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Prolonged Stimulation of μ-Opioid Receptors Produces β-Arrestin-2-Mediated Heterologous Desensitization of α_2-Adrenoceptor Function in Locus Ceruleus Neurons

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ABSTRACT

Prolonged agonist stimulation of the μ-opioid receptor (MOR) initiates receptor regulatory events that rapidly attenuate receptor-mediated signaling (homologous desensitization). Emerging evidence suggests that persistent MOR stimulation can also reduce responsiveness of effectors to other G-protein-coupled receptors, termed heterologous desensitization. However, the mechanisms by which heterologous desensitization is triggered by MOR stimulation are unclear. This study used whole-cell patch-clamp recordings of ligand activated G-protein-activated inwardly rectifying potassium channel currents in mouse brain slices containing locus ceruleus (LC) neurons to determine the effects of prolonged stimulation of MOR on α_2-adrenoceptor (α_2-AR) function. The results show distinct and sequential development of homologous and heterologous desensitization during persistent stimulation of MOR in LC neurons with Met^5-enkephalin (ME). ME stimulation of MOR promoted rapid homologous desensitization that reached a steady state after 5 min and partially recovered over 30 min. Longer stimulation of MOR (10 min) induced heterologous desensitization of α_2-AR function that exhibited slower recovery than homologous desensitization. Heterologous (but not homologous) desensitization required β-arrestin-2 (βarr-2) because it was nearly abolished in βarr-2-knockout (ko) mice. Heterologous (but not homologous) desensitization was also prevented by inhibition of ERK1/2 and c-Src signaling in wild-type (wt) mouse LC neurons. Heterologous desensitization may be physiologically relevant during exposure to high doses of opioids because α_2-AR-mediated slow inhibitory postsynaptic currents were depressed in wt but not βarr-2 ko LC neurons after prolonged exposure to opioids. Together, these findings demonstrate a novel mechanism by which βarr-2 can regulate postsynaptic responsiveness to neurotransmitter release.

Introduction

Opioid drugs such as morphine are effective analgesics, but their use is limited because of the propensity to induce tolerance and dependence (Williams et al., 2001). Most clinically used opioids mediate their analgesic and rewarding affects via activation of the MOR (Kieffer and Gavériaux-Ruff, 2002). Sustained agonist stimulation of MOR also initiates receptor regulatory events that rapidly attenuate receptor-mediated signaling, known as homologous desensitization, and these mechanisms may be involved in opioid tolerance (Dang and Christie 2012). The process of acute MOR desensitization is regulated by multiple mechanisms that may include phosphorylation by G-protein-coupled receptor kinases (GRKs) and binding of β-arrestins, as well as ERK1/2 and protein kinase C activation and possibly other mechanisms (Zhang et al., 1998; Bohn et al., 2000; Li and Wang, 2001; Dang et al., 2011; Groer et al., 2011). In brief, MOR phosphorylation by GRK increases its affinity for β-arrestins, which contributes to homologous desensitization and initiates receptor sequestration and endocytosis. Many studies have reported that rapid desensitization of MOR in neurons is largely homologous because activation of other GPCRs coupled to the same effector mechanisms remains largely unaffected (e.g., Harris and Williams, 1991; Connor et al., 1996; Fiorillo and Williams, 1996; Bailey et al., 2004, 2009a,b; Dang et al., 2009, 2011). However, other evidence

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ABBREVIATIONS: MOR, μ-opioid receptor; GRK, G-protein-coupled receptor kinases; ERK1/2, extracellular signal regulated kinases 1 and 2; GIRK, G protein-activated inwardly rectifying potassium channel; α_2-AR, α_2-adrenergic receptor; GPCR, G protein-coupled receptor; ME, Met^5-enkephalin; LC, locus ceruleus; βarr-2, β-arrestin-2; c-Src, cellular Src; NA, noradrenaline; wt, wild type; MES, 2-(morpholino)-ethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; sIPSC, slow inhibitory postsynaptic current; MK801, dizocilpine maleate; UK14304, brimonidine tartrate; PP2, 4-amino-5-(4-chlorophenyl)-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PD98059, 2′-amino-3′-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; ko, knockout; DYNi, dynamin inhibitory peptide.

from neurons and cultured cells suggests that persistent MOR stimulation can also cause heterologous desensitization, reducing cellular responsiveness to subsequent agonist stimulation of α_2-ARs or other GPCRs (Blanchet and Lüscher, 2002; Tan et al., 2003, 2009; Chu et al., 2010). These discrepancies might be attributed to differences in experimental conditions and designs including cell types investigated, species, age of animals, duration of agonist exposure, and endpoints used to measure desensitization. It is of interest that studies reporting substantial MOR-induced heterologous desensitization of other GPCRs have usually exposed cells to high concentrations of MOR agonists for periods of >15 min (e.g., Blanchet and Lüscher, 2002) to several hours (e.g., Tan et al., 2009). In contrast, studies reporting largely homologous desensitization of MOR have usually examined brief periods of MOR stimulation with ME, usually for 5 to 10 min, at which times homologous desensitization has reached steady state (Harris and Williams, 1991; Osborne and Williams, 1995; Connor et al., 1996; Fiorillo and Williams, 1996; Alvarez et al., 2002; Bailey et al., 2004). Most of these studies have tested for the presence of heterologous desensitization using supramaximal concentrations of α_2-AR or other GPCR agonists, which clearly establishes sustained sensitivity of the effector mechanism (usually GIRK channels) but could be insensitive to moderate loss of α_2-AR sensitivity (Connor et al., 2004). Although contributions of other experimental differences cannot be ruled out, a possible explanation for discrepant findings of homologous versus heterologous desensitization is that homologous desensitization is induced initially during exposure to high concentrations of ME, but this is followed by heterologous desensitization mediated by distinct mechanisms (e.g., Tan et al., 2009).

To test whether both homologous and heterologous desensitization of MOR can be detected sequentially in LC neurons, whole-cell patch-clamp recordings were made from mouse brain slices. The LC contains a relatively homogeneous population of noradrenergic neurons expressing one type of opioid receptor, the MOR, and other G_i-coupled receptors including α_2-ARs that modulate the same population of GIRK channels (North and Williams, 1985). Desensitization of MOR-activated GIRK currents in LC neurons provides a rapid, reliable assay of G-protein activation that does not involve loss of channel function because agonists at other GPCRs are still able to almost fully activate the same population of GIRK currents when MOR is desensitized in these cells (Connor et al., 2004). The results show that exposure to high concentrations of ME sequentially produces homologous desensitization of MOR followed by heterologous desensitization of α_2-ARs in LC neurons. Distinct mechanisms are responsible for each, with dependence of heterologous but not homologous desensitization on βarr-2, ERK1/2, and c-Src. It is noteworthy that MOR induction of heterologous desensitization attenuates not only G_i-mediated signaling elicited by exogenous agonist but also synaptically evoked release of noradrenaline (NA).

Materials and Methods

Electrophysiology. All experiments were approved by the Royal North Shore Hospital/University of Technology Sydney Ethics Committee, which complies with National Health and Medical Research Council of Australia guidelines. Whole-cell recordings of membrane currents were made from 167 routinely genotyped, 5- to 12-week-old βarr-2 knockout mice from Drs. Lefkowitz and Caron (Duke University) (Bohn et al., 2000) or from their wild-type (wt) littermates (C57BL6 background). LC slices were prepared as described previously (Dang et al., 2011). In brief, mice were anesthetized with isoflurane (4% in air) and decapitated; brains were removed and blocked. Horizontal Vibratome (Leica1000) sections (200–220 μm) were prepared at 4°C and then were incubated in extracellular solution (artificial cerebrospinal fluid, 60 min, 35°C). Artificial cerebrospinal fluid contained 126 mM NaCl, 2.5 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 21.4 mM $NaHCO_3$, and 10 mM glucose with 95% O_2-5% CO_2 at 37°C. Pipette (2–4 MΩ) solution contained 115 mM K-MES, 20 mM NaCl, 1.5 mM $MgCl_2$, 10 mM BAPTA, 5 mM HEPES, 4 mM Mg^+-ATP, and 0.4 Na-GTP, pH 7.3 to 7.4. Voltage-clamp recordings of visualized LC neurons (infrared Nomarski optics, holding potential −55 to −60 mV) were acquired using Axograph X (Axograph Scientific, Sydney, NSW, Australia) and filtered at 20 to 100 Hz. All drugs were applied by superfusion of the recording chamber (~2 ml/min, 35°C). Bestatin (10 μM) and thiorphan (1 μM) were included in all experiments using ME to limit degradation of the peptide (Williams et al., 1987). Cocaine (3 μM) and prazosin (1 μM) were included in all NA applications, and the superfusion solution was equilibrated with prazosin (1 μM) for at least 5 min before application of NA to eliminate actions on α_1-ARs on GIRK in LC neurons (Osborne et al., 2002). All applications of NA were prepared as fresh solutions when reapplication time exceeded 5 min to avoid oxidative decomposition.

Slow Inhibitory Postsynaptic Currents. A pair of bipolar tungsten stimulating electrodes (220 μm apart) were placed at the edge of the LC approximately 100 μm from the recorded neuron to evoke a slow inhibitory postsynaptic current (sIPSC) while holding the membrane potential at −60 mV. Using a pulse generator (Digitimer, Welwyn Garden City, UK), a train of stimuli (four pulses of 1-ms duration at 17 Hz) was adjusted between 20 and 40 V to generate sIPSCs of 20 to 50 pA in amplitude. sIPSCs were evoked every 90 s, sampled at 10 kHz, and filtered online at 5 kHz (Axograph). Four consecutive traces in each condition were averaged to determine sIPSC amplitude. The superfusion solution was equilibrated with 10 μM 6-cyano-2,3-dihydroxy-7-nitroquinoxaline, 10 μM dizocilpine maleate (MK801), 100 μM picrotoxin, and 1 μM prazosin for at least 10 min before examining synaptic currents.

Statistics and Curve Fitting. Data were analyzed using Prism (GraphPad Software, Inc., San Diego, CA). Values are presented as means ± S.E.M. Statistical comparisons were made with *t* tests (for two groups) or two-way analysis of variance with Bonferroni post hoc tests and were considered significant if $P < 0.05$. When possible, rate constants were estimated using Axograph X simple exponential fit of the decline of the ME (30 μM) current.

Reagents were from the following: $(Met)^5$-enkephalin, thiorphan, bestatin, and prazosin-HCl from Sigma-Aldrich (St. Louis, MO); cocaine HCl and morphine HCl from GlaxoSmithKline Australia (Melbourne, VIC, Australia); naloxone HCl, brimonidine tartrate (UK14304), 4-amino-5-(4-chlorophenyl)-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), 2′-amino-3′-methoxyflavone (PD98059), 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0126), and somatostatin from Tocris (Bristol, UK). The GRK2 inhibitory peptide sequence Trp643 to Ser670 (Ac-WKKELRDAVREA-QQLVQRVPKMKNKPRS-NH_2, >95% purity) (Li and Wang, 2001) was custom synthesized by AUSPEP (Parkville, VIC, Australia).

Results

Homologous MOR Desensitization Precedes Heterologous Desensitization of α_2-Adrenergic Receptors. Desensitization was defined as the loss of MOR function that develops rapidly within several minutes during sustained

application of high concentrations of agonists (Connor et al., 2004). Activation of MOR with a supramaximal concentration of the endogenous opioid, ME (10 μM, 5 min) has been widely reported to produce maximal desensitization and robust MOR endocytosis in LC neurons (Osborne and Williams, 1995; Bailey et al., 2004; Dang and Williams, 2004; Arttamangkul et al., 2006; Dang et al., 2009, 2011). The present experiments were designed to test whether the sensitivity of α_2-AR coupling to GIRK currents (rather than capacity for GIRK channel activation) is reduced after a different duration of MOR stimulation with supramaximal concentrations of ME (10–30 μM). MOR and α_2-ARs activate the same population of GIRK channels (North and Williams, 1985). As reported previously (Christie et al., 1987; Dang et al., 2009, 2011), a supramaximal concentration of the specific α_2-AR agonist, brimonidine (3 μM), produced an outward current with amplitude similar to that for a supramaximal concentration of ME (30 μM), whether it was applied immediately before (74 ± 12 pA, $n = 11$) or after (80 ± 14 pA, $n = 5$) ME (Fig. 1, A, i and ii, and C). As reported previously (Dang et al., 2009, 2011), desensitization of the response to ME was near maximal after 5 (Fig. 1, A, iii, and B) or 10 min (Fig. 1B, ii) exposure, and the response to brimonidine showed only a modest trend for desensitization after 10 min of exposure to ME (not significant, $P = 0.53$) (Fig. 1C). These findings are consistent with previous reports of largely homologous desensitization in both rat (Fiorillo and Williams, 1996; Connor et al., 1996; Bailey et al., 2004, 2009a,b) and mouse LC neurons (Dang et al., 2009).

Although these findings show that the capacity of GIRK channels to respond to GPCR activation is not diminished during desensitization of MOR for up to 10 min and clearly replicate previous work demonstrating little heterologous desensitization in LC neurons, activation of GIRK with supramaximal concentrations of full agonists such as brimonidine is not a sensitive measure of receptor desensitization (Connor et al., 2004; Dang and Williams, 2004). To examine a more sensitive measure of heterologous desensitization, LC neurons were tested with a submaximal concentration of NA (3 μM) (Fig. 1, B and C) in the presence of cocaine (30 μM) and prazosin (1 μM). NA-induced (3 μM) activation of α_2-AR elicited a GIRK current of 30 ± 4 pA ($n = 12$), approximately half of the maximal current activated by brimonidine. In LC neurons from wt mice, the NA (3 μM) elicited a GIRK current of 55 ± 5% of the maximal GIRK current subsequently produced by ME (30 μM), which is most suitable for determination of α_2-AR desensitization (Connor et al., 2004). It should be noted that the GIRK current activated by NA (3 μM) expressed as raw current or percentage of subsequent exposure to ME (30 μM) was not affected by any subsequent treatments including βarr-2 knockout, dynamin inhibition, GRK inhibition, ERK1/2 inhibition, or c-Src inhibition (one-way analysis of variance: $P = 0.50$ for raw current and $P = 0.31$ for percentage of ME current).

NA (3 μM) was therefore applied before and after MOR desensitization treatment to test for loss of α_2-AR function. The results show that a 5-min desensitization treatment with ME (30 μM) produced primarily homologous desensitization. After exposure to ME (30 μM) for 5 min, the GIRK current by NA (3 μM) was reduced by 19 ± 6% ($n = 5$; $P = 0.057$), demonstrating only a trend for heterologous desensitization. When MOR desensitization treatment with ME (30

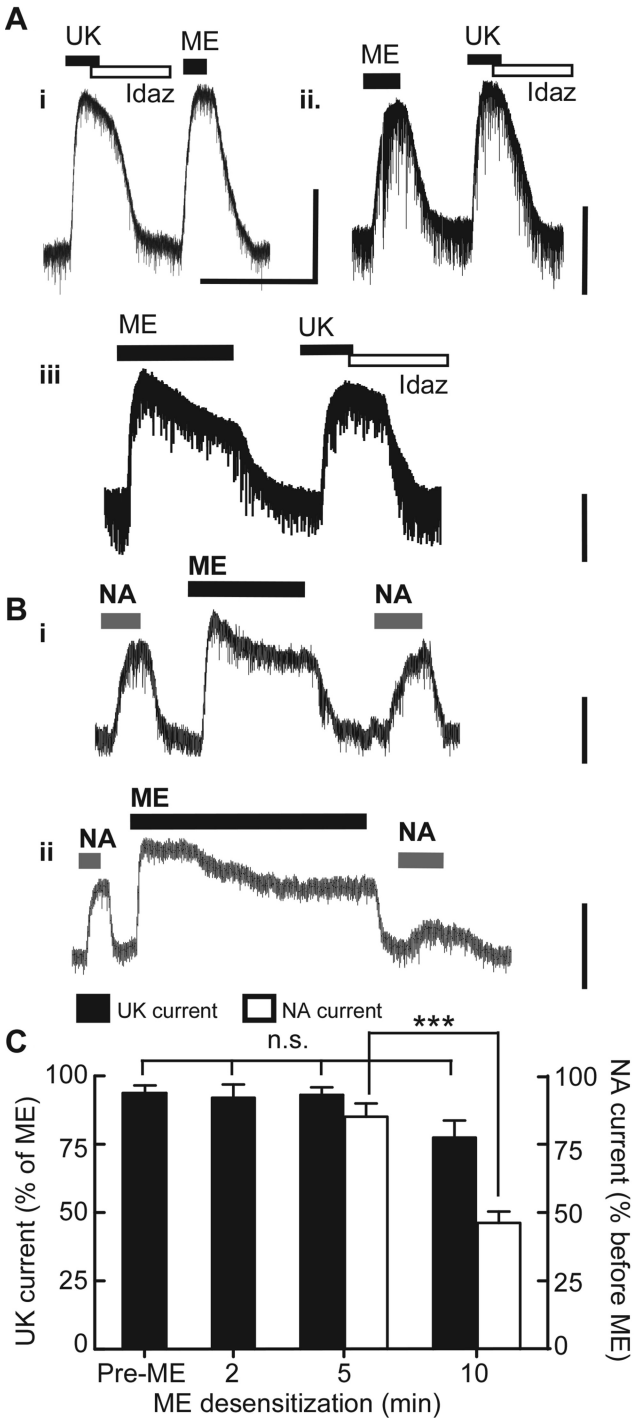

Fig. 1. Heterologous desensitization of α_2-AR requires more prolonged exposure to ME than does homologous desensitization of MOR. A, the full α_2-AR agonist, brimonidine (UK) (1 μM), reversed by idazoxan (Idaz) (3 μM), and MOR agonist ME (30 μM) produce similar outward currents, regardless of order of exposure for 1 to 2 min (i and ii) or after exposure to ME for 5 min (drug application time shown by bars) (iii). B, the outward current produced by a submaximal concentration of NA is unaffected by ME-induced desensitization of MOR for 5 min (i) but is substantially reduced after a 10-min exposure to ME (ii). C, exposure to a desensitizing concentration of ME for 2, 5, or 10 min does not significantly reduce the response to a supramaximal concentration of brimonidine but a submaximal concentration of NA (3 μM) displays significant heterologous desensitization after a 10-min exposure (4–10 cells/group). Scale bars, 5 min, 50 pA. ***, $P < 0.001$. n.s., not significant.

μM) was extended to 10 min, subsequent stimulation of α_2-AR with NA (3 μM) elicited a GIRK current that was significantly smaller than that produced by a 5-min exposure to ME (Fig. 1, B, ii, and C). Before desensitization of MOR, NA (3 μM) activation of the α_2-adrenergic receptor elicited an outward current of 46 ± 10 pA (55 ± 5% of ME current, n = 12), but after a 10-min desensitization treatment with ME (30 μM), the NA (3 μM)-activated GIRK current in the same neurons was reduced to 23 ± 6 pA (−53 ± 5%, $P < 0.001$). More prolonged exposure to ME did not further increase heterologous desensitization. These findings suggest that substantial heterologous desensitization of α_2-ARs can be observed in mouse LC neurons and that this develops more slowly than homologous desensitization.

Recovery from Heterologous Desensitization Is Slower Than That from Homologous Desensitization. Previous studies have reported that recovery from homologous MOR desensitization proceeds gradually for more than 1 h after washing out of a desensitizing concentration of ME (Dang and Williams, 2004; Dang et al., 2011). To determine the rate and extent of recovery of α_2-AR function after induction of heterologous desensitization, NA (3 μM)-activated GIRK currents were assessed 10, 20, and 30 min after a desensitization treatment with ME (30 μM for 10 min). Immediately after washing out of ME, heterologous desensitization was assessed with NA (3 μM) (t = 5 min). As shown above, the NA (3 μM)-evoked response was reduced by 58 ± 5% after ME (30 μM; 10 min) desensitization treatment (35 ± 7 pA before and 17 ± 4 pA after ME treatment; n = 5, $P <$ 0.002). Subsequent assessment of α_2-AR function with NA (3 μM) at 10, 20, and 30 min after ME desensitization treatment showed that recovery was very slow and not significant for at least 30 min after washout of ME (Fig. 2). When recovery from homologous desensitization was assessed at corresponding time points, ME (300 nM)-mediated GIRK conductance increased more rapidly and had reversed significantly by 20 min after washout of ME (Fig. 2). These results show that the recovery from homologous desensitization occurs more rapidly and is more complete than recovery from heterologous desensitization.

Heterologous Desensitization Is Dependent on β-Arrestin-2. Activation of MOR by efficacious agonists such as ME leads to rapid and robust recruitment of βarr-2 to the plasma membrane, contributing to desensitization by uncoupling MOR from G-proteins, and mediates receptor endocytosis (Cen et al., 2001; Bohn et al., 2004). We therefore explored the possibility that signaling events related to βarr-2 recruitment may mediate heterologous desensitization using βarr-2 ko mice. As reported previously (Dang et al., 2009), deletion of βarr-2 did not inhibit homologous desensitization of MOR, but it nearly abolished heterologous desensitization of α_2-ARs after a 10-min MOR desensitization treatment in LC neurons (Fig. 3). Activation of α_2-ARs with NA (3 μM) in LC neurons from ko mice before MOR desensitization treatment evoked an outward current of 40 ± 6 pA (68 ± 11% of ME current, n = 6), but after MOR desensitization, the NA (3 μM)-evoked current was 33 ± 4 pA (Fig. 3B). This reduction of receptor function was significantly smaller than that observed in LC neurons from wt mice (Fig. 3, A and E).

To confirm that βarr-2 regulation of MOR function contributes to heterologous desensitization rather than other adaptations that could be present in the germline knockout, manipulations were performed to disrupt βarr-2-dependent regulation of MOR in LC neurons from wt mice, as reported previously (Dang et al., 2009, 2011). GRK2 inhibitory peptide (100 μM) was dialyzed into neurons from wt mice to disrupt βarr-2 interaction with the MOR at the plasma membrane. This manipulation has previously been reported to disrupt βarr-2-dependent endocytosis of MOR (Zhang et al., 1998; Li and Wang, 2001; Dang et al., 2009). Disruption of GRK2 function with GRK2 inhibitory peptide had no effect on homologous desensitization but nearly abolished heterologous desensitization: NA-evoked GIRK current was 40 ± 11 pA before or 57 ± 7% of ME current and 36 ± 9 pA after ME (30 μM, 10 min) desensitization treatment, respectively, a reduction of 8 ± 5% (n = 6) (Fig. 3, C and E). The findings that heterologous desensitization in LC neurons was prevented both by deletion of βarr-2 and disruption of GRK2 function in wt mice suggests that βarr-2 recruitment to MOR contributes to the attenuation of α_2-AR function after ME (30 μM) desensitization treatment.

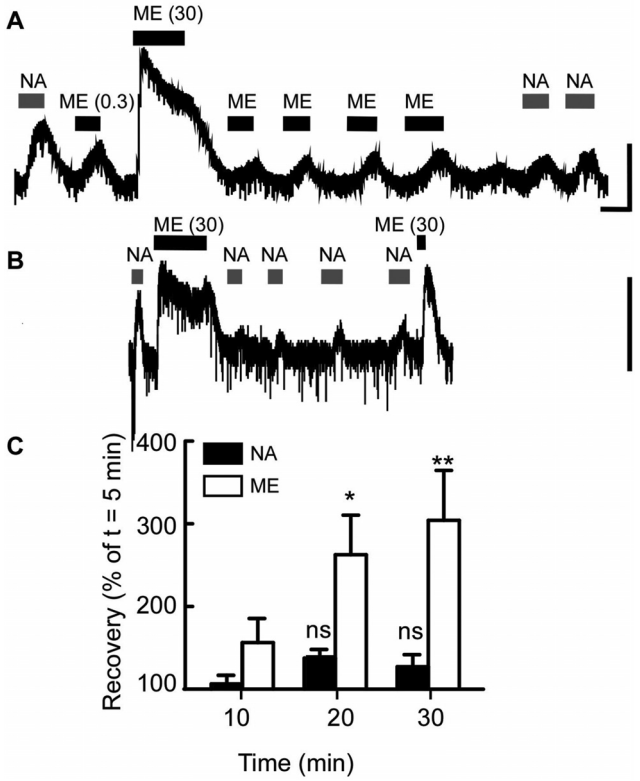

Fig. 2. Homologous desensitization recovers more rapidly than heterologous desensitization. A, recovery of the response to a submaximal probe concentration of ME (300 nM) develops slowly but consistently after a 10-min exposure to ME, but the response to NA (3 μM) remains depressed more than 40 min after washout of ME. B, the response to a probe concentration of NA shows little recovery during repeated application for 30 min after exposure to a desensitizing concentration of ME for 10 min. C, response to a submaximal probe concentration of ME shows significant recovery 20 and 30 min after ME-induced desensitization for 10 min, but the response to NA does not (n = 4–8 cells/group). Scale bars, 5 min, 50 pA. *, $P < 0.05$; **, $P < 0.01$. ns, not significant.

Heterologous Desensitization Does Not Require Dynamin-Dependent Endocytosis. Tan et al. (2009) reported that cointernalization of MOR and α_2-ARs, when stimulated by either agonist, mediates heterologous desensitization in cultured sensory neurons. To determine whether or not

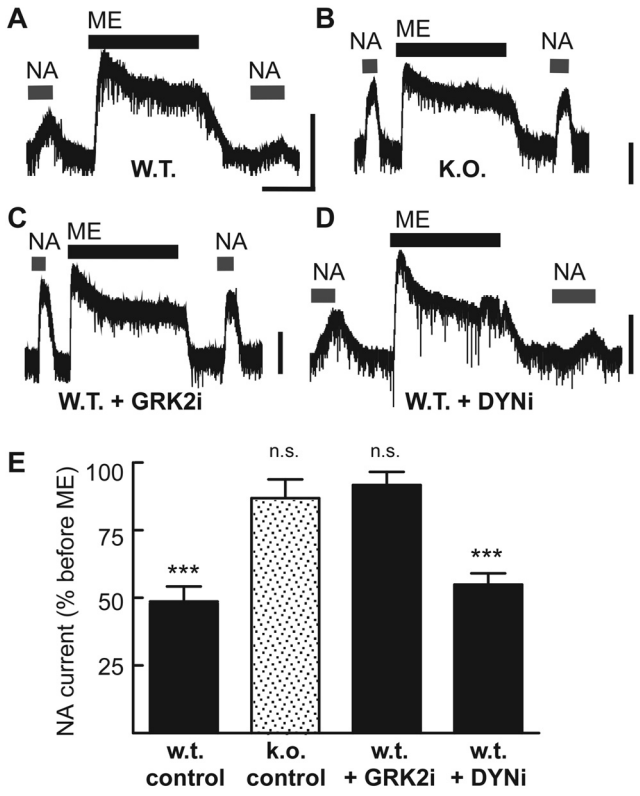

Fig. 3. Disruption of βarr-2 signaling but not dynamin blocks heterologous desensitization. A, heterologous desensitization of response to a probe concentration of NA (3 μM) after a 10-min exposure to ME (30 μM) in LC from a wt mouse. Heterologous desensitization is nearly abolished in LC from a βarr-2 ko mouse (B) and when GRK2 inhibitor is included in the recording pipette (C) but not when dynamin inhibitory peptide is included in the recording pipette (D). E, summary of heterologous desensitization under these conditions (n = 6–12 cells/group). Scale bars, 5 min, 50 pA. ***, $P < 0.001$ versus pre-ME response to NA. n.s., not significant.

endocytosis of MOR is required for development of heterologous desensitization of α_2-ARs, LC neurons were dialyzed with dynamin inhibitory peptide (DYNi) (100 μM) via the recording electrode. This procedure was previously shown to block MOR endocytosis (Dang et al., 2009, 2011). Impairing dynamin function with DYNi had no effect on ME-induced heterologous desensitization of α_2-ARs. Activation of α_2-ARs with NA (3 μM) elicited a GIRK conductance of 32 ± 3 pA before (50 ± 6% of ME current, n = 8) and 17 ± 2 pA after ME desensitization treatment (10 min) in LC neurons dialyzed with DYNi (Fig. 3, D and E). This reduction of 45 ± 4% (n = 8) was similar to that seen in untreated LC neurons (see above), suggesting that endocytosis is not required to produce heterologous desensitization in LC neurons.

ERK1/2 Is Required for Heterologous Desensitization. Having found that dynamin-dependent endocytosis is unlikely to be responsible for heterologous desensitization in LC neurons, experiments were conducted to determine the mechanism by which βarr-2 might promote the attenuation of α_2-AR function after MOR desensitization. βarr-2 mediates internalization of MOR and also acts as a scaffold protein in the ERK1/2 cascade (Miller and Lefkowitz, 2001). Possible mechanisms may therefore involve the phosphorylation and activation of ERK1/2, a process facilitated by βarr-2 (Macey et al., 2006; Zheng et al., 2008) that is c-Src-dependent (e.g., Walwyn et al., 2009). Experiments were therefore performed to determine ERK1/2 involvement in the induction heterologous desensitization of α_2-adrenergic receptor function. Before ME desensitization treatment (30 μM; 10 min), specific ERK1/2 inhibitors (PD98059 or U0126; 10 μM) were dialyzed into LC neurons via the recording electrode to block ERK1/2 activation. As reported previously (Dang et al., 2009), inhibition of ERK1/2 had only a small effect on homologous desensitization, but it almost completely prevented heterologous desensitization of α_2-AR function (Fig. 4, A, B, and D). Before the desensitization with ME (30 μM; 10 min) activation of α_2-adrenergic receptor by NA (3 μM) elicited a GIRK current of 36 ± 6 pA (44 ± 7% of ME current, n = 8), which was reduced to 33 ± 4 pA after MOR desensitization (Fig. 2C) (−7 ± 4%; n = 8, not significant). These results suggest that ERK1/2 activation is required for heterologous desensitization of α_2-adrenergic receptor function after MOR desensitization treatment.

c-Src activates ERK1/2 by phosphorylating ras protein kinase, initiating the ras-raf cascade, a process potentiated by βarr-2 (Luttrell et al., 1999; Miller and Lefkowitz, 2001). To determine whether c-Src is required for βarr-2-dependent heterologous desensitization (e.g., Walwyn et al., 2009), the c-Src inhibitor PP2 (10 μM) was dialyzed into LC neurons via the recording electrode. Intracellular application of PP2 prevented ME-mediated heterologous desensitization of α_2-AR function. In the presence of the c-Src inhibitor, the NA-activated GIRK conductance was 36 ± 16 pA before (48 ± 12% of ME current, n = 3) and 37 ± 17 pA after ME desensitization treatment (Fig. 4, C and D). Taken together, these results suggest that βarr-2 promotes heterologous desensitization by facilitating c-Src-ERK1/2 signaling cascades.

α_2-AR-Mediated sIPSCs Are Depressed after ME Desensitization Treatment. The results from this study show

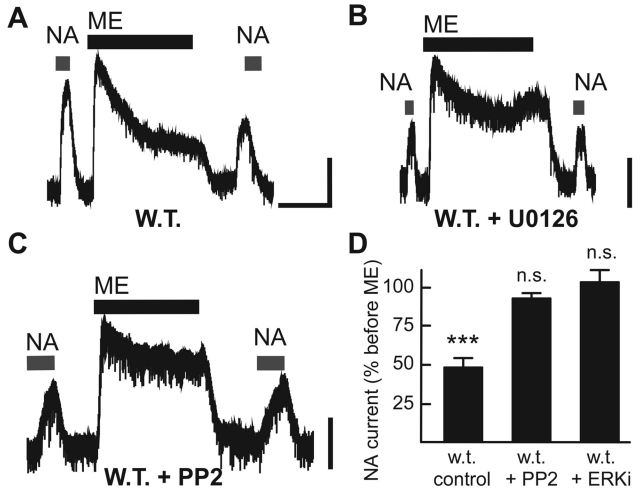

Fig. 4. Inhibition of ERK1/2 or c-Src activity inhibits heterologous desensitization. A, heterologous desensitization of response to a probe concentration of NA (3 μM) after a 10-min exposure to ME (30 μM) in LC. Heterologous desensitization is nearly abolished in the presence of the ERK1/2 inhibitor U0126 (10 μM in the recording pipette) (B) or in the presence of the c-Src inhibitor PP2 (10 μM in the recording pipette) (C). D, summary of heterologous desensitization under these conditions [n = 4–12 cells/group; the ERK1/2 group includes PD98059 (n = 4) or U0126 (n = 4), both at 10 μM]. Scale bars, 5 min, 50 pA. ***, $P < 0.001$ versus pre ME response to NA. n.s., not significant.

that persistent MOR desensitization treatment promotes robust heterologous desensitization that is mediated by a βarr-2-, c-Src-, and ERK1/2-dependent mechanism that exhibits slow and incomplete recovery. Reduced responsiveness of G_i-coupled GPRC signaling could therefore have profound effects on neuronal responsiveness to exogenous agonists at α_2-ARs and possibly synaptically released neurotransmitters. Electrical stimulation in the vicinity of the noradrenergic LC induces sIPSCs onto LC neurons that are mediated by postsynaptic α_2-ARs and modulate neuronal excitability (Egan et al., 1983). To test the hypothesis that MOR-mediated heterologous desensitization inhibits the sIPSC in LC, sIPSCs were measured in LC neurons before and after ME desensitization treatment (30 μM; 10 min) and after ME had completely washed from the slices (10-min washout). Figure 5A, i, shows that α_2-adrenergic receptor-mediated sIPSCs were significantly reduced after ME desensitization treatment (30 μM ME; 10 min). Before ME desensitization treatment, α_2-ARs mediated evoked sIPSCs (18 ± 2 pA, $n = 7$). After prolonged MOR desensitization treatment, evoked sIPSCs were significantly reduced (Fig. 5B). To test whether this depression of α_2-AR-mediated sIPSCs was caused by heterologous desensitization, experiments were conducted in LC neurons from βarr-2 ko mice, in which heterologous desensitization of exogenously applied NA is largely prevented. As shown in Fig. 5, α_2-AR-mediated evoked sIPSCs were nearly the same before and after MOR desensitization treatment (12 ± 3% reduction, $n = 7$). These results suggest that heterologous desensitization can reduce the effect of synaptically released NA onto opioid-sensitive neurons such as LC neurons.

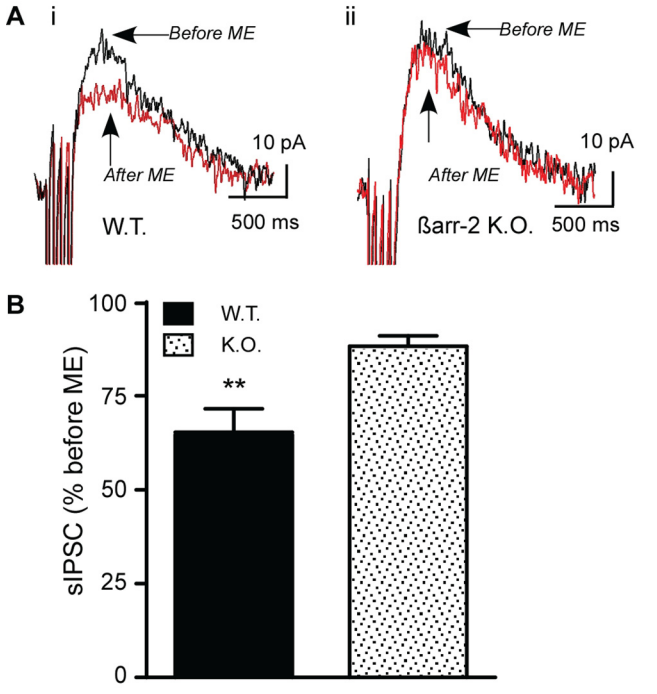

Fig. 5. Heterologous desensitization of synaptically released NA in LC neurons. A, electrically evoked sIPSCs (four consecutive traces averaged) are depressed after washout from a 10-min exposure to a desensitizing concentration of ME (30 μM), in a wild-type LC neuron (I), but the sIPSC is unaffected in a LC neuron from a βarr-2 ko mouse (II). B, heterologous desensitization of the amplitude of the sIPSC is significant in wt but not βarr-2 ko mice. LC neurons ($n = 7$ per group). **, $P < 0.01$.

Discussion

This study shows that persistent stimulation of LC neurons with the endogenous opioid peptide ME (30 μM) produces rapid cellular desensitization that consists of two components mediated by distinct mechanisms: rapid homologous desensitization of MOR followed by more slowly developing heterologous desensitization of α_2-AR function. Although stimulation of MOR with a supramaximal concentration of ME for 5 min produces maximal steady-state homologous desensitization, a significant reduction in α_2-AR function requires at least a 10-min exposure to ME. The two processes can also be distinguished by their rate of recovery. Partial recovery from homologous desensitization is significant after 20 min (Fig. 2) but is not complete for more than 1 h (Dang et al., 2011). In contrast, heterologous desensitization shows no significant recovery observed up to 30 min after washout of ME. The molecular mechanisms also differ. βarr-2-dependent mechanisms contribute to homologous desensitization but are not essential (Dang et al., 2009). However, βarr-2 is required for heterologous desensitization because it is absent in βarr-2 ko and when GRKs are blocked pharmacologically. The heterologous signaling mechanism does not require dynamin-dependent endocytosis but is dependent on ERK1/2 activity. It is noteworthy that βarr-2-dependent heterologous desensitization inhibits NA-mediated sIPSCs in LC neurons, suggesting that it can affect neuronal signaling by attenuating the responsiveness of opioid-sensitive neurons to synaptically released NA. Although heterologous desensitization of sIPSCs was less than that observed for superfused NA (3 μM), it should be noted that the concentration of NA in the vicinity of synaptic release sites during sIPSCs is unknown and could approach α_2-AR saturating concentrations.

Unlike homologous desensitization, which occurs rapidly during MOR stimulation with an endogenous opioid peptide, heterologous desensitization develops more slowly when probed with a submaximal concentration of NA (a sensitive measure of receptor desensitization) (Connor et al., 2004) but is not significant when tested with a supramaximal concentration of the efficacious α_2-AR agonist, brimonidine. This finding suggests that α_2-AR sensitivity is reduced after a prolonged (10-min) exposure to ME, but the capacity of GIRK channels to respond to agonists is not affected. This finding also suggests that heterologous desensitization is less pronounced than homologous desensitization because the latter produces substantial reductions in responses to both probe concentrations and the maximum response (Dang et al., 2009, 2011).

These findings are consistent with earlier reports that MOR stimulation with ME mediated rapid MOR desensitization that is primarily homologous when probed with supramaximal concentrations of α_2-AR agonists (Harris and Williams, 1991; Osborne and Williams, 1995; Connor et al., 1996; Fiorillo and Williams, 1996; Alvarez et al., 2002; Bailey et al., 2004). Others have suggested that MOR-mediated desensitization occurs at the GIRK channels to cause both homologous and heterologous desensitization after exposure to the MOR agonist for 15 min (Blanchet and Lüscher, 2002). The basis for the discrepancy with the above (and present) studies is unknown but could involve other variables such as age of animals (postnatal days 10 to 21 rats in Blanchet and

Lüscher (2002) versus adult rats and mice in most studies) or perhaps other experimental differences.

βarr-2 has been shown to function as a scaffolding protein for localization and activation of protein kinases including c-Src and ERK1/2 (Luttrell and Lefkowitz, 2002; Luttrell, 2005; Macey et al., 2006; Zheng et al., 2008). Figure 3 shows that disruption of βarr-2 function through germline deletion of βarr-2 or by inhibition of GRK2 function prevented heterologous desensitization. In addition, blocking ERK1/2 activation directly (PD98059 or U0126) or via inhibition of c-Src activity (PP2) blocked heterologous desensitization. Taken together, these results suggest that ME-mediated heterologous desensitization is caused by βarr-2 activation of ERK1/2 or as scaffolding protein for ERK1/2 near their effectors or both (Luttrell and Lefkowitz, 2002; Luttrell, 2005; Macey et al., 2006; Zheng et al., 2008). It is of interest that this mechanism does not require endocytosis of the MOR-βarr-2 complex because it is not affected by intracellular application of dynamin inhibitors that we have shown to block MOR endocytosis (Dang et al., 2011) and regulation of homologous desensitization (Dang et al., 2009, 2011), which is consistent with evidence that β-arrestin recruitment and ERK1/2 activation can occur independently of endocytosis (Whistler and von Zastrow, 1999; Kramer and Simon, 2000).

The putative mechanism of heterologous desensitization involving βarr-2-dependent activation of ERK1/2 as a scaffolding protein is unlikely to contribute to homologous MOR desensitization, which reaches steady state within 5 min (Dang et al., 2009). It is likely that the ERK1/2-dependent mechanism of homologous desensitization previously reported (Dang et al., 2009) is due to direct G-protein βγ subunit activation of ERK1/2 because it is observed when either GRK or βarr-2 mechanisms are blocked (DeWire et al., 2007). If G-protein βγ subunit activation of ERK1/2 wanes during prolonged exposures that produce heterologous desensitization, it would be expected that βarr-2-independent components of homologous desensitization would become insensitive to ERK1/2 inhibition.

Prolonged exposure to [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (4 h) in cultured neonatal sensory neurons induced both desensitization and endocytosis of MOR, as well as of α_2-ARs, and vice versa (Tan et al., 2009). However, morphine-induced desensitization, which was not associated with MOR endocytosis, was not accompanied by α_2-AR internalization or α_2-AR desensitization. These findings were interpreted to suggest that hetero-oligomers of MOR and α_2-AR cointernalize to produce heterologous desensitization after stimulation by either agonist. Although it is conceivable that a similar mechanism might be important in LC neurons after exposure to ME or [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin for several hours (Bailey et al., 2009b), it is unlikely to account for the heterologous desensitization observed in LC neurons in the present study after exposure to ME for 10 min, as follows. First, brief exposure to high concentrations of ME for ≤5 min is known to induce nearly maximal endocytosis of MOR in LC neurons (Arttamangkul et al., 2006, 2008), but more prolonged exposure is required to produce heterologous desensitization. It should also be noted that ME-induced endocytosis of MOR is not abolished in βarr-2 ko mice (Arttamangkul et al., 2008; Quillinan et al., 2011). Second, deletion of βarr-2 does not prevent homologous desensitization of MOR (Dang et al., 2009, 2011) but blocks heterologous desensitization of α2-ARs. Finally, direct block of endocytosis by dynamin inhibition, which is not expected to disrupt βarr-2-signaling (see above), did not affect heterologous desensitization in LC neurons. It is therefore likely that homologous MOR desensitization and heterologous desensitization of α_2-AR in LC neurons does not involve endocytosis of hetero-oligomers.

Attenuation of GIRK channel activation by G_i-coupled GPCRs after MOR desensitization treatment could conceivably result from reduced receptor-G-protein coupling or inhibition of GIRK channel activation. One study suggested that MOR-mediated desensitization occurs at the GIRK channels, causing both homologous and heterologous desensitization (Blanchet and Lüscher, 2002). However, such a mechanisms is not consistent with the present results. If desensitization is caused by inhibition of GIRK channels per se, then heterologous and homologous desensitization should share the same kinetics. In addition, manipulations that affect heterologous desensitization should have the same affect on homologous desensitization or vice versa. To the contrary, the present study has established (as widely reported) that maximal steady-state desensitization of MOR activated GIRK current after a 5-min treatment with ME (30 μM), but, concurrently, NA (3 μM) activation of GIRK channels was marginally affected. Furthermore, extending ME desensitization treatment to 10 min did not increase the magnitude of homologous desensitization but significantly increased the magnitude of heterologous desensitization. Finally, disruption of βarr-2 function (GRK inhibition) or expression (βarr-2 ko mice) and inhibition of ERK1/2 activation prevented heterologous desensitization but did not affect homologous desensitization. These results are not compatible with a single mechanism mediated by suppression of GIRK channel activity. Rather they suggest that homologous desensitization and heterologous desensitization both occur upstream of GIRK channel activation.

The present finding of βarr-2-dependent heterologous desensitization predicts that agonists that fail to efficaciously recruit βarr-2 to MOR should produce little heterologous desensitization. Unfortunately, this could not be tested directly in the present study because the amplitude of morphine-induced GIRK currents is too small to reliably determine homologous desensitization (Dang et al., 2009). However, the possibility is supported by studies that have reported little or no loss of α2-AR function if heterologous desensitization was significant after prolonged [several hours in vitro (Bailey et al., 2009b)] or long-term [6 days in vivo (Christie et al., 1987)] exposure of LC to morphine, an agonist that does not efficiently recruit βarr-2 or induce MOR endocytosis in LC (Arttamangkul et al., 2006, 2008).

In conclusion, activation of MOR elicits receptor regulatory events that lead to rapid homologous desensitization. Results presented here show that persistent MOR stimulation also promotes βarr-2-dependent heterologous desensitization. Although heterologous desensitization develops more slowly than homologous desensitization, its recovery is slow and incomplete and can have a profound effect on neuronal signaling by reducing postsynaptic responsiveness to neurotransmitter release. The physiological significance of heterologous desensitization of α2-AR function during opioid treatment in vivo remains uncertain because it was observed

only after prolonged exposure to a high concentration of an efficacious opioid agonist.

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Authorship Contributions

Participated in research design: Dang, Chieng, and Christie.
Conducted experiments: Dang and Chieng.
Performed data analysis: Dang, Chieng, and Christie.
Wrote or contributed to the writing of the manuscript: Dang and Christie.

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