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Prolonged stimulation of μ-opioid receptors produces ß-arrestin-2 mediated heterologous desensitization of α_2-adrenoceptor function in locus coeruleus neurons

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Running title: Heterologous desensitization of μ-opioid receptors

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Abbreviations: ACSF – artificial cerebrospinal fluid, α_2-AR - α_2-adrenergic receptor, BAPTA - 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, βarr-2 – β-arrestin-2, cSrc.-cellular sarc, DYNi – dyanmin inhibitory peptide, ERK1/2 – extracellular signal regulated kinases 1 and 2 , GIRK – G protein activated inwardly rectifying potassium channel, GPCR – G-protein coupled receptor, LC - locus coeruleus, ME - Met^5-enkephalin, MOR - μ-opioid receptor, NA- noradrenaline, PKC – protein kinase C, PP2 - 4-amino-5-(4-chlorophenyl)-(t-butyl)pyrazolo[3,4-D]pyrimidine , sIPSC – slow inhibitory postsynaptic current.

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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 (GPCRs), 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 GIRK currents in mouse brain slices containing 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 ß-arrestin-2 (ßarr-2) because it was nearly abolished in ßarr-2-knockout (k.o.) mice. Heterologous (but not homologous) desensitization was also prevented by inhibition of ERK1/2 and c-Src signaling in wild-type (w.t.) mouse LC neurons. Heterologous desensitization may be physiologically relevant during exposure to high doses of opioids because α_2-AR mediated slow inhibitory post-synaptic currents were depressed in w.t. but not ßarr-2 k.o. LC neurons after prolonged exposure to opioids. Together, these findings demonstrate a

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novel mechanism by which ßarr-2 can regulate post-synaptic responsiveness to neurotransmitter release.

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Introduction

Opioid drugs such as morphine are effective analgesics but their use is limited due to 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 Gaveriaux-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 (GRK) and binding of β-arrestins, as well ERK1/2 and PKC activation and possibly other mechanisms (Bohn et al., 2000; Dang et al., 2011; Groer et al., 2011; Li and Wang, 2001; Zhang et al., 1998). Briefly, 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 (eg. Harris and Williams, 1991; Fiorillo and Williams, 1996; Connor et al., 1996; Bailey et al., 2004, 2009 a,b; Dang et al., 2009, 2011). However, other evidence 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 Luscher, 2002; Chu et al. 2010; Tan et al., 2003; Tan et al., 2009). These discrepancies might be attributed to differences in experimental conditions and designs including cell types investigated, species, age of animals, duration

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of agonist exposure and end-points 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 (eg. Blanchet and Luscher, 2002) to several hours (eg. Tan et al., 2009). By contrast, studies reporting largely homologous desensitization of MOR have usually examined brief periods of MOR stimulation with ME, usually for 5-10 min, at which times homologous desensitization has reached steady state (Harris and Williams, 1991; Osborne &Williams, 1995; Fiorillo and Williams, 1996; Connor et al 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 chanels) 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 may be that homologous desensitization is induced initially during exposure to high concentrations of ME but this is followed by heterologous desensitization mediated by distinct mechanisms (eg. 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 homogenous population of noradrenergic neurons expressing one type of opioid receptor, the MOR, and other Gi coupled receptors including α_2-ARs that modulate the same population of GIRK channels

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(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 heterologous desensitization of α_2-ARs in LC neurons. Distinct mechanisms are responsible for each, with dependence of heterologous but not homologous desensitization on ßarr-2, ERK1/2 and c-Src. Importantly, MOR induction of heterologous desensitization not only attenuates Gi mediated signaling elicited by exogenous agonist but also synaptically evoked release of noradrenaline (NA).

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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, 5-12 weeks old βarr-2 knockout mice from Drs Lefkowitz and Caron (Duke University, see Bohn et al., 2000), or their wild-type littermates (w.t., C57Bl6 background) . LC slices were prepared as previously described (Dang et al., 2011) . Briefly, mice were anesthetized with isoflourane (4% in air), decapitated, brains were removed and blocked. Horizontal vibratome (Leica1000) sections (200-220 µm) were prepared at 4^0C, then incubated in extracellular solution (ACSF, 60 min, 35˚C). ACSF contained (mM): 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 10 glucose, 95%O_2-5%CO_2 at 37˚C. Pipette (2-4 MΩ) solution contained (in mM): 115 K MES (2-[morpholino]-ethane-sulfonic acid), 20 NaCl, 1.5 $MgCl_2$, 10 BAPTA, 5 HEPES, 4 Mg-ATP and 0.4 Na-GTP, pH= 7.3-7.4. Voltage-clamp recordings of visualized LC neurons (infrared Nomarski optics, holding potential -55 to -60 mV) were acquired using Axograph X (Axograph Scientific, Australia) and filtered at 20-100 Hz. All drugs were applied by superfusion of the recording chamber (~2 ml/min, 35˚C). Bestatin (10µM) 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

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al., 2002). All applications of NA were prepared as fresh solutions when re-application 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 LC about 100 µm from the recorded neuron to evoke slow inhibitory postsynaptic current (sIPSC) while holding membrane potential at -60 mV. Using a pulse generator (Digitimer, Welwyn Garden City, UK), a train of stimuli (4 pulses of 1 ms duration at 17 Hz) was adjusted between 20-40 V to generate sIPSCs of 20-50 pA in amplitude. sIPSCs were evoked every 90 s, sampled at 10 kHz and filtered online at 5 kHz (Axograph 4.6, Molecular Devices, Sunnyvale, CA). Four consecutive traces in each condition were averaged to determine sIPSC amplitude. The superfusion solution was equilibrated with CNQX (10 µM), MK801 (10 µM), picrotoxin (100 µM) and prazosin (1 µM) for at least 10 min before examining synaptic currents.

Statistics and curve fitting: Data were analysed using PRISM (GraphPad Software, Inc, San Diego, CA). Values are presented as mean ± SEM. Statistical comparisons were made with t-tests (for 2 groups) or two-way ANOVA with Bonferroni's post-hoc tests and 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, prazosin-HCl; Sigma (St. Louis, MO). Cocaine HCl (Glaxo Australia), naloxone HCl, brimonidine tartrate (UK14304), PP2 (4-amino-5-(4-chlorophenyl)-(t-butyl)pyrazolo[3,4-D]pyrimidine) PD98059, U0126, somatostatin; Tocris (Bristol, UK), morphine HCl;

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GSK (Melbourne, Vic, Australia). The GRK2 inhibitory peptide sequence W643-S670 (Ac-WKKELRDAVREAQQLVQRVPKMKNKPRS-NH2, > 95% purity; Li et al., 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 sensitivity of α_2-AR coupling to GIRK currents (rather than capacity for GIRK channel activation) is reduced after 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 previously reported (Christie et al., 1987; Dang et al., 2009, 2011), a supramaximal concentration of the specific α_2-AR agonist, brimonidine (UK14034, 3 μM), produced an outward current of similar amplitude to 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. 1Ai,ii, C). As previously reported (Dang et al., 2009, 2011),

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desensitization of the response to ME was near maximal after 5 (Fig. 1Aiii, B) or 10 min (Fig 1Bii) exposure and the response to brimonidine showed only a modest trend for desensitization after 10 min exposure to ME (not significant, Fig. 1C, P = 0.53). 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).

Whilst 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). In order to examine a more sensitive measure of heterologous desensitization, LC neurons were tested with a sub-maximal concentration of NA (3 µM, Figure 1B,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 w.t. 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 ßarr-2 knockout,

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dynamin inhibition, GRK inhibition, ERK1/2 inhibition or cSrc inhibition (One way ANOVA $p = 0.50$ for raw current and $p = 0.31$ for % 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 5 min desensitization treatment with ME (30μM) 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 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 5 min exposure to ME (Figure 1Bii, C). Prior to 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 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 homologous desensitization

Previous studies have reported that recovery from homologous MOR desensitization proceeds gradually for over one hour after washing out a desensitizing concentration of

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ME (Dang et al., 2011; Dang and Williams, 2004). To determine the rate and extent of recovery of α_2-AR function after induction of heterologous desensitization, NA (3μM) 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 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, recovery was very slow and not significant for at least 30 min after washout of ME (Figure 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 (Figure 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 ßarr-2 to the plasma membrane contributing to desensitization by uncoupling MOR from G-proteins and mediates receptor endocytosis (Bohn et al., 2004; Cen et al., 2001). We therefore explored the possibility that signaling events related to ßarr-2 recruitment may mediate heterologous desensitization using ßarr-2 k.o. mice. As

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previously reported (Dang et al., 2009) deletion of ßarr-2 did not inhibit homologous desensitization of MOR but it nearly abolished heterologous desensitization of α_2-ARs following 10 min MOR desensitization treatment in LC neurons (Figure 3). Activation of α_2-ARs with NA (3 µM) in LC neurons from k.o. mice before MOR desensitization treatment evoked an outward current of 40 ± 6 pA (68 ±11% of ME current, n = 6), but following MOR desensitization, the NA (3 µM) evoked current was 33 ± 4 pA (Figure 3B). This reduction of receptor function was significantly smaller than that observed in LC neurons from w.t. mice (Figure 3A,E).

To confirm that ßarr-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 ßarr-2 dependent regulation of MOR in LC neurons from w.t mice, as previously reported (Dang et al., 2009, 2011). GRK2 inhibitory peptide (GRK2i; 100 µM) was dialyzed into neurons from w.t. mice to disrupt ßarr-2 interaction with the MOR at the plasma membrane. This manipulation has previously been reported to disrupt ßarr-2-dependent endocytosis of MOR (Dang et al., 2009; Li and Wang, 2001; Zhang et al., 1998). Disruption of GRK2 function with GRK2i 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 (30µM, 10 min) desensitization treatment respectively; a reduction of 8 ± 5 % (n = 6), Figure 3C,E). The findings that heterologous desensitization in LC neurons was prevented by both by deletion of ßarr-2 and disruption of GIRK2 function in w.t. mice suggests that ßarr-2 recruitment to MOR

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contributes to the attenuation of α_2-AR function following ME (30 µM) desensitization treatment.

Heterologous desensitization does not require dynamin-dependent endocytosis

Tan et al. (2009) reported that co-internalization of MOR and α_2-ARs when stimulated by either agonist mediates heterologous desensitization in cultured sensory neurons. To determine whether or not 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 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 (Figure 3 D, 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 ßarr-2 might promote the attenuation of α_2-AR function following MOR desensitization. ßarr-2 mediates internalization of MOR and also acts as a scaffold protein in the extracellular signal-regulated kinase (ERK 1/2) cascade (Miller

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and Lefkowitz, 2001). Possible mechanisms may therefore involve the phosphorylation and activation of ERK1/2, a process facilitated by ßarr-2 (Macey et al., 2006; Zheng et al., 2008) that is cSrc-dependent (eg. Walwyn et al., 2009). Experiments were therefore performed to determine ERK1/2 involvement in the induction heterologous desensitization of α_2 adrenergic receptor function. Prior to 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 previously reported (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 (Figure 4 A, B, 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 ± 4pA after MOR desensitization (Figure 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 following MOR desensitization treatment.

cSrc activates ERK1/2 by phosphorylating ras protein kinase, initiating the ras-raf cascade, a process potentiated by ßarr-2 (Luttrell et al., 1999; Miller and Lefkowitz, 2001). To determine whether or not cSrc is required for ßarr-2-dependent heterologous desensitization (eg. Walwyn et al., 2009), the cSrc 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 cSrc inhibitor, the NA activated GIRK conductance was 36 ± 16 pA before (48 ± 12% of

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ME current, n = 3) and 37 ±17 pA after ME desensitization treatment (Figure 4C, D). Together, these results suggest that ßarr-2 promotes heterologous desensitization by facilitating cSrc-ERK1/2 signaling cascades.

α_2-AR mediated sIPSCs are depressed following ME desensitization treatment.

The results from this study show that persistent MOR desensitization treatment promotes robust heterologous desensitization that is mediated by a ßarr-2, cSrc and ERK1/2 dependent mechanism that exhibits slow and incomplete recovery. Reduced responsiveness of G_i-couple GPRCs 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 slow inhibitory postsynaptic currents (sIPSCs) onto LC neurons that are mediated by post-synaptic 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), after ME had completely washed from slices (10 min washout). Figure 5Ai shows that α_2 adrenergic receptor mediated sIPSCs were significantly reduced after ME desensitization treatment (30 µM ME; 10 min). Prior to ME desensitization treatment, α_2-AR mediated evoked sIPSCs (18 ± 2 pA, n = 7). After prolonged MOR desensitization treatment, evoked sIPSCs were significantly reduced (Figure 5 B). To test whether this depression of α_2-AR mediated sIPSC was caused by heterologous desensitization, experiments were conducted in LC neurons from ßarr-2 k.o. mice, where heterologous desensitization of exogenously applied NA is largely

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prevented. As shown in Figure 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.

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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 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 (Figure 2) but is not complete for more than one hour (Dang et al., 2011). By contrast, heterologous desensitization recovers shows no significant recovery observed up to 30 min after washout of ME. The molecular mechanisms also differ. ßarr-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 k.o.s and when GRKs are blocked pharmacologically. The heterologous signaling mechanism does not require dynamin-dependent endocytosis but is dependent on ERK 1/2 activity. Importantly, βarr-2-dependent heterologous desensitization inhibits NA mediated sIPSCs in LC neurons, suggesting it can affect neuronal signaling by attenuating the responsiveness of opioid sensitive neurons to synaptically released NA. Although heterologous desensitization of sIPSCs was smaller 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.

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Unlike homologous desensitization, which occurs rapidly during MOR stimulation with an endogenous opioid peptide, heterologous desensitization develops more slowly when probed with a sub-maximal 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 suggests that α_2-AR sensitivity is reduced following prolonged (10 min) exposure to ME but the capacity of GIRK channels to respond to agonists is not affected. This finding also suggests that heterolgous 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; Fiorillo and Williams, 1996; Connor et al 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 MOR agonist for 15 min (Blanchet and Luscher, 2002). The basis for the discrepancy with the above (and present) studies is unknown but could involve other variables such as age of animals (P10-P21 rats in Blanchet and Luscher, 2002, versus adult rats and mice in most studies) or perhaps other experimental differences.

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ßarr-2 has been shown to function as scaffolding protein for localization and activation of protein kinases including including cSrc and ERK1/2 (Luttrell, 2005; Luttrell and Lefkowitz, 2002; Macey et al., 2006; Zheng et al., 2008). Figure 3 shows that disruption of ßarr-2 function through germline deletion of ßarr-2 or by inhibition of GRK2 function prevented heterologous desensitization. Additionally, blocking ERK1/2 activation directly (PD98059 or U0126) or via inhibition of cSrc activity (PP2) blocked heterologous desensitization. Taken together, these results suggest ME mediated heterologous desensitization is caused by ßarr-2 activation of ERK1/2 or as scaffolding protein for ERK1/2 near their effectors, or both (Luttrell, 2005; Luttrell and Lefkowitz, 2002; Macey et al., 2006; Zheng et al., 2008). It is of interest that this mechanism does not require endocytosis of the MOR-βarr2-complex because it is not affected by intracellular application of dynamin inhibitors that we have been 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 ßarr-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 ßarr-2 mechanisms are blocked (DeWire et

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al., 2007). If G-protein βγ subunit activation of ERK1/2 wanes during prolonged exposures that produce heterologous desensitization it would be expected that ßarr-2-independent components of homologous desensitization would become insensitive to ERK1/2 inhibition.

Prolonged exposure to DAMGO (4 hours) in cultured neonatal sensory neurons induced both desensitization and endocytosis of MOR, as well as α_2-ARs, and vice versa (Tan et al., 2009). However, morphine-induced desensitization, which was not associated with MOR endocytosis, was not accompanied α_2-AR internalization or α_2-AR desensitization. These findings were interpreted to suggest that hetero-oligomers of MOR and α_2-AR co-internalize 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 DAMGO for several hours (but see Bailey et al., 2009b), it is unlikely to account for heterologous desensitization observed in LC neurons in the present study after exposure to ME for 10 min, as follows. Firstly, brief exposure to high concentrations of ME for $\leq$ 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 k.o.s (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 βarr2-signaling (see above), did not affect heterologous desensitization in LC

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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 following 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 Luscher, 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. Additionally, 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 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 ßarr-2 function (GRK inhibition) or expression (βarr-2 k.o.s), as well as 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

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GIRK channel activity. Rather they suggest that homologous desensitization and heterologous desensitization both occur upstream of GIRK channels activation.

The present finding of βarr2-dependent heterologous desensitization predicts that agonist which 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 chronic (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 βarr2-dependent heterologous desensitization. Although heterologous desensitization develops more slowly than homologous desensitization, its recovery is slow and incomplete and can have profound affect on neuronal signaling by reducing post-synaptic responsiveness to neurotransmitter release. The physiological significance of heterologous desensitization of α2-AR function during opioid treatment *in vivo* remains uncertain because 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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Figure 1.

Heterologous desensitization of α_2-AR requires more prolonged exposure to ME than does homologous desensitization of MOR. A, (i-ii)The full α_2-AR agonist, brimonidine (UK14304, 1 μM; UK, reversed by idazoxan (3 μM; Idaz) and MOR agonist, ME (30 μM) produce similar outward currents, regardless of order of exposure for 1-2 min or (iii) after exposure to ME for 5 min (drug application time shown by bars). B, The outward current produced by a submaximal concentration of NA is (i) unaffected by ME-induced desensitization of MOR for 5 min but is substantially reduced after 10 min exposure to ME. 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 10 min exposure (4-10 cells per group). Scale bars; 5 min, 50 pA. *** - $P < 0.001$.

Figure 2

Homologous desensitization recovers more rapidly than heterologous desensitization. A, Recover of the response to a submaximal probe concentration of ME (300 nM) develops slowly but consistently after 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

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ME-induced desensitization for 10 min but the response to NA does not (n = 4-8 cells per group). Scale bars 5 min, 50 pA. * - $P < 0.05$, ** $P < 0.01$.

Figure 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 10 min exposure to ME (30 μM) in LC from a w.t. mouse. B, Heterologous desensitization is nearly abolished in LC from a βarr-2 k.o. and C, when GRK2 inhibitor is included in the recording pipette but, D, not when dynamin inhibitory peptide is included in the recording pipette. E. Summary of heterologous desenstization under these conditions (n = 6-12 cells per group). Scale bars 5 min, 50 pA. ***- $P < 0.001$ versus pre-ME response to NA.

Figure 4

Inhibition of ERK1/2 or cSrc activity inhibits heterologous desensitization. A, Heterologous desensitization of response to a probe concentration of NA (3 μM) after 10 min exposure to ME (30 μM) in LC. B, Heterologous desensitization is nearly abolished in the presence of the ERK1/2 inhibitor U0126 (10 μM in the recording pipette) or C, in the presence of the cSrc inhibitor, PP2 (10 μM in the recording pipette). D, Summary of heterologous desenstization under these conditions (n = 4-12 cells per group; the ERK1/2 group includes PD98059 (n = 4) or U0126 (n = 4) both 10 μM). Scale bars 5 min, 50 pA. ***- $P < 0.001$ versus pre-ME response to NA.

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Figure 5.

Heterologous desensitization of synaptically released NA in LC neurons. A, Electrically-evoked low IPSCs (4 consecutive traces averaged) are depressed following washout from 10 min exposure to a desensitizing concentration of ME (30 μM), (i) in a wild type LC neuron but (ii) the IPSC is unaffected in a LC neuron from a βarr-2 k.o. mouse. B, Heterologous desensitization of the amplitude of the sIPSC is significant in w.t. but not βarr-2 k.o. LC neurons (n = 7 per group). ** - $P < 0.01$.

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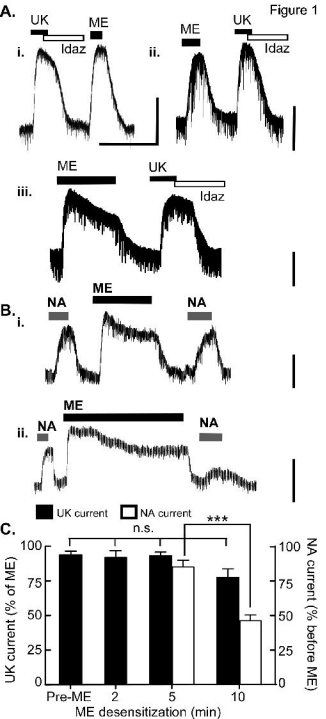
Figure 1


A.
UK
ME
Idaz
i.
ii.
iii.
B.
NA
ME
i.
ii.
UK current
NA current
C.
n.s.
***
UK current (% of ME)
NA current (% before ME)
100
75
50
25
0
Pre-ME
2
5
10
ME desensitization (min)

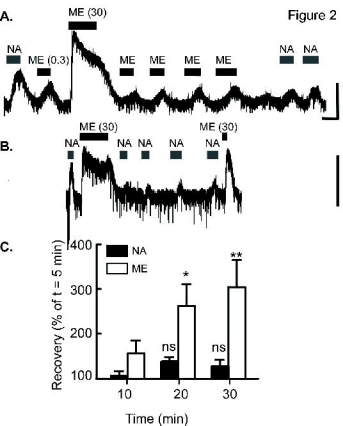
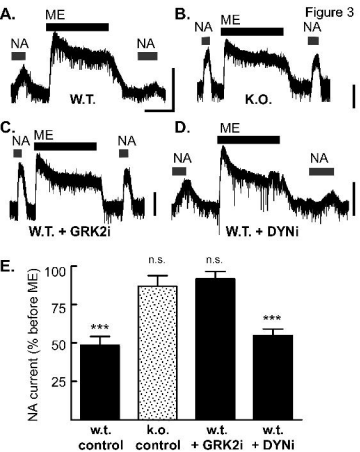

A.
ME (30)
NA
ME (0.3)
ME
ME
ME
ME
NA
NA
B.
ME (30)
ME (30)
NA
NA
NA
NA
NA
C.
NA
ME
Recovery (% of t = 5 min)
400
300
200
100
ns
*
ns
**
10
20
30
Time (min)

Figure 2


A.
ME
NA
NA
W.T.
B.
Figure 3
NA
ME
NA
K.O.
C.
NA
ME
NA
W.T. + GRK2i
D.
ME
NA
NA
W.T. + DYNi
E.
NA current (% before ME)
100
75
50
25
n.s.
n.s.
***
***
w.t. control
k.o. control
w.t. + GRK2i
w.t. + DYNi

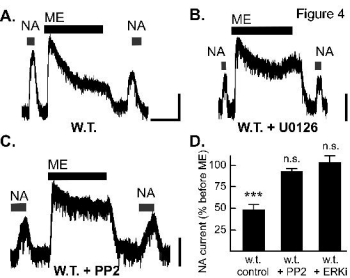

A.
NA
ME
NA
W.T.
B.
ME
NA
NA
W.T. + U0126
C.
ME
NA
NA
W.T. + PP2
D.
NA current (% before ME)
100
75
50
25
***
n.s.
n.s.
w.t. control
w.t. + PP2
w.t. + ERKi

Figure 4

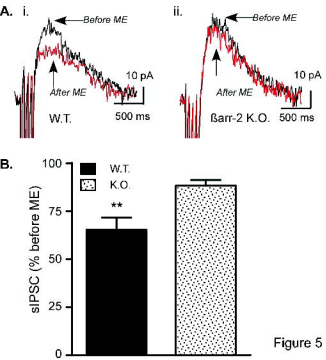

A.
i.
Before ME
After ME
10 pA
500 ms
W.T.
ii.
Before ME
After ME
10 pA
500 ms
ßarr-2 K.O.
B.
W.T.
K.O.
**
sIPSC (% before ME)
100
75
50
25
0

Figure 5