

MOL #109603

Desensitization and tolerance of mu opioid receptors on pontine Kölliker-Fuse neurons.

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MOL #109603

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Number of:	Pages	30
	Figures	4
	References	45
Words in:	Abstract	153
	Introduction	473
	Discussion	896

Abbreviations

ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; DNQX, 6,7-dinitroquinoxaline-2,3-dione; GIRK, G protein-coupled inwardly rectifying potassium; KF, Kölliker-Fuse; LC, locus coeruleus; ME, [Met]⁵enkephalin; MTR, morphine-treated rat; NLX, naloxone; PDBu, phorbol 12,13-dibutyrate

MOL #109603

Abstract

Acute desensitization of mu opioid receptors is thought to be an initial step in the development of tolerance to opioids. Given the resistance of the respiratory system to develop tolerance, desensitization of neurons in the Kölliker-Fuse (KF), a key area in the respiratory circuit, was examined. The activation of G protein-coupled inwardly rectifying potassium (GIRK) current was measured using whole-cell voltage-clamp recordings from KF and locus coeruleus (LC) neurons contained in acute rat brain slices. A saturating concentration of the opioid agonist [Met⁵]-enkephalin (ME) caused significantly less desensitization in KF neurons compared to LC neurons. In contrast to LC, desensitization in KF neurons was not enhanced by activation of PKC or in slices from morphine-treated rats. Cellular tolerance to ME and morphine was also lacking in KF neurons from morphine-treated rats. The lack of cellular tolerance in KF neurons correlates with the relative lack of tolerance to the respiratory depressant effect of opioids.

MOL #109603

Introduction

The incidence of prescription opioid overdose has been increasing at a rapid rate in the United States (Centers for Disease Control and Prevention, 2016; Rudd *et al.*, 2016). The primary cause of death from opioid overdose is respiratory depression. The mu opioid receptor is responsible for both the analgesic and respiratory depressant effects caused by morphine (Matthes *et al.*, 1996; Dahan *et al.*, 2001). Individuals at risk of overdosing on opioids are those using large doses due to the development of tolerance to the desired effect (analgesia or euphoria), while tolerance to the respiratory depressant effect develops less readily (Ling *et al.*, 1989; Paronis and Woods, 1997; Athanasos *et al.*, 2006; Hill *et al.*, 2016; Emery *et al.*, 2016).

The development of opioid tolerance is likely a multistep process involving many pathways (Williams *et al.*, 2013). Desensitization of the opioid receptor itself is thought to be an initial step in the development of tolerance but to date there is no concrete evidence linking the two processes. Most of what is known about the process of desensitization of endogenous, somatodendritic mu opioid receptors comes from locus coeruleus (LC) neurons (Harris and Williams, 1991; Blanchet and Lüscher, 2002; Alvarez *et al.*, 2002; Bailey *et al.*, 2004; Virk and Williams, 2008; Bailey, Oldfield, *et al.*, 2009; Bailey, Llorente, *et al.*, 2009; Quillinan *et al.*, 2011; Arttamangkul *et al.*, 2012; Dang *et al.*, 2012; Williams, 2014; Lowe *et al.*, 2015). A 5 – 10 min exposure to saturating concentrations of opioid agonists leads to a reduction in the activation of G protein-coupled inwardly rectifying potassium (GIRK) conductance. Given that tolerance to respiratory depressant effects of opioids is less than to anti-nociceptive effects, mu opioid receptors on neurons of the central respiratory network may not desensitize. In the present study, this hypothesis was tested by measuring desensitization and tolerance of mu opioid receptors on Kölliker-Fuse (KF) neurons using whole-cell voltage-clamp recordings in rat brain slices.

The Kölliker-Fuse (KF) is located in the pontine pneumotaxic center and controls upper airway patency and regulates respiration, in particular the inspiratory/expiratory phase transition. Activation of mu opioid receptors in the KF

MOL #109603

leads to a reduction in respiratory rate in anesthetized animals (Prkic et al., 2012; Levitt et al., 2015) and disrupts the inspiratory/expiratory phase transition in arterially perfused preparations of rat (Levitt et al., 2015). Mu opioid receptors on KF neurons activate GIRK conductance (Levitt et al., 2015), which was used to examine acute desensitization and tolerance. Acute desensitization in KF and LC neurons were compared under identical experimental conditions. Tolerance in KF neurons was measured in brain slices from rats that were treated chronically with morphine using a dosing paradigm that produces tolerance in LC neurons (Quillinan *et al.*, 2011; Levitt and Williams, 2012). The results show that opioid-sensitive neurons in the KF displayed less desensitization and did not develop long-term tolerance.

MOL #109603

Materials and Methods

Ethical Approval

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).

Animals

Rats (n = 110 total, Sprague-Dawley, male, 4 – 7 weeks old, Charles River Laboratories) were used for all experiments. Seventeen rats were treated with morphine sulfate continuously released from osmotic pumps as described previously (Levitt and Williams, 2012). Five rats were implanted with pumps containing vehicle (water) alone. Osmotic pumps (2ML1; Alzet, Cupertino, CA) were filled with the required concentration of morphine sulfate in water to deliver 50 mg/kg/day. Each 2ML1 pump has a 2 ml reservoir that releases 10 ul/h for up to 7 days. 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. This dosing paradigm produces tolerance in LC neurons (Quillinan et al., 2011; Levitt and Williams, 2012).

Brain slice electrophysiology

Recording conditions and acute brain slice preparation was performed as described previously (Levitt and Williams, 2012; Levitt et al., 2015). Rats (Sprague-Dawley, male, 4 - 7 weeks) were killed and the brain was removed, blocked and mounted in a vibratome chamber (Leica VT 1200S). Horizontal (LC or KF) or coronal (KF) slices (240 μ m) were prepared in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 2.6 CaCl₂, 1.2 NaH₂PO₄, 11 D-glucose and 21.4 NaHCO₃ (equilibrated with 95% O₂/ 5% CO₂). Slices were stored at 32°C in glass vials with oxygenated (95% O₂/ 5% CO₂) ACSF. MK801 (10 μ M) was included in the cutting and initial incubation solution to block NMDA

MOL #109603

receptor-mediated excitotoxicity. After an incubation period of at least 30 min, slices were hemisected and transferred to a recording chamber which was perfused with 34°C ACSF (95% CO₂/5% O₂) at a rate of 1.5 – 3 ml/min.

Coronal and horizontal brain slices containing KF were used. Opioid-mediated currents and intrinsic cell properties are similar for KF neurons in coronal and horizontal slices (Levitt et al., 2015). The location of KF neurons in coronal and horizontal slices was consistent with the rat brain atlas (Paxinos and Watson, 1998). KF neurons with opioid-mediated outward current (~60 % of KF neurons) fired action potentials with slower frequency after injection of current steps (50 – 250 pA, 2 s) and had smaller amplitude AHP (Levitt et al., 2015).

Whole-cell recordings were made from LC and KF neurons with an Axopatch 1D amplifier in voltage-clamp mode ($V_{\text{hold}} = -60$ mV). Recording pipettes (1.5 – 2.2 M Ω) were filled with internal solution containing (in mM): 115 potassium methanesulfonate, 20 NaCl, 1.5 MgCl₂, 5 HEPES(K), 2 BAPTA, 2 Mg-ATP, 0.2 Na-GTP, pH 7.4, 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). Series resistance was monitored without compensation and remained < 15 M Ω for inclusion.

Drugs (including ME, morphine, naloxone, baclofen and CGP55845) were applied by bath perfusion at the indicated concentrations. Bestatin (10 μ M) and thiorphan (1 μ M) were included with ME to prevent degradation.

Drugs

ME ([Met⁵]-enkephalin acetate salt), bestatin HCl, DL-thiorphan, (\pm)-baclofen and phorbol 12,13-dibutyrate (PDBu) were from Sigma-Aldrich (St. Louis, MO). Morphine sulfate and morphine alkaloid were obtained from the National Institute on Drug Abuse Neuroscience Center (Bethesda, MD). Naloxone and MK801 were from Abcam (Cambridge, MA). CGP55845 was from Tocris (Minneapolis, MN).

Data Analysis

MOL #109603

Statistical analysis was performed using GraphPad Prism 6.0f software (La Jolla, CA). Values are presented as mean \pm SEM. Recordings were made from one neuron per slice with 1- 2 slices per animal. Data sets with $n > 8$ were tested for normality using the D'Agostino-Pearson normality test. Nonparametric statistical tests were used if one data set of a comparison failed the normality test and was not well fit by a Gaussian frequency distribution. Comparisons between two values were made using two-tailed t-test or Mann-Whitney test. Comparisons between three or more values were made with one-way ANOVA and Tukey's multiple comparisons test or Kruskal-Wallis test and Dunn's multiple comparisons test. Comparisons between two groups with two or more values per group were made using two-way ANOVA and Sidak's multiple comparisons test. Plateau values were from one phase decay nonlinear fit of data from each neuron. Statistical tests used are listed in the figure legends or in the text. Comparisons with $p < 0.05$ were considered significant.

MOL #109603

Results

Acute desensitization of opioid receptors on KF and LC neurons

Whole-cell voltage-clamp recordings were made from Kölliker-Fuse (KF) and locus coeruleus (LC) neurons in acute rat brain slices. The opioid agonist [Met⁵]-enkephalin (ME) produced an outward current in KF and LC neurons, which is mediated by activation of G protein-coupled inwardly rectifying potassium (GIRK) conductance (Torrecilla et al., 2002; Levitt et al., 2015). A saturating concentration of ME (30 μ M) induced a current that was 80 ± 6 pA (2.4 ± 0.1 pA/pF; $n = 38$) in KF neurons and 356 ± 28 pA (5.2 ± 0.6 pA/pF; $n = 14$) in LC neurons. In both LC and KF, the current produced by ME is due to activation of mu opioid receptors, rather than delta opioid receptors (Williams and North, 1984; Levitt *et al.*, 2015).

Acute desensitization of the opioid-mediated GIRK current was evaluated using two measures, which may be mediated by different processes (Birdsong *et al.*, 2015). The first measure was the decline in current that occurred during an 8-minute continuous application of a saturating concentration of ME (30 μ M). The second measure was the reduction in the current produced by a sub-saturating concentration of ME (300 nM) that was applied as a probe before and 10 minutes after washout of the saturating concentration of ME (30 μ M).

During an 8-minute application of ME (30 μ M) the current declined in both LC and KF neurons (Figure 1A,B). At the end of the application, the current had declined significantly less in KF neurons compared to LC neurons (Figure 1C; KF = 28 ± 1.4 % decline ($n = 21$); LC = 44 ± 2.7 % decline ($n = 13$); $p < 0.0001$ unpaired t-test). When the data were normalized to the peak and fit to a single exponential, the half life was less for KF (1.6 ± 0.2 min; $n = 10$) compared to LC (2.5 ± 0.3 min; $n = 11$; $p = 0.0297$ unpaired t-test), and the plateau of the decay curve was significantly less desensitized for KF neurons (65.2 ± 4.5 % of peak; $n = 10$) compared to LC neurons (46.0 ± 4.3 % of peak; $n = 11$; $p = 0.0061$ unpaired t-test). The data became significantly different 5 minutes into perfusion of ME (30 μ M) (Figure 1D; $p < 0.05$ repeated-measures two-way ANOVA and Sidak post-test). The half life and plateau values for LC was similar to previously reported values (half life = 2.3 min (95%

MOL #109603

confidence interval 2.0 – 2.7 min); plateau = 36.2 ± 1.6 % ($p = 0.2058$ unpaired t-test)) (Bailey *et al.*, 2003).

Since the amplitude of currents were smaller in KF neurons, the correlation between current amplitude and degree of desensitization was examined. The amount of decline in KF neurons was not correlated with the amplitude of current (Figure 1E; slope of linear regression (1.4 ± 1.4 pA/% decline) did not significantly deviate from zero ($p = 0.314$)). The decline was also not correlated with amplitude in LC neurons (slope of linear regression (3.8 ± 2.9 pA/% decline) did not significantly deviate from zero ($p = 0.215$)).

The second measure of desensitization was to use a sub-saturating concentration of ME (300 nM) as a probe before and 10 minutes after exposure to the saturating concentration of ME. This second measure is a sensitive assay since ME (300 nM) is on the steep part of the ME concentration-response curve. ME (300 nM) is near the EC_{50} in both LC and KF neurons (Quillinan *et al.*, 2011; Levitt *et al.*, 2015). The ratio of current produced by ME (300 nM) and ME (30 μ M) was used to approximate the sensitivity of mu opioid receptors on individual neurons. This ratio was 61 ± 3 % for LC neurons and 62 ± 3 % for KF neurons. Repeated application of ME (300 nM; 5-30 min apart) produced currents with similar amplitude in KF neurons (101 ± 3 % of the first application ($n = 18$), $p = 0.6614$ paired t-test).

In LC neurons, the current produced by ME (300 nM) was robustly reduced (32 ± 4 % of pre-desensitized; $n = 9$) when tested 10 minutes after an 8-minute application of a desensitizing concentration of ME (Figure 2A,C). In KF neurons, the current produced by ME (300 nM) was significantly less affected (68 ± 2 % of pre-desensitized; $n = 18$) 10 minutes following application of a desensitizing concentration of ME (Figure 2B,C; $p < 0.0001$ unpaired t-test).

There was no correlation between the amplitude of current (ME peak) and the reduction of ME (300 nM) probe current (% of pre-desensitized) in KF or LC (Figure 2D; KF, slope of linear regression = -0.85 ± 0.87 pA/% (not significantly different from zero, $p = 0.3428$); LC, slope of linear regression = 1.88 ± 3.01 pA/% (not significantly different from zero $p = 0.5517$)). For both LC and KF, there was no correlation between the amount of desensitization measured with prolonged

MOL #109603

application and the probe concentration, indicating that these two assays are independent measures (LC, slope of linear regression = -0.20 ± 0.19 % of probe/% decline ($p = 0.336$); KF, slope of linear regression = -0.24 ± 0.37 % of probe/% decline ($p = 0.5271$)). Thus, using two measures, in KF neurons there was less desensitization than in LC neurons.

Recovery from desensitization

To measure recovery from desensitization, the ME (300 nM) probe was applied at 10, 20 and 30 minutes following desensitization (Figure 3A). The small reduction of probe current observed in KF neurons partially recovered over the course of 30 minutes, reaching 83 ± 7 % ($n = 7$) of the initial amplitude (Figure 3C). This is unlike the LC in which the probe current recovers to 60 – 70 % of the initial amplitude after 30 minutes (Quillinan et al., 2011; Levitt and Williams, 2012).

Modulation by PKC

In LC neurons, activation of PKC enhanced the decline in current induced by a saturating concentration of ME (30 μ M) (Bailey *et al.*, 2004). To investigate this in KF neurons, PKC was activated by including the phorbol ester PDBu (100 nM) in the perfusion solution for 10 minutes prior to and throughout the experiment. PDBu caused a robust increase in spontaneous synaptic currents, which were blocked by the AMPA and GABA-A blockers DNQX (10 μ M) and gabazine (1 μ M), respectively. The decline in current during application of ME (30 μ M, 8 min) was not affected by PDBu (Figure 3B; PDBu = 32 ± 3.2 % ($n = 9$) vs. untreated = 28 ± 1.4 % ($n = 21$); $p = 0.2399$ unpaired t-test).

The current produced by ME (300 nM) was significantly reduced following perfusion of PDBu (100 nM, 10 min, 72 ± 3 % of baseline; $n = 10$; $p < 0.0001$ paired t-test). However, the ratio of the current produced by ME (300 nM) relative to the saturating concentration of ME (30 μ M) was unchanged by PDBu treatment (untreated = 61.5 ± 2.9 % ($n = 20$); PDBu = 59 ± 4 % ($n = 12$); $p = 0.5506$ unpaired t-test). This indicates that the reduction in current produced by PDBu is downstream of activation of the opioid receptor by agonist.

MOL #109603

The desensitization protocol was then performed with PDBu (100 nM) included in the perfusion solution. Desensitization measured at 10 minutes using ME (300 nM) as a probe was unaffected by PDBu treatment ($67 \pm 3\%$ ($n = 6$) of pre-desensitized, compared to $68 \pm 2\%$ ($n = 18$) of pre-desensitized in untreated slices; Figure 3C; $p = 0.7728$ unpaired t-test). Recovery from this small amount of desensitization over 30 minutes was not different from control (Figure 3C; $n = 4$; two-way ANOVA: $F_{(1,43)} = 0.00126$, $p = 0.9719$).

Modulation by chronic morphine treatment

Decline of the current produced by a saturating concentration of ME (30 μ M) in LC neurons is enhanced in slices from morphine-treated rats (Dang and Williams, 2004; Quillinan *et al.*, 2011). To determine if decline was enhanced in KF neurons, rats were treated with morphine (50 mg/kg/day) continuously released from osmotic pumps for 6 – 7 days. Brain slices from morphine-treated rats were incubated in morphine-free solution for at least 2 hours to allow morphine to wash from the slice (Levitt and Williams, 2012). The decline in current produced by the saturating concentration of ME (30 μ M) was not different from untreated rats (Figure 3B; morphine-treated = $28 \pm 5\%$ ($n = 7$) vs. untreated = $28 \pm 1\%$ ($n = 21$); $p = 0.9442$ unpaired t-test). These results were unlike what has been reported in the LC where the decline was greater after chronic morphine treatment (control = $35 \pm 2\%$ decline; morphine-treated = $47 \pm 2\%$ decline) (Quillinan *et al.*, 2011).

The recovery from desensitization is significantly attenuated in LC neurons in slices from morphine-treated rats (Dang and Williams, 2004; Quillinan *et al.*, 2011). The recovery from desensitization in KF neurons was measured in slices from morphine-treated rats using ME (300 nM) as a probe before and 10, 20 and 30 minutes after an 8-minute application of a saturating concentration of ME (30 μ M). The current produced by ME (300 nM) at 10 minutes was $74 \pm 5\%$ ($n = 9$) of the pre-desensitized current, which was not different from experiments from untreated rats (Figure 3C; untreated rats = $68 \pm 2\%$ of pre-desensitized ($n = 18$); $p = 0.2740$ unpaired t-test). The current produced by ME (300 nM) recovered over the course of 30 minutes similar to experiments from untreated rats (Figure 3C; $n = 3-5$; two-

MOL #109603

way ANOVA: $F_{(1, 46)} = 0.0056$, $p = 0.9405$). Thus unlike LC, desensitization of opioid receptors on KF neurons was not enhanced in slices from morphine-treated rats.

Cellular tolerance

Chronic morphine treatment leads to the development of cellular tolerance in LC neurons (Christie *et al.*, 1987; Levitt and Williams, 2012). Cellular tolerance in KF neurons was investigated in slices from naïve, vehicle or morphine-treated rats (50 mg/kg/day for 6-7 days) that had been incubated for at least 2 hours in morphine-free solution. A saturating concentration of ME (30 μ M) produced the same amplitude currents in slices from naïve and morphine-treated rats (naïve = 80 ± 6 pA ($n = 38$); morphine-treated = 80 ± 12 pA ($n = 13$); $p = 0.8854$ Mann-Whitney test). The percentage of neurons with opioid-mediated currents was similar in slices from vehicle and morphine-treated rats (vehicle-treated 12/18 neurons = 67%; morphine-treated 23/36 neurons = 64 %).

In opioid-sensitive KF neurons, bath perfusion of the GABA-B receptor agonist baclofen (30 μ M) also produced an outward current (Figure 4A). The amplitude of baclofen-mediated currents was not significantly different in slices from naïve, vehicle-treated or morphine-treated rats (naïve = 69 ± 7 pA ($n = 35$); vehicle-treated = 78 ± 14 ($n = 9$); morphine-treated = 73 ± 8 pA ($n = 16$); $p > 0.05$ Kruskal-Wallis test, Dunn's multiple comparisons). In slices from naïve rats, the amplitude of the baclofen-mediated current was positively correlated with the amplitude of current produced by a sub-saturating concentration of ME (300 nM) (Figure 4B; linear regression: $r^2 = 0.77$; $n = 35$). Therefore, the ME-mediated current was normalized to the baclofen-mediated current for comparisons between cells. The current produced by ME (300 nM) was not different in morphine-treated rats compared to naïve rats or vehicle-treated rats (Figure 4C; naïve = 78 ± 4 % of baclofen ($n = 31$); vehicle-treated = 78 ± 8 % of baclofen ($n = 7$); morphine-treated = 78 ± 6 % of baclofen ($n = 16$); $p > 0.05$ one-way ANOVA and Tukey's post-test).

Morphine is a partial agonist in KF neurons (Levitt *et al.*, 2015) and was used as a sensitive measure to determine the extent of cellular tolerance. The current produced by bath perfusion of a sub-saturating concentration of morphine (1 μ M)

MOL #109603

was similar in slices from naïve, vehicle-treated and morphine-treated rats (Figure 4C; naïve= 62 ± 8 % of baclofen (n = 7); vehicle-treated = 76 ± 4 % of baclofen (n = 8); morphine-treated = 72 ± 5 % of baclofen (n = 9); $p > 0.05$ one-way ANOVA and Tukey's post-test). Thus, using both ME and morphine as an indicator, there was no evidence of opioid receptor tolerance on KF neurons.

MOL #109603

Discussion

The main finding of this study was that compared to LC neurons, prolonged activation of mu opioid receptors on KF neurons resulted in less desensitization. Manipulations that have been shown to enhance desensitization in LC neurons failed to alter desensitization in KF neurons. Desensitization and long-lasting cellular tolerance can be distinguished and are likely mediated by different mechanisms (Levitt and Williams, 2012). Cellular tolerance was not evident in KF neurons from morphine-treated rats. The lack of tolerance occurred even when using a sub-saturating concentration of morphine, which is a sensitive assay for tolerance.

Somatodendritic mu opioid receptors on neurons in several areas, including LC (Harris and Williams, 1991; Blanchet and Lüscher, 2002; Alvarez *et al.*, 2002; Bailey *et al.*, 2004; Virk and Williams, 2008; Bailey, Oldfield, *et al.*, 2009; Bailey, Llorente, *et al.*, 2009; Quillinan *et al.*, 2011; Arttamangkul *et al.*, 2012; Dang *et al.*, 2012; Williams, 2014; Lowe *et al.*, 2015), periaqueductal gray (PAG) (Ingram *et al.*, 2007), hypothalamic proopiomelanocortin (POMC) (Pennock *et al.*, 2012) and ventral tegmental area (VTA) (Lowe and Bailey, 2015) desensitize to various degrees. Desensitization of somatodendritic opioid receptors on KF neurons was less than LC neurons (directly compared here), and less than previously reported for LC, POMC neurons or VTA dopamine neurons (Bailey *et al.*, 2003; Pennock *et al.*, 2012; Lowe and Bailey, 2015). The results in the KF are more similar to the lack of desensitization of presynaptic opioid receptors in LC, PAG, POMC and VTA (Blanchet and Lüscher, 2002; Fyfe *et al.*, 2010; Pennock *et al.*, 2012; Lowe and Bailey, 2015). The mechanism underlying the differential regulation of desensitization of pre- and post-synaptic receptors is unknown. The KF could serve as a tool to identify kinases or other signaling molecules that mediate acute opioid receptor desensitization. The additional lack of cellular tolerance in KF indicates either a lack of other factors that mediate cellular tolerance or that desensitization necessarily precedes cellular tolerance.

Mechanisms of desensitization

MOL #109603

The precise mechanisms that cause opioid receptor desensitization are not completely known, although many have been proposed (Williams *et al.*, 2013). Evidence suggests that phosphorylation of the C-terminal tail of the mu opioid receptor is necessary for desensitization (Birdsong *et al.*, 2015; Yousuf *et al.*, 2015). However, the kinase(s) responsible are not known. Leading candidates are GRK2 and 3 (Lowe *et al.*, 2015). The enhancement of desensitization observed in LC neurons after morphine-treatment was absent in mice lacking beta-arrestin 2 or with inactive GRK2 (Quillinan *et al.*, 2011). The lack of this morphine-induced enhancement of desensitization in KF neurons indicates that KF neurons may have reduced levels of either beta-arrestin 2 and/or GRK2/3.

Activation of PKC enhances desensitization of the opioid-mediated current, but this may be independent of phosphorylation of the receptor itself (Arttamangkul *et al.*, 2015; Yousuf *et al.*, 2015). In the LC, the enhancement of opioid-mediated desensitization appears to be specifically due to PKC α (Bailey, Oldfield, *et al.*, 2009; Bailey, Llorente, *et al.*, 2009). PKC can also reduce GIRK activity directly (Stevens *et al.*, 1999; Mao *et al.*, 2004; Adney *et al.*, 2015). The KF highly expresses PKC γ (Lein *et al.*, 2007), which could mediate the heterologous reduction in GIRK current after PDBu, but does not enhance opioid-mediated desensitization. PKC-dependent tolerance may occur in other places in the respiratory network, since tolerance to morphine-induced respiratory depression can be reversed by PKC inhibition in awake mice (Withey *et al.*, 2017).

Amplitude of current does not explain resistance to desensitization

The difference in desensitization between LC and KF neurons is not likely due to differences in the amplitude of GIRK current produced by ME. There was no correlation between amplitude of current and amount of desensitization in KF or LC neurons (Figure 1E, 2D), similar to previous reports in LC neurons (Virk *et al.*, 2009). In the LC, when opioid currents were artificially reduced using an irreversible opioid antagonist, then smaller amplitude currents were correlated with greater (rather than smaller) apparent amounts of desensitization (Virk *et al.*,

MOL #109603

2009). Finally, the KF has a similar level of receptor reserve to LC based on ME 300 nM/ME 30 μ M ratio and the observation that morphine is a partial agonist (Levitt et al., 2015).

Physiological relevance

Circulating concentrations of morphine following systemic treatment varies widely. In a report of cancer patients receiving continuous infusion of morphine sulfate, steady state plasma morphine concentrations were 267 nM – 105 μ M (n = 8; median = 479 nM) (Portenoy *et al.*, 1991). In a report of heroin overdoses, total morphine levels ranged from 35 nM – 17 μ M (n = 234), with significant overlap between fatal and non-fatal cases (Meissner et al., 2002).

The chronic morphine treatment strategy used in this study creates a circulating morphine concentration of 1 μ M (Quillinan et al., 2011) and was sufficient to induce desensitization and tolerance of LC neurons (Levitt and Williams, 2012). This concentration is sub-maximal, but above the EC₅₀ for morphine to activate GIRK currents in KF neurons (EC₅₀ = 0.4 μ M; Levitt et al., 2015). In KF neurons from morphine-treated rats, there was no tolerance at this clinically relevant concentration of morphine (1 μ M). Thus, KF neurons would remain sensitive to morphine during dose escalation and could contribute to the reduced (but not absent) development of tolerance to the life-threatening respiratory depressant effects of opioids. Whether opioid receptors on neurons in respiratory nuclei distinct from KF develop tolerance remains to be determined.

MOL #109603

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Author contributions:

Participated in research design: Levitt and Williams

Conducted experiments: Levitt

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Wrote or contributed to the writing of the manuscript: Levitt and Williams

MOL #109603

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MOL #109603

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MOL #109603

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MOL #109603

Footnotes

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MOL #109603

Legends for Figures

Figure 1. Decline in peak ME current was less in KF neurons.

Whole-cell voltage-clamp recording from LC (A) or KF (B) neuron in brain slice.

Opioid agonist ME (30 μ M) was applied by bath perfusion. C, Summary of decline in ME (30 μ M)-mediated current measured at the end of 7-9 min of ME perfusion. Line and error are mean \pm SEM. Scatter are individual data points (one neuron per slice). *** p < 0.0001 unpaired t-test.

D, Summary of ME (30 μ M)-mediated current each minute normalized to the peak in LC and KF neurons. KF declined less (repeated-measures two-way ANOVA: interaction $F_{(8,152)} = 7.020$, $p < 0.0001$; treatment $F_{(1,19)} = 7.532$, $p = 0.0129$; * p < 0.05, ** p < 0.01, *** p < 0.0001 Sidak post-test; $n = 11$ (LC) and 10 (KF)). Points and error are mean \pm SEM. Data were fit to a single exponential.

E, In LC and KF neurons, correlation between the amount of decline (%) and peak ME (30 μ M)-mediated current (pA) was not significant (ns; LC, slope of linear regression = 3.8 ± 2.9 pA/% decline, $p = 0.215$; KF, slope of linear regression = 1.4 ± 1.4 pA/% decline, $p = 0.314$). Each data point is from a single cell in a separate slice.

Figure 2. Desensitization of ME (300 nM)-mediated current was less in KF neurons.

Whole-cell voltage-clamp recording from LC (A) or KF (B) neuron in brain slice. ME (0.3 μ M) was bath perfused before and 10 minutes following application of ME (30 μ M, 7-9 min). C, Summary of the current produced by ME (0.3 μ M) after desensitization normalized to the current produced by ME (0.3 μ M) before desensitization. Line and error are mean \pm SEM. Scatter are individual data points (one neuron per slice). *** p < 0.0001 unpaired t-test.

D, Correlation between the reduction of ME (0.3 μ M) current (% of pre-desensitized) and peak ME (30 μ M)-mediated current (pA) was not significant (ns; slope of linear regression = -0.85 ± 0.87 pA/% prepulse, $p = 0.3428$). Each data point is from a single cell in a separate slice.

Figure 3. Manipulations that enhance desensitization fail in KF.

A, Whole-cell voltage-clamp recording from a KF neuron in control brain slice. ME

MOL #109603

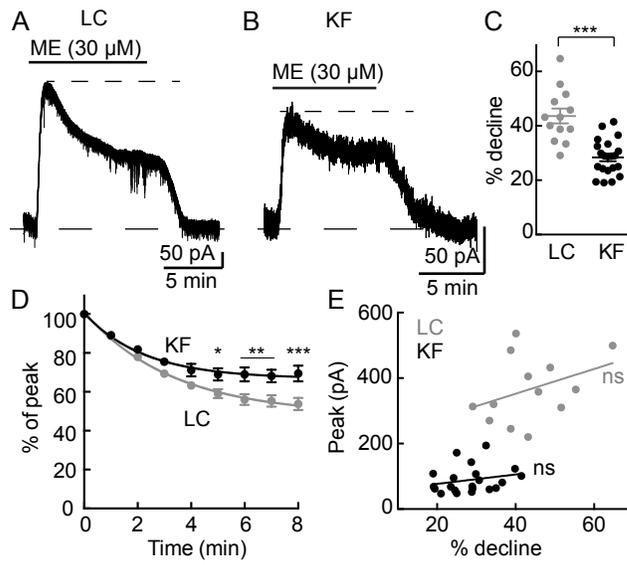
(0.3 μ M) was perfused before and 10, 20 and 30 minutes following application of ME (30 μ M, 7-9 min). Experiments using untreated slices from naïve rats (control) were compared to slices from naïve rats treated with PKC activator PDBu (100 nM) or to slices from morphine (50 mg/kg/day, 6-7 days)-treated rats (MTR). B, Summary of the decline of the ME (30 μ M)-mediated current in KF neurons. Control data are repeated from Figure 1C. Decline was not different from control for either manipulation ($p > 0.05$ one-way ANOVA and Tukey post-test). Line and error are mean \pm SEM. Scatter are individual data points (one neuron per slice). C, Recovery of ME (0.3 μ M)-mediated currents in control slices, PDBu-treated slices and slices from morphine-treated rats (MTR) was not different (two-way ANOVA: PDBu vs control, $F_{(1,43)} = 0.001$, $p = 0.9719$; morphine-treated rat vs control, $F_{(1,46)} = 0.006$, $p = 0.9405$). Data are mean \pm SEM ($n = 3-17$).

Figure 4. Lack of cellular tolerance in KF neurons.

A, Whole-cell voltage-clamp recording from a KF neuron in a slice from a morphine (50 mg/kg/day, 6-7 days)-treated rat. Opioid agonists ME (0.3 μ M) and morphine (1 μ M), and GABA-B agonist baclofen (30 μ M) produced outward currents. Agonists that wash slowly were reversed by antagonists naloxone (NLX, 1 μ M) and CGP55845 (300 nM). B, Correlation of the amplitude of current produced by baclofen (30 μ M) and ME (0.3 μ M) in neurons from naïve rats. $R^2 = 0.77$ by linear regression. C, ME (0.3 μ M) and morphine (1 μ M)-mediated currents were normalized to the amplitude of current produced by baclofen (30 μ M). The amplitude of current was not different between vehicle (Veh)- and morphine-treated rats (MTR) for morphine (1 μ M; $p = 0.5177$ Mann-Whitney test) or ME (0.3 μ M; $p = 0.9740$ Mann-Whitney test). Line and error are mean \pm SEM. Scatter are individual data points (one neuron per slice).

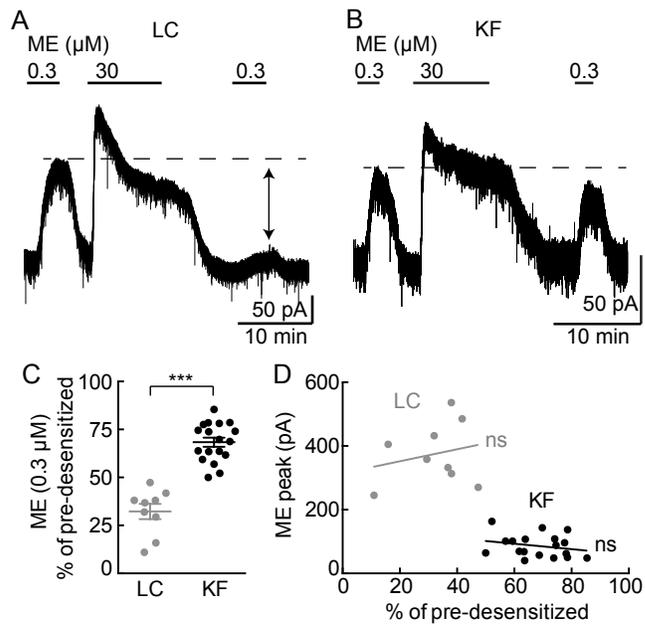
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Figure 1



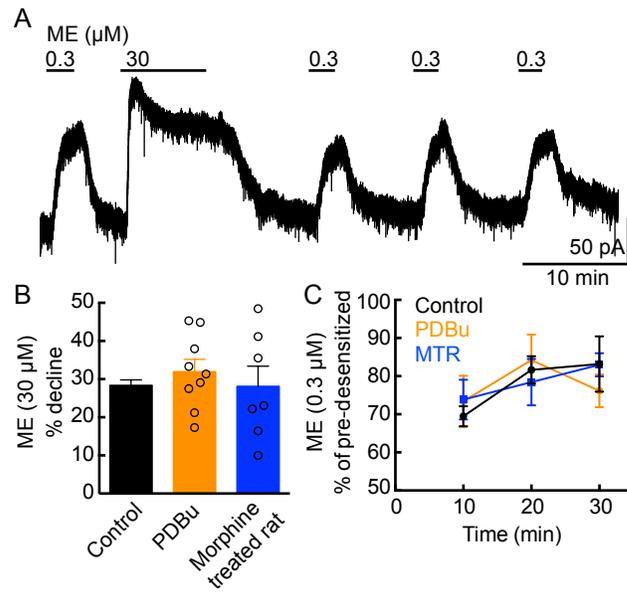
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Figure 2



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Figure 3



MOL #109603

Figure 4

