed with the slowed recovery from desensitization, the acute decline in the current induced by ME (30 μ M, following treatment with β -CNA) was not solely dependent on GRK2/3 in slices taken from morphine-treated animals.

Conclusions

This study demonstrated that chronic morphine treatment induced heterologous adaptations in the kinase regulation of acute desensitization for two GPCRs. This may be one adaptation responsible for the augmentation of desensitization seen in animals chronically treated with morphine and

therefore may contribute to behavioral tolerance. The surprising adaptive change in kinase regulation of GPCRs may have significant functional consequences that are not directly related to opioid receptors.

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Authorship Contributions

Participated in research design: Leff, Arttamangkul, Williams.

Conducted experiments: Leff, Arttamangkul.

Performed data analysis: Leff, Arttamangkul

Wrote or contributed to writing of the manuscript: Leff, Williams.

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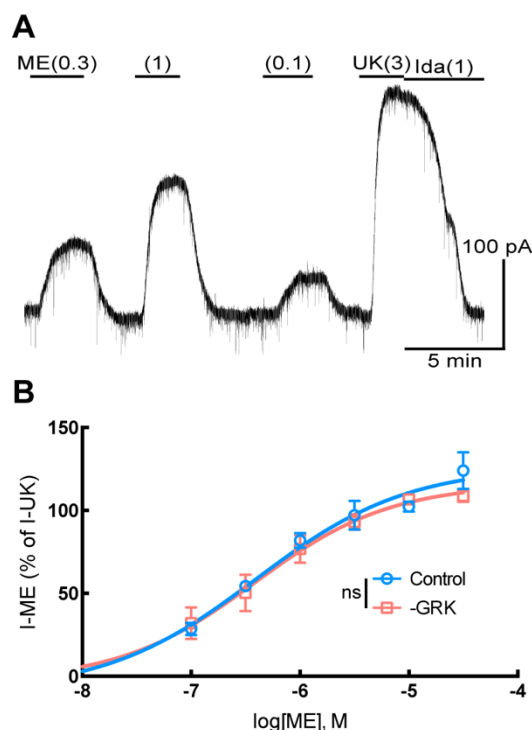
Address correspondence to: John T. Williams, Vollum Institute L474, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239. E-mail: williamj@ohsu.edu

Chronic treatment with morphine disrupts acute kinase-dependent desensitization of GPCRs

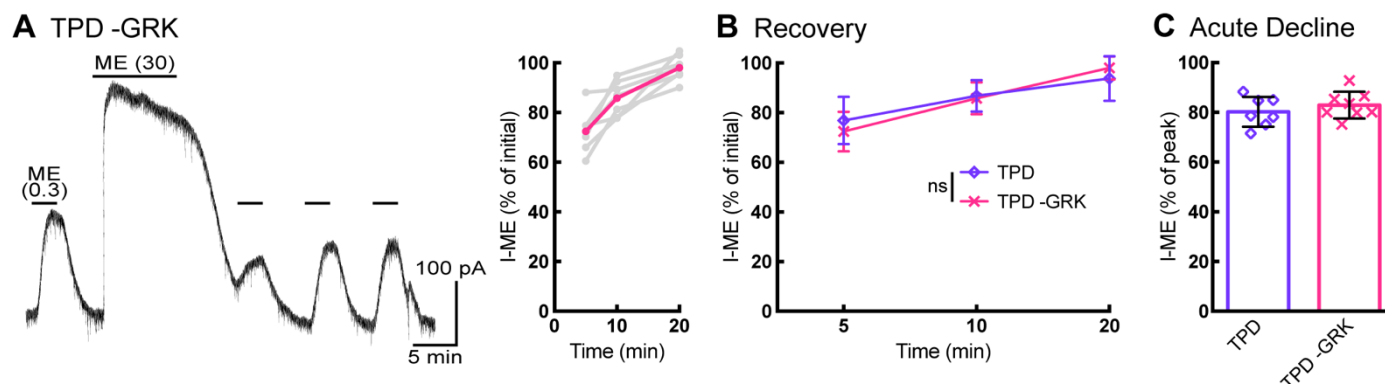
Emily R Leff, Seksiri Arttamangkul, John T Williams

Vollum Institute, Oregon Health and Science University, Portland, OR 97239

Molecular Pharmacology

Supplemental Figure 1:

Supplemental Figure 1: GRK2/3 inhibitor does not alter MOR sensitivity to ME. (A) Example trace showing currents induced by different concentrations of the MOR agonist ME (0.3, 1, and 0.1 μ M) and a saturating concentration of the α 2-adrenergic receptor agonist, UK14304 (UK, 3 μ M), which was reversed by the antagonist Idazoxan (Ida, 1 μ M). (B) Concentration-response curves for WT controls (light blue open circles, $n = 3-4$ /concentration) and WT incubated in CMP101 (30 μ M, 1 hour, salmon open squares, $n = 3-4$ /concentration). CMP101 was also included in the bath (1 μ M) and drug solutions (10 μ M). ME-induced current responses were normalized as a percentage of the current induced by UK. Curves were fit using nonlinear regression (log[agonist] vs. response – variable slope (four parameters)). There was no significant difference between the curves for any of the parameters (extra sum-of-squares F test, $P = 0.5207$). Data presented as average \pm S.D.

Supplemental Figure 2:

Supplemental Figure 2: GRK2/3 inhibitor does not further block desensitization in slices from animals expressing TPD MORs. Example trace showing the currents induced by ME (0.3 μ M) before and following application of ME (30 μ M, 10 min) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes post desensitization in slices from (A) MOR KO animals virally expressing TPD MORs in the LC and incubated in CMP101 (30 μ M, 1 hour, $n = 7$ slices, 5 animals). CMP101 was also included in the bath (1 μ M) and drug solutions (10 μ M). Grey lines indicate individual n 's and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (B) recovery from desensitization (2way ANOVA, Tukey's post hoc) and (C) acute decline (one-way ANOVA, Tukey's post hoc) for TPD controls (purple open diamonds) and TPD incubated in CMP101 (pink x's). Data presented as average \pm S.D.