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Agonist-Specific Regulation of G Protein–Coupled Receptors after Chronic Opioid Treatment

Sweta Adhikary, Omar Koita, Joseph J. Lebowitz, William T. Birdsong, and John T. Williams

Vollum Institute, Oregon Health and Science University, Portland, Oregon (S.A., O.K., J.J.L., J.T.W.) and Department of Pharmacology, University of Michigan, Ann Arbor, Michigan (W.T.B.)

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ABSTRACT

Chronic treatment of animals with morphine results in a long lasting cellular tolerance in the locus coeruleus and alters the kinase dependent desensitization of opioid and nonopioid G protein–coupled receptors (GPCRs). This study examined the development of tolerance and altered regulation of kinase activity after chronic treatment of animals with clinically relevant opioids that differ in efficacy at the μ-opioid receptors (MOR). In slices from oxycodone treated animals, no tolerance to opioids was observed when measuring the MOR induced increase in potassium conductance, but the G protein receptor kinase 2/3 blocker, compound 101, no longer inhibited desensitization of somatostatin (SST) receptors. Chronic fentanyl treatment induced a rightward shift in the concentration response to [Met^5]enkephalin, but there was no change in the kinase regulation of desensitization of the SST receptor. When total phosphorylation deficient MORs that block desensitization, internalization, and tolerance were virally expressed, chronic treatment with fentanyl resulted in the altered kinase regulation of SST receptors. The results suggest that sustained opioid receptor signaling initiates the process that results in altered kinase regulation of not only opioid receptors, but also other GPCRs. This study highlights two very distinct downstream adaptive processes that are specifically regulated by an agonist dependent mechanism.

SIGNIFICANCE STATEMENT

Persistent signaling of MORs results in altered kinase regulation of nonopioid GPCRs after chronic treatment with morphine and oxycodone. Profound tolerance develops after chronic treatment with fentanyl without affecting kinase regulation. The homeostatic change in the kinase regulation of nonopioid GPCRs could account for the systems level in vivo development of tolerance that is seen with opioid agonists, such as morphine and oxycodone, that develop more rapidly than the tolerance induced by efficacious agonists, such as fentanyl and etorphine.

Introduction

Opioids are widely used for pain management and they exert both their analgesic and rewarding effects through the activation of the μ-opioid receptors (MOR) (Matthes et al., 1996). Agonist-bound MORs undergo regulatory processes that include desensitization and internalization that are thought to be proximal to the development of cellular tolerance. Both desensitization and internalization of MORs are agonist-specific. High efficacy ligands like [Met^5]enkephalin (ME) and fentanyl cause robust desensitization and receptor internalization, whereas low efficacy agonists like morphine and oxycodone cause less desensitization and internalization but may induce counter-regulatory and homeostatic adaptations (Harris and Williams, 1991; Fiorillo and Williams, 1996; Alvarez et al., 2002; Bailey et al., 2003; Dang and Williams, 2004; Johnson et al., 2006; Virk and Williams, 2008). Consequently, long-term treatment with agonists of different efficacy could result in unique adaptations at the cellular level, leading to differences in cellular tolerance.

Cellular tolerance is defined by a loss of receptor function after long-term agonist exposure. This loss of function can result from a decrease in receptor/effector coupling, internalization, or down regulation. In locus coeruleus (LC) neurons, chronic morphine treatment results in increased desensitization of MORs and a long lasting reduction in MOR efficacy that persists even in the absence of morphine (Bailey et al., 2009a; Levitt and Williams, 2012). Additionally, chronic morphine treatment alters the kinase regulation of MORs and somatostatin (SST) receptors, both of which are class A GPCRs. In opioid naïve animals, inhibition of G protein receptor kinase 2/3 (GRK2/3) with compound 101 inhibits SST receptor desensitization; however, in chronic morphine treated animals, the concurrent inhibition of GRK2/3, protein kinase C (PKC), and c-Jun kinase (JNK) was required to prevent desensitization of SST receptors (Leff et al., 2020). These findings suggest that chronic morphine treatment alters kinase regulation of LC neurons such that additional kinases are upregulated to induce desensitization of both MORs and SST receptors. The mechanism that underlies the altered kinase regulation is not known.

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ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; CMP101, compound 101; GPCR, G protein–coupled receptor; JNK, c-Jun N-terminal kinase; LC, locus coeruleus; ME, [Met^5]enkephalin; MOR, μ-opioid receptor; OTA, oxycodone treated animals; PKC, protein kinase C; SST, somatostatin; TPD, total phosphorylation deficient.

The present study aimed to evaluate the differential effects of chronic treatment by opioids that are commonly used in clinic. Animals were treated for 6–7 days with one of several opioid agonists that had varying efficacies to investigate the role of efficacy in the development of cellular tolerance and kinase dependent regulation of desensitization. Cellular tolerance was assessed using whole cell voltage clamp recordings from LC neurons. The results show that chronic treatment with oxycodone does not induce cellular tolerance to opioids, whereas chronic treatment with fentanyl results in profound opioid tolerance. The altered regulation of the ability of kinase inhibitors to block desensitization of the SST receptor was examined with the GRK2/3 inhibitor compound 101. The results demonstrate that chronic treatment with both morphine and oxycodone, but not fentanyl or buprenorphine, altered the kinase regulation of SST receptor desensitization. Thus, not only was there an agonist selective action on the development of cellular tolerance and the induction of an altered kinase regulation of desensitization, but there was a distinct difference in the ability of agonists to affect the two processes.

Materials and Methods

Drugs. Morphine sulfate, Oxycodone, Fentanyl, and Buprenorphine were obtained from the National Institute on Drug Abuse, Neuroscience Center (Bethesda, MD). [Met]5 enkephalin (ME), bestatin, thiorphan, and idazoxan were from Sigma-Aldrich (St. Louis, MO). SST was from ProSpec (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel). MK-801 (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten5,10-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); UK14304 tartate (5-Bromo6-(2-imidazolin-2-ylamino)quinoxaline) was from Tocris (Bio-Techne Corp., Minneapolis, MN). Potassium methanesulfonate was acquired from Alfa Aesar (Ward Hill, MA). SST, ME, UK, and idazoxan were dissolved in water, diluted to the appropriate concentration in artificial cerebrospinal fluid (ACSF), and applied by superfusion. CMP101 was first dissolved DMSO (10% of final volume), sonicated, and then brought to its final volume with 20% (2-Hydroxypropyl)-b-cyclo-dextrin (Sigma-Aldrich) and sonicated again to create a 10mM solution. Slices were incubated in CMP101 (30 μM) diluted in ACSF for at least 1 hour prior to recording, and CMP101 included in the bath (1 μM) and drug solutions (10 μM).

Animals. Adult rats of both sexes were used with ages between 5 and 8 weeks for all experiments. Wild-type Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). MOR knockout Sprague-Dawley rats were 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).

Viral Injections. MOR knockout animals (P24-30) were anesthetized with isoflurane and placed in a stereotaxic frame for microinjection of adenoassociated virus type 2 (AAV2) encoding virally expressed total phosphorylation deficient MORs (TPD, AAV2- CAG-SS-GFP-MOR-TPD-WPRE-SV40pA, Arttamangkul et al., 2018; 2019) in the LC. A total of 200 nl of virus was injected at 0.1 μl/min bilaterally in the LC (anteroposterior: -9.72 mm, mediolateral: ± 1.25 mm, dorsoventral: -6.95 mm, from bregma). Both viruses were obtained from Virovek (Hayward, CA). Electrophysiology experiments were carried out at least two weeks after injection.

Chronic Opioid Treatment. Rats were treated with either morphine sulfate, oxycodone, fentanyl, or buprenorphine continuously released from osmotic pumps as described previously (Quillinan et al., 2011). To avoid overdose deaths, the treatment of animals with oxycodone and fentanyl required priming doses with intraperitoneal injections prior to implantation of the osmotic mini pumps. Twice daily doses oxycodone: 5 mg/kg or fentanyl: 0.5 mg/kg were given for 2 days before implantation of the pumps. This procedure dramatically reduced the number of overdose deaths after implantation of the pumps. Thus, by this very basic protocol it seems reasonable to conclude that at least acutely the dose of the two drugs was close to functionally equivalent.

Osmotic pumps (2ML1; Alzet, Cupertino, CA) were filled with the required concentration of each drug in water to deliver 80 mg/kg/d morphine, 30 mg/kg/d oxycodone, 1.5 mg/kg/d fentanyl, or 10 mg/kg/d buprenorphine. Each pump has a 2-ml reservoir that releases 10 μl/h for up to 7 days. The dose of each agonist was chosen to induce the maximum amount of tolerance over the relatively short duration of application. Briefly, rats were anesthetized with isoflurane, and an incision was made in the midscapular region for subcutaneous implantation of osmotic pumps. Pumps remained until animals were used for experiments 6 or 7 days later.

Tissue Preparation. Acute brain slice perpetration was performed as previously described (Levitt and Williams, 2012). Briefly, rats were deeply anesthetized using isoflurane and euthanized by cardiac percussion. Brains were removed, blocked, and mounted in a vibratome chamber (VT 1200S; Leica, Nussloch, Germany). Horizontal LC slices (260 μm) were prepared in warm (34°C) ACSF containing (in mM) 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, and 11 D-glucose with +MK-801 (10 μM). Slices were allowed to recover in warm ACSF containing +MK-801 (10 μM) for at least 30 minutes or 2 hours for treated animals and then stored in glass vials with warm (34°C) oxygenated (95% O_2/5% CO_2) ACSF until used.

Electrophysiology. Slices were hemisected and then transferred to the recording chamber, which was continuously superfused with ACSF (1.5–2 ml/min, at 34°C). Whole cell recordings from LC neurons were obtained with an Axopatch 200B amplifier (Axon Instruments) in voltage clamp mode holding potential (V_{hold} = -55 mV). Recording pipettes (World Precision Instruments, Saratosa, FL) with a resistance of 1.0–1.5 MΩ were filled with an internal solution of (mM) 115 potassium methanesulfonate or potassium methyl sulfate, 20 NaCl, 1.5 MgCl2, 5 HEPES(K), 10 BAPTA, 2 Mg-ATP, 0.2 NaGTP, pH 7.4, and 275–280 mOsM. Liquid junction potential (10 mV) was not corrected. Data were filtered at 10 kHz and collected at 20 kHz with AxographX or 400 Hz with PowerLab (Chart version 5.4.2; AD Instruments, Colorado Springs, CO). Only recordings in which the series resistance remained < 14 MΩ were included.

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 9 (GraphPad Software, version 9.0d; San Diego, CA) based on number of technical replicates (N = number of cells). Values are presented as mean ± S.D. Statistical comparisons were made using unpaired T-test, one-way or two-way ANOVA, as well as multiple comparison adjusted Tukey's post hoc tests, as appropriate. Concentration response curves were constructed using GraphPad Prism 9 and the confidence interval (CI, 95%) for each curve was determined. For all experiments, $P < 0.05$ was used to define statistical significance.

Results

Tolerance Induced by Chronic Opioid Treatment. Chronic treatment of rats with morphine has been shown to induce a 2-fold rightward shift of the concentration response curve to the full MOR agonist DAMGO (D-Ala^2-N-Me-Phe^4-$glycol^5$-enkephalin) and a decrease in the maximum current induced by the partial agonist, normorphine in locus coeruleus neurons (Christie et al., 1987). The present results aimed to determine the degree of tolerance induced by chronic treatment

with two agonists with varying efficacies. Rats were implanted with osmotic pumps that continuously released oxycodone (30 mg/kg/d) or fentanyl (1.5 mg/kg/d). Brain slices were prepared in opioid free solution and washed for 90 minutes before making whole cell recordings.

Tolerance Measured with ME. Concentration response curves were created by measuring the outward current induced by ME in brain slices obtained from untreated, oxycodone, and fentanyl treated animals. The current induced by ME was normalized to that induced by a saturating concentration of the α_2-adrenergic receptor agonist, UK14304 (3 μM). In slices from opioid naïve rats, a saturating concentration of ME caused an outward current that was 128.9 ± 19% of the current produced by UK14304 (Fig. 1, A and C). The concentration of ME that produced a half maximal outward current (EC_{50}) in slices from control was 166 nM, 95% CI = 152–297 nM, N = 3 or 4 cells per concentration from 4 male and 5 female; Fig. 1, A and C). In slices from oxycodone treated rats, the concentration response curve to ME was not different from that obtained in naive animals (EC_{50} 179 nM, 95% CI = 117–280 nM; N = 3 to 5 cells per concentration from 6 male and 8 female; Fig. 1C, two-way ANOVA, P = 1.0). In slices taken from fentanyl treated animals, however, there was a 2-fold rightward shift in ME concentration response curve (EC_{50} 518 nM, 95% CI = 496–646 nM; N = 3 to 5 cells per concentration from 6 male and 8 female; Fig. 1, B and C, two-way ANOVA, P = 0.002). The maximum current induced by ME was not different in slices from untreated, oxycodone treated, and fentanyl treated animals (125.0 ± 3.4%, 126.4 ± 4.1%, and 134.5 ± 7.1%, respectively, one-way ANOVA, P = 0.13). By this measure, the results indicated that treatment with fentanyl but not oxycodone resulted in tolerance to ME dependent activation of MORs in the LC. As previously reported for morphine treated animals, the current induced by UK14304 (3 μM) was the same among the treatment groups (untreated: 235.5 ± 99.7 pA n = 10; oxycodone treated: 221.0 ± 90.0 pA, n = 17; fentanyl treated: 198.2 ± 87.8 pA, n = 19; one-way ANOVA, P = 0.19).

Oxycodone Treatment - Tolerance Measured with Oxycodone. It is possible that tolerance to ME was not observed after oxycodone treatment due to the high efficacy of ME or a potential agonist-specific action. Thus concentration response curves to oxycodone were constructed in slices from untreated or oxycodone treated animals. The oxycodone concentration response curves were created by normalizing the peak outward current amplitude induced by oxycodone to the current induced by a saturating concentration of UK14304 (3 μM). A single concentration of oxycodone was tested in each slice. In slices from untreated animals, a saturating concentration of oxycodone caused an outward current that was 99.1 ± 3.1% of the current produced by UK14304, compared with 125.0 ± 3.4% for ME, demonstrating that oxycodone is a partial agonist in this assay (Fig. 2, A and C). The EC_{50} of oxycodone was 2.5 μM (95% CI = 1.1–5.5 μM; N = 3 to 5 cells per concentration from 4 male and 6 female; Fig. 2, A and C). In slices taken from oxycodone treated animals (OTA), the concentration response to oxycodone was unchanged (saturating current 97.1 ± 12.5% of UK14304, EC_{50} 4.5 μM, 95% CI = 1.4–16.1 μM; N = 3 or 4 cells per concentration from 3 males and 6 female; Fig. 2C, two-way ANOVA: main effect of concentration, $P < 0.01$, no effect of drug treatment, P = 0.46). Oxycodone treatment did not alter the average current induced by UK14304 (untreated: 194.6 ± 61.9, N = 6, pA, OTA: 257.9 ± 50.9 pA, N = 13 cells, unpaired t test, $P > 0.05$). Thus, chronic treatment with oxycodone did not alter the sensitivity of MORs to either a subsequent challenge with ME or oxycodone, suggesting that there was minimal cellular tolerance induced by 6–7 days of treatment with oxycodone.

In addition to long lasting cellular tolerance induced by chronic morphine treatment, there is a short lasting morphine induced desensitization found in slices from morphine treated animals that were maintained in a morphine solution (Bailey et al., 2009b; Levitt and Williams, 2012). To determine whether chronic oxycodone treatment could induce this short-term desensitization, LC brain slices were prepared and maintained in a solution containing oxycodone (1 μM). Recordings were made in the continued presence of oxycodone, and the opioid antagonist naloxone (1 μM) was applied to determine the steady state oxycodone current (N = 8 cells from 1 male and 2 female; Fig. 2, B and C). The amplitude of this oxycodone mediated current was not different from the current produced by oxycodone (1 μM) in slices from oxycodone treated animals that were prepared and maintained in drug free solution or in slices from untreated animals (OTA: Oxycodone 34.5 ± 11.3% of UK14304; OTA, wash: 34.9 ± 6.1% of UK14304;

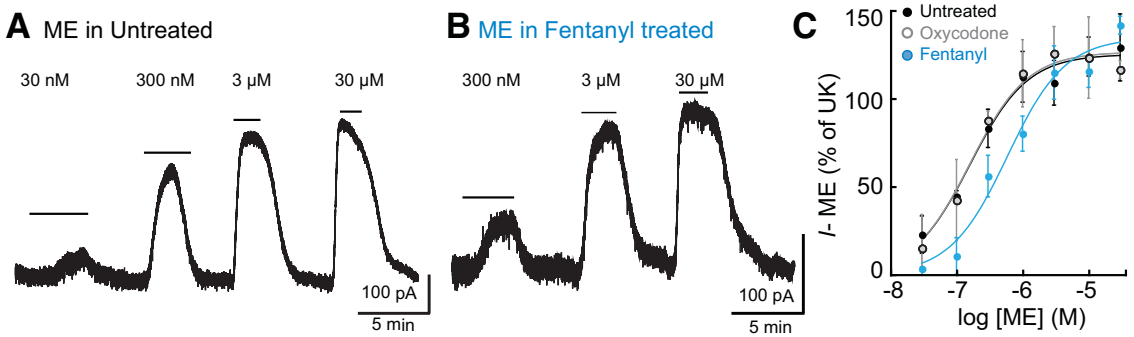

Fig. 1. Decreased sensitivity to ME after chronic fentanyl but not oxycodone treatment. (A) Representative trace showing outward currents induced by ME in a slice from an untreated animal. (B) Representative trace from a fentanyl treated animal. (C) Concentration response curve of ME normalized to UK14304 (3 μM) in slices from untreated (black circle), oxycodone treated (gray circle), and fentanyl treated animals (blue circle). In slices from untreated animals the EC_{50} was 166 nM (95% CI = 152–297 nM, N = 3, 5 cells/concentration). There was a 2-fold rightward shift in the concentration response curve in slices from fentanyl treated (EC_{50} fentanyl treated animals: 518 nM, 95% CI = 496–644 nM, N = 3, 5 cells per concentration). The concentration response in slices from oxycodone treated animals was not changed relative to untreated animals (179 nM, 95% CI = 117–280 nM, N = 3, 5 cells/concentration).

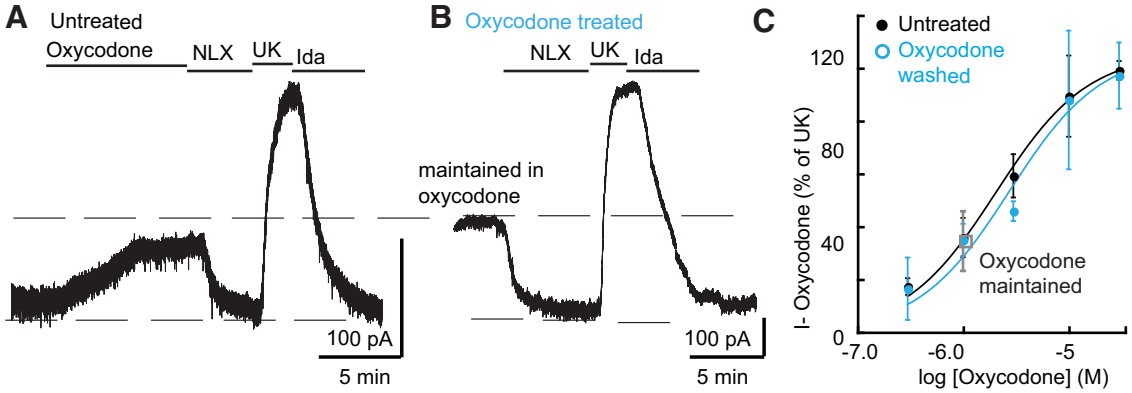

Fig. 2. Oxycodone concentration response curves. (A) Representative trace showing G protein–coupled inwardly rectifying potassium channel channel currents induced by oxycodone (1 μM) and reversal by naloxone. (1 μM), followed by UK14304 (3 μM) and reversal by idaxozan (1 μM). (B) Representative trace showing oxycodone induced current in a slice from oxycodone treated and oxycodone maintained animal. Application of naloxone (1 μM) revealed the oxycodone current, followed by UK14304 (3 μM) and reversal by idaxozan (1 μM). (C) Oxycodone concentration response curves normalized to UK14304 (3 μM) current in slices from untreated animals (black circle) and oxycodone treated animals (gray circle). There were no differences in oxycodone induced currents between untreated and oxycodone treated animals, (EC_{50} untreated: 2.5 μM, 95% CI = 1.1–5.5 μM, N = 3, 5 cells/concentration; EC_{50} oxycodone treated animals: 4.5 μM; 95% CI = 1.4–16.1 μM, N = 3, 4 cells/concentration). Additionally, summary of oxycodone induced current in slices maintained in oxycodone treatment normalized to UK14303 (3 μM; gray square). The current induced by oxycodone (1 μM) was not different between slices from untreated, oxycodone treated, and oxycodone treated maintained in oxycodone (untreated, 35.8 ± 7.4% of UK14304; oxycodone treated, 34.9 ± 6.1% OF UK14304; oxycodone treated maintained in oxycodone, 34.5 ± 11.3% of UK14304).

untreated: 35.8 ± 7.4% of UK14304; Fig. 1G, one-way ANOVA, $P > 0.05$).

Oxycodone Treatment - Tolerance Measured with Morphine. As a partial agonist, the current induced by morphine has been used as more sensitive assay to detect tolerance than higher efficacy agonists (Christie et al., 1987). Therefore, the outward current induced by morphine (1 μM) was examined in slices from animals treated with oxycodone or fentanyl. In slices from untreated animals, morphine (1 μM) induced an outward current that was 66.7 ± 14.1% of that produced by UK14304 (N = 11 cells from 3 male and 3 female; Fig. 3, A and D). In slices from oxycodone treated animals, morphine (1 μM) induced outward current was not different as in slices from untreated animals (54.7 ± 9.0%, one-way ANOVA, P = 0.16; N = 7 cells from 3 male and 2 female; Fig. 3, B and D). Thus, chronic treatment with oxycodone did not change the sensitivity of MORs to morphine. In slices from fentanyl treated animals, however, the morphine induced current was reduced to 18.9 ± 13.8% of the current induced by UK14304 (one-way ANOVA, $P < .001$; N = 10 cells from 4 male and 3 female; Fig. 3, C and D). Thus, like the rightward shift in the concentration response curve to ME after treatment with fentanyl, there was a robust decrease in the current induced by morphine.

Increased Acute ME Induced MOR Desensitization after Chronic Oxycodone Treatment. An increase in MOR desensitization after chronic morphine treatment is a measure of cellular tolerance (Quillinan et al., 2011). Therefore, experiments were designed to test whether chronic oxycodone and fentanyl treatment increased MOR desensitization. A brief application of a saturating concentration of ME (10 μM, 2 minutes) is enough to desensitize MORs measured using a subsequent application of morphine (Dang and Williams, 2005). The present experiments examined the current induced by

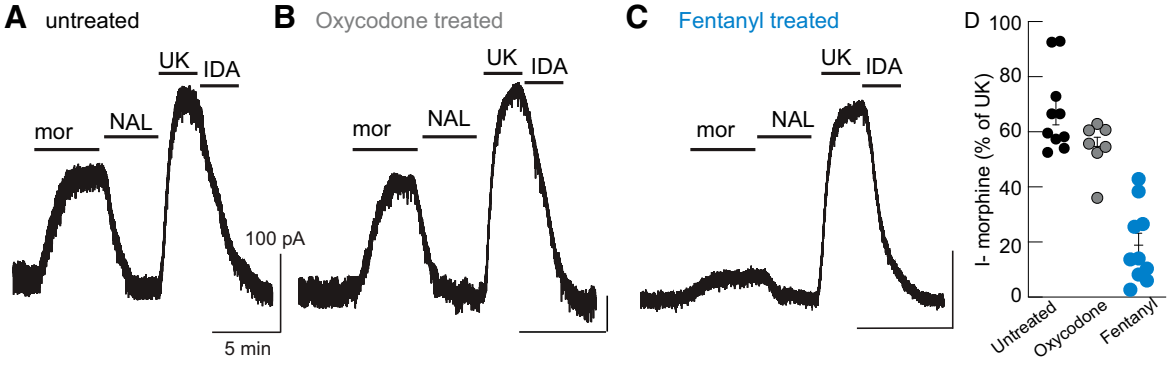

Fig. 3. Morphine induced current is decreased in slices from fentanyl but not oxycodone treated animals. (A) Representative trace showing the current induced by morphine (1 μM), reversed by naloxone (1 μM), followed by UK14303 (3 μM), reversed by idaxozan (1 μM) in a slice from an untreated animal. (B) Representative trace showing the morphine induced current in a slice from an oxycodone treated animal. (C) Representative trace showing the current induced by morphine in a slice from a fentanyl treated animal. (D) Summary data of morphine induced currents normalized to UK14303 induced currents from untreated animals (black circle, N = 11 cells from 3 male and 3 female), oxycodone treated animals (gray circle, N = 7 cells from 3 male and 2 female), and fentanyl treated animals (blue circles, N = 10 cells from 4 male and 3 female). Chronic fentanyl treatment, but not chronic oxycodone treatment, significantly reduced morphine induced current, $P < .05$, one-way ANOVA followed by a Tukey test).

oxycodone (10 μM) or morphine (10 μM) in slices from oxycodone or fentanyl treated animals, respectively, after application of saturating concentration of ME. After the application of ME (10 μM, 2 minutes), oxycodone (10 μM) or morphine (10 μM) were applied. The morphine or oxycodone induced current was normalized to that induced by UK14304 (3 μM). In slices from untreated animals, the current induced by oxycodone (10 μM) after ME application was decreased to 54.0 ± 10% of UK14304 (Fig. 4C, $N = 6$ cells from 3 male and 2 female) from 89.4 ± 15.4% without preapplication of ME (Fig. 2C, $N = 5$ cells from 2 male and 2 female). In slices from oxycodone treated animals the current after application of ME was the same as that in untreated animals (OTA: 54.0 ± 10.0% of UK14304; $n = 5$ cells from 1 male and 3 female; Fig. 4C, $P > 0.05$, unpaired t test).

In slices from fentanyl treated animals, morphine (10 μM) was applied after the application of ME (10 μM, 2 minutes). In slices from untreated animals, the current induced by morphine (10 μM) decreased to 58.5 ± 7.9% of UK14304 (Fig. 4, A and C; $N = 6$ cells from 3 male and 2 female rats) relative to the published value of 76 ± 3% (Levitt and Williams, 2012). In slices from fentanyl treated animals, after ME preapplication the morphine current decreased to 32.7 ± 5.2% of UK14304 (Fig. 4, B and C; $N = 6$ cells from 3 male and 2 female; $P < 0.05$, unpaired t test). The results indicated that chronic fentanyl treatment decreases MOR-effector coupling, unlike after chronic oxycodone treatment. The current induced by a saturating concentration of ME (10 μM) was not different in slices from untreated, oxycodone treated, and fentanyl treated animals (untreated: 120.5 ± 11.6%, $N = 6$, oxycodone treated: 132.9 ± 22.0%, $N = 5$, fentanyl treated: 119.3 ± 17.7%, $N = 6$; $P > 0.05$; one-way ANOVA Dunnett post hoc). Thus, as determined by several measures, the results indicate that the degree of tolerance induced by chronic treatment with oxycodone and fentanyl differ substantially.

Agonist-Specific Kinase Adaptations. The SST receptor activates the same potassium conductance as opioids in LC neurons (Fiorillo and Williams, 1996). The current induced by SST (10 μM, 10 minutes) peaks and declines and was taken as a measure of acute desensitization. Like MORs, SST receptors are phosphorylated by GRK2/3 (Günther et al., 2018) and inhibition of GRK2/3 with compound 101 decreased SST receptor desensitization (Leff et al., 2020). In slices taken from animals chronically treated with morphine, compound 101 alone was no longer sufficient to block acute SST induced desensitization (Leff et al., 2020). Instead, the combination of kinase inhibitors, acting at GRK2/3, PKC, and JNK were required to block SST induced desensitization. Thus, chronic morphine treatment altered the kinase dependence of GPCRs in LC neurons.

In the present study, animals were chronically treated with morphine, oxycodone, fentanyl, or buprenorphine to investigate the agonist-specific regulation of SST desensitization by kinases. In untreated animals, the current induced by SST (10 μM) declined to 32.7 ± 13.1% of the peak after 10 minutes ($N = 14$ cells from 5 male and 7 female; Fig. 5C). In slices that were incubated with compound 101 the decline was reduced to 63.7 ± 6.8% of the peak ($N = 11$ cells from 5 male and 5 female; Fig. 5C). Thus, the inhibition of GRK2/3 alone is sufficient to decrease acute SST induced desensitization in untreated animals. (Fig. 5C; $P < 0.01$, unpaired t test). As previously reported (Leff et al., 2020), in slices from morphine treated animals, the decline in the current induced by SST was insensitive to compound 101 (without compound 101: 35.8 ± 8.0% percent of peak, $N = 7$ cells from and 3 male and 3 female; in compound 101: 33.2 ± 12.2%, $N = 7$ cells from 3 male and 3 female; Fig. 5C; $P > 0.05$, unpaired t test).

As was found with the morphine treated animals, in slices from oxycodone treated animals, compound 101 alone did not block the SST induced desensitization (without compound 101: 37.07 ± 15.9%, $N = 7$ cells from 3 male and 1 female; in compound 101: 38.1 ± 15.5%, $N = 6$, 3 male and 1 female; Fig. 5, A and C, $P > 0.05$, unpaired t test). However, in slices from buprenorphine treated animals, similar to untreated animals, compound 101 inhibited SST receptor desensitization (without compound 101: 30.4 ± 15.8%, $N = 9$ cells from 3 male and 3 female; in compound 101: 64.7 ± 7.7, $N = 13$ cells in 5 male and 4 female; Fig. 5C; $P < 0.05$, unpaired t test). The same result was obtained using slices from fentanyl treated animals. Compound 101 inhibited SST receptor

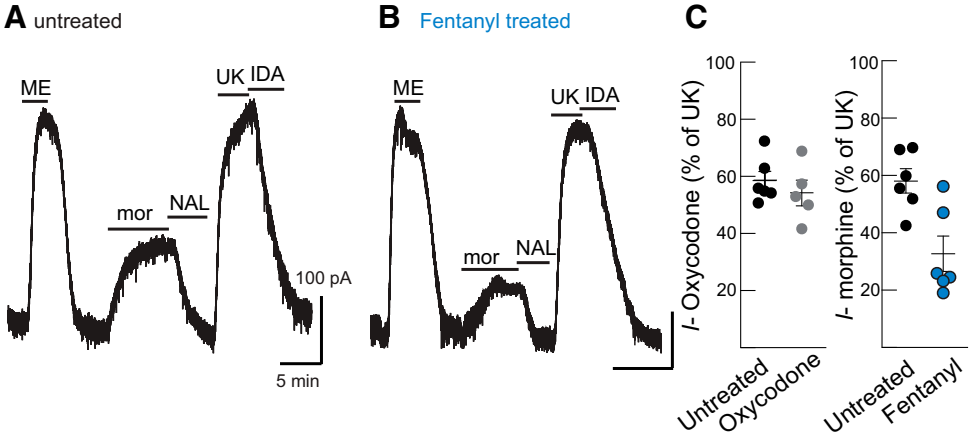

Fig. 4. Increased MOR desensitization by ME after chronic fentanyl but not oxycodone. (A) Representative trace showing the current induced by ME (10 μM; 2 minutes) followed by morphine (10 μM) and reversed by naloxone (1 μM). Morphine induced current is normalized to current induced by UK14303 (3 μM). (B) Representative trace showing the morphine induced current after ME (10 μM; 2 minutes) in a slice from a fentanyl treated animal. (C) Summary showing oxycodone induced current after ME (10 μM; 2 minutes) in slices from untreated animals (black circle, $N = 6$) and oxycodone treated animals (gray circles, $N = 5$) and fentanyl treated animals (blue circles, $N = 6$). There was no change in desensitization MORs between untreated and oxycodone treated animals ($P > 0.05$, unpaired t test), but the morphine induced current was decreased in slices from fentanyl treatment animals compared with untreated controls ($P < 0.05$, unpaired t test).

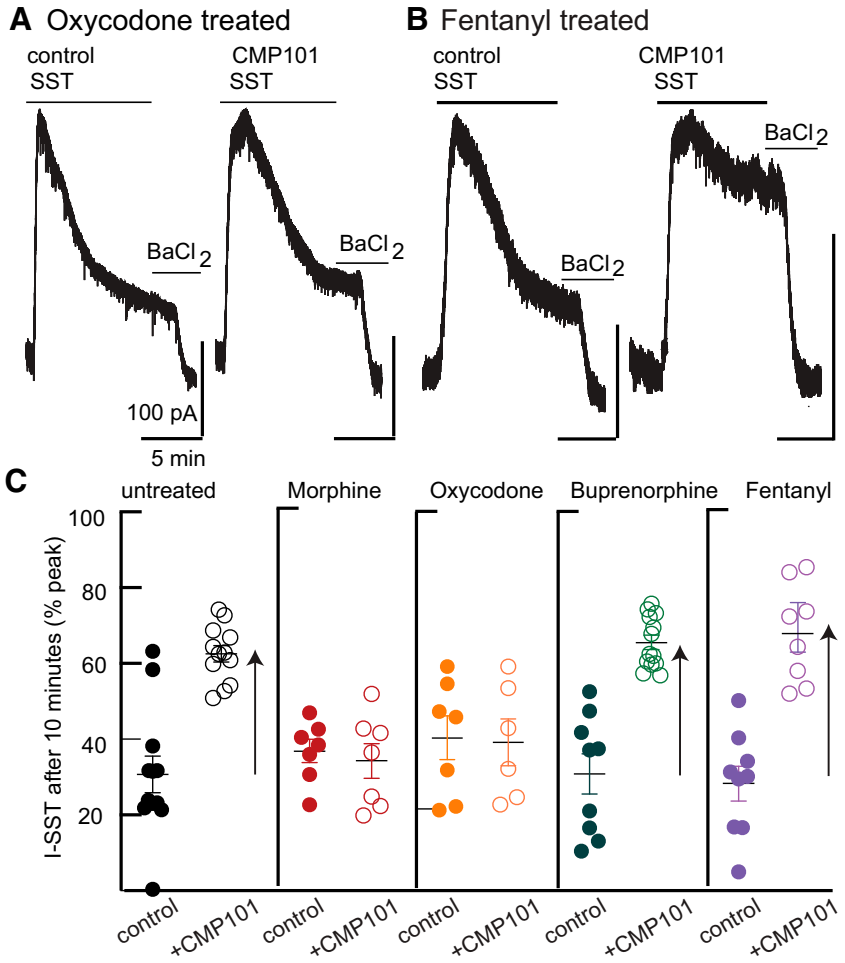

Fig. 5. Compound 101 blocks desensitization induced by SST in slices from buprenorphine and fentanyl treated animals but not after treatment with morphine or oxycodone. (A) Representative trace of the current induced by SST (10 μM; 10 minutes) in a slice from an oxycodone treated animal in control (left) and after treatment with compound 101 (right). (B) Representative trace of the SST induced current in a slice from a fentanyl treated animal in control (left) and after treatment with compound 101 (right). (C) Summary of the % decline. From the peak current induced by SST (10 μM; 10 minutes) in slices from (from left to right) untreated, morphine treated, oxycodone treated, buprenorphine treated, and fentanyl treated animals with and without compound 101. Compound 101 was effective in reducing the decline in current in slices from untreated, buprenorphine, and fentanyl treated animals but not in morphine or oxycodone treated animals.

desensitization (without compound 101: 27.5 ± 3.6%, $N = 9$ cells from 4 male and 3 female; in compound 101: 67.1 ± 13.0, $N = 8$ cells in 3 male and 6 female; Fig. 5, B and C; $P < 0.05$, unpaired t test). Therefore, the opioid induced regulation of kinase signaling in the locus coeruleus after chronic treatment with morphine and oxycodone is agonist-specific. These agonists have moderate efficacy and are inefficient at the induction of receptor internalization. However, chronic treatment with buprenorphine and fentanyl result in no change in kinase regulation in spite of the fact that the efficacy of these agonists is very different.

Sustained Signaling of MORs and GRK2/3 Dependent SST Receptor Desensitization. The opioid agonist dependent changes to LC kinase activity measured with the desensitization induced by SST could result from at least two mechanisms: 1) continued opioid receptor activation or 2) opioid receptor internalization. Current interest in agonist selective actions has centered on agonists that selectively activate G protein or arrestin dependent pathways (biased signaling, reviewed Birdsong and Williams, 2020). A number of biased agonists have been reported; however, no agonist has been found to be completely biased (Gillis, Gondin et al., 2020; Gillis, Kliewer et al., 2020). One potential way to distinguish the two pathways is through the elimination of MOR receptor phosphorylation (Kliewer et al., 2019). A receptor with alanine mutation of all 11 phosphorylation sites of the c-terminal tail of MOR, termed the total phosphorylation deficient (TPD) receptor, eliminated MOR desensitization and internalization without attenuating G-protein mediated signaling (Arttamangkul et al., 2018; Kliewer et al., 2019). To address the two potential mechanisms that induce alterations in kinase activity after chronic opioids, the phosphorylation deficient MOR (TPD-MOR) was expressed in the LC of MOR knockout rats.

First, MOR knockout animals were treated with morphine, and the desensitization induced by SST in the absence and presence of compound 101 was examined. In these experiments compound 101 was still able to inhibit SST receptor desensitization, indicating that the change in kinase regulation was dependent on the activation of MORs (without 101: 38.0 ± 10.2% percent of peak, $N = 6$ cells from and 2 male and 3 female; in compound 101: 63.0 ± 9.1%, $N = 6$ cells from 2 male and 3 female; Fig. 6).

Next, TPD-MOR expressing MOR knockout animals were treated with fentanyl, and the action of compound 101 on SST receptor desensitization was assessed. In untreated MOR knockout animals expressing the TPD-MOR, SST induced desensitization was robust and sensitive to compound 101 (without compound 101: percent peak 26.6 ± 10.8%, $N = 9$ cells from 2 male and 3 female; in compound 101: 67.0 ± 5.8%, $N = 9$ cells in 2 male and 1 female; Fig. 7, A and C; $P > 0.05$, unpaired t test). In fentanyl treated animals, however, the SST receptor desensitization was insensitive to compound 101 (without compound 101: percent peak 29.6 ± 11.4%, $N = 6$ cells from 3 male and 1 female; in compound 101:

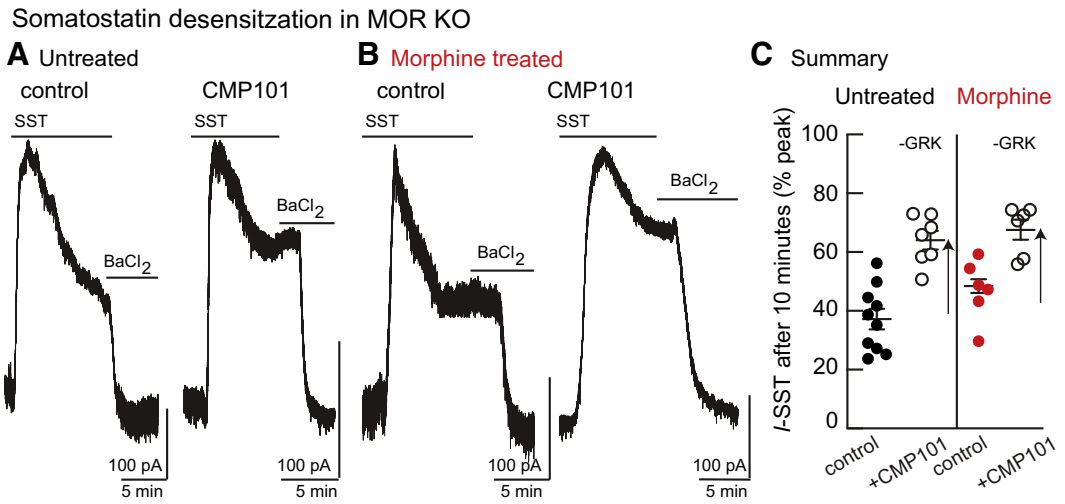

Fig. 6. Compound 101 is effective after morphine treatment in MOR knockout animals. (A) Representative trace the current induced by SST (10 μM; 10 minutes) in a slice from an untreated animal in control (left) and after treatment with compound 101 (right) in a MOR knockout animal. (B) Representative trace of the current induced by SST (10 μM; 10 minutes) in a slice from a morphine treated animal in control (left) and after treatment with compound 101 (right). (C) Summary of % decline from the peak current induced by SST (10 μM; 10 minutes) in slices from an untreated (left) and morphine treated animals. In MOR knockout animals compound 101 reduced SST induced desensitization in both untreated animals (open circles, $N = 7$) and morphine related animals (open circles, $N = 6$).

31.5 ± 11.9%, $N = 7$ cells in 2 male and 2 female; Fig. 7, B and C; $P > 0.05$, unpaired t test). This result is the same as the results obtained using wild-type animals treated with morphine or oxycodone. Thus, sustained signaling of MORs was sufficient to alter the kinase regulation that modulated SST receptor desensitization and suggests that desensitization and/or internalization may prevent these adaptations. Taken together, the results indicate that treatment with fentanyl in wild-type animals induced cellular tolerance to reduce signaling such that downstream adaptive mechanisms underlying the altered kinase regulation were not engaged.

Discussion

The present study found that chronic treatment of rats with different opioid agonists had markedly different actions that were dependent on the agonist. Both the degree of tolerance measured by MOR activation of potassium conductance and the GRK2/3 dependent desensitization of a nonopioid receptor were affected differently depending on the agonist treatment. Chronic treatment with oxycodone did not induce desensitization or measures of tolerance, but resulted in a decrease in the ability of the GRK2/3 inhibitor compound 101 to block acute desensitization of the SST receptor. Chronic treatment with fentanyl induced robust tolerance and increased MOR desensitization, but had no effect on the ability of compound 101 to block desensitization of the SST receptor. Previous work has shown that with viral expression of TPD-MORs in MOR knockout rats, acute desensitization, trafficking, and tolerance are drastically decreased (Arttamangkul et al., 2018). In this study, treatment of MOR knockout animals expressing the TPD receptor with fentanyl resulted in an inability GRK2/3 inhibition alone to regulate SST receptor desensitization. This observation is the opposite of that seen

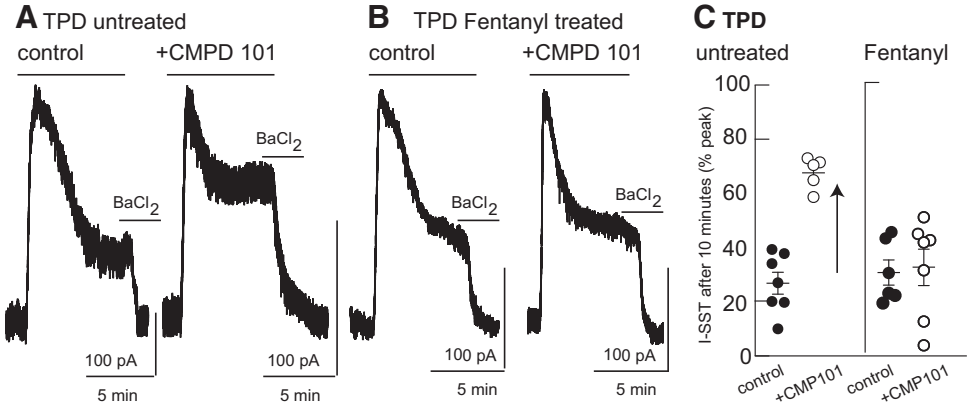

Fig. 7. Compound 101 no longer blocks the decline in SST current in animals that express the TPD-MOR. (A) Representative trace the current induced by SST (10 μM; 10 minutes) in a slice from an untreated animal in control (left) and after treatment with compound 101 (right) in a MOR knockout animal expressing TPD-MORs. (B) Representative trace of the current induced by SST (10 μM; 10 minutes) in a slice from a fentanyl treated animal in control (left) and after treatment with compound 101 (right). (C) Summary of % decline from the peak current induced by SST (10 μM; 10 minutes) in slices from an untreated (left) and fentanyl treated animals. In animals expressing the TPD-MOR, compound 101 reduced SST induced desensitization in untreated animals (open circles, $N = 5$) and compound 101 was ineffective at blocking SST induced desensitization in slices from fentanyl treated animals (open circles, $N = 7$).

in fentanyl treated wild-type animals and is the same as in animals treated with oxycodone and morphine. Taken together, the results suggest that sustained signaling from the plasma membrane is the underlying process that initiates the altered LC kinase signaling.

Agonist-Specific Regulation after Chronic Treatment. Chronic opioid treatment results in a variable amount of tolerance that is dependent on agonist efficacy, the measure of tolerance, and details of the treatment protocol (reviewed – Williams et al., 2013; reviewed – Morgan and Christie, 2011). When measuring the antinociceptive action of opioids using the tail flick assay, chronic treatment with oxycodone or etorphine administered with osmotic mini pumps induced tolerance to oxycodone more quickly than that to etorphine (Pawar et al., 2007; Madia et al., 2009). Similar results were obtained in animals treated with a variety of opioids, where morphine and oxycodone resulted in a larger shift in the dose response curve than fentanyl measured also using a tail flick assay (He et al., 2021). The conclusion was the analgesic tolerance induced by high efficacy agonists developed slower than with treatment with lower efficacy agonists. Using a paw withdrawal assay, however, chronic treatment with fentanyl induced a larger rightward shift in the dose response than morphine (Kliewer et al., 2019).

A large component of the analgesic tolerance induced by chronic opioid treatment results from homeostatic compensatory mechanisms (reviewed – Christie, 2008; reviewed – Williams et al., 2013). These compensatory mechanisms underlie acute withdrawal that is induced by treatment with naloxone. Previous studies have suggested that the compensatory mechanisms are more prevalent with the use of agonists that are less effective at the induction of desensitization or tolerance or when MOR phosphorylation is inhibited (Kliewer et al., 2019). Thus, when comparing in vivo tolerance and cellular tolerance, compensatory mechanisms likely underlie difference in measures between the two assays.

Agonist Regulation of Kinase Dependent Desensitization. The interaction between arrestin, GRK2/3, and MOR signaling has received considerable interest as one mechanism that underlies biased signaling. Phosphorylation of the receptor is the first and necessary step in the recruitment of arrestin to MOR (Gillis, Kliewer et al., 2020). The GRK/GPCR interaction is transient and dependent on agonist/receptor association, and phosphorylation of the receptor at multiple sites most likely requires multiple binding interactions between GRK and the receptor (Gurevich and Gurevich, 2019). The inability of morphine and oxycodone to recruit arrestin is certainly dependent on the lack of receptor phosphorylation induced by morphine and oxycodone (Gillis, Gondin et al., 2020). In fact, with the overexpression of GRK2, the recruitment of arrestin by all agonists is augmented (Gillis, Gondin et al., 2020). Thus, the regulation of GRK2/3 activity, along with PKC and JNK, will have a dramatic action on desensitization that is dependent on the recruitment of arrestin.

Persistent Signaling Gates Adaptive Mechanisms. Although chronic oxycodone did not induce cellular tolerance, the continued activation of the receptor resulted in a downstream alteration of kinase activity. The combination of kinase inhibitors that acted on PKC, JNK, and GRK2/3 was required to block desensitization of the SST receptor after chronic morphine treatment (Leff et al., 2020). Given that both morphine and oxycodone do not effectively recruit GRK2/3, the fact that other kinases are upregulated after chronic treatment is consistent with work indicating that morphine induces receptor phosphorylation by PKC (Bailey et al., 2004; Johnson et al., 2006; Bailey, Llorente et al., 2009; Bailey, Oldfield et al., 2009; Levitt and Williams, 2012). This modulation is surely dependent on persistent activation of MORs, as TPD-MOR after treatment with fentanyl also alters the kinase signaling in the LC. Thus, agonist efficacy alone does not account for the downstream adaptation of kinase activity.

Buprenorphine is a weak partial agonist of the MORs that induced a hyperpolarization in LC neurons (Virk et al., 2009). Buprenorphine, like morphine and oxycodone, does not recruit GRK and therefore arrestin to induce acute desensitization or receptor internalization. It appears that although the outcomes of chronic treatment with buprenorphine and fentanyl are the same, it is likely that this results from different mechanisms. The low intrinsic efficacy of buprenorphine results in a low level of receptor activation that may not effectively result in downstream regulation of kinases. In that way it may be similar to results obtained with fentanyl treated animals where the receptor signaling is compromised by the development of tolerance (He et al., 2021).

Although inhibition of GRK2/3 alone in slices from morphine treated animals was not sufficient to block desensitization of the SST receptor, the inhibition of a series of other kinases, including GRK2/3, did block desensitization (Leff et al., 2020). Thus, GRK2/3 remained active, but inhibition of it alone was ineffective in blocking desensitization after an apparent upregulation of PKC and JNK. The change in kinase regulation was dependent on an increase in the ability of other kinases that may phosphorylate MORs and SST receptors or an auxiliary protein that mediates desensitization (Yousuf et al., 2015). In our hands, inhibition of PKC had little or no effect on desensitization of MORs in untreated animals (Levitt and Williams, 2012; Arttamangkul et al., 2015); however, there was a component of short-term tolerance that was sensitive to inhibition of PKC in slices from morphine treated animals (Levitt and Williams, 2012). In addition, the inhibition of JNK resulted in a block in acute desensitization at the spinal level (Melief et al., 2010). Thus, there is evidence that multiple kinases can underlie acute MOR desensitization. However, neither PKC nor JNK alone had an effect in MOR or SST receptor desensitization in LC neurons from untreated or morphine treated animals (Leff et al., 2020). Therefore, the conclusion of the present work is that after treatment of animals with moderate efficacy opioids, multiple kinases are engaged to mediate acute desensitization of MORs and other GPCRs, and the engagement of these kinases is dependent on sustained signaling.

Summary

The present work distinguishes both receptor level regulation and a downstream adaptation induced by opioid agonists with varying efficacies after chronic treatment. Chronic oxycodone treatment did not induce cellular tolerance, unlike the profound tolerance induced by chronic treatment with fentanyl. Alternatively, chronic oxycodone treatment, but not chronic fentanyl treatment, induced a decrease in the ability of GRK2/3 inhibition to prevent acute desensitization, and this adaptation included the desensitization of the SST

receptor. Although dose equivalence of different opioid agonists used for chronic treatment was not established, the experiments with the TPD-MOR expressing neurons after chronic treatment with fentanyl indicates that the adaptive changes (measures of tolerance and altered kinase regulation) were receptor dependent rather than the dose used for treatment. When internalization was blocked using expression of the TPD-MOR and animals were treated with fentanyl, sustained signaling promoted the altered kinase regulation of SST receptors. Taken together the results suggest that: 1) agonists with different efficacy differentially regulate MOR and thus cellular tolerance, and 2) although inhibiting phosphorylation may reduce one measure of tolerance, persistent signaling induced by these receptors can lead to downstream adaptations like upregulation of PKC and JNK that promote other measures of cellular and systemic tolerance that may underlie opioid withdrawal. The consequence is that signaling at the cellular level after chronic opioid treatment affects desensitization of GPCRs beyond the opioid receptors.

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Author Contributions

Participated in research design: Adhikary, Birdsong, Williams.

Conducted experiments: Adhikary, Koita, Lebowitz, Williams.

Performed data analysis: Adhikary.

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

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Address correspondence to: John T. Williams, Oregon Health Science University, 3181 Sam Jackson Park Road, Portland, OR 97201. Email: williamj@ohsu.edu