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Tolerance to Repeated Morphine Administration Is Associated with Increased Potency of Opioid Agonists

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Tolerance to the pain-relieving effects of opiates limits their clinical use. Although morphine tolerance is associated with desensitization of μ-opioid receptors, the underlying cellular mechanisms are not understood. One problem with the desensitization hypothesis is that acute morphine does not readily desensitize μ-opioid receptors in many cell types. Given that neurons in the periaqueductal gray (PAG) contribute to morphine antinociception and tolerance, an understanding of desensitization in PAG neurons is particularly relevant. Opioid activity in the PAG can be monitored with activation of G-protein-mediated inwardly rectifying potassium (GIRK) currents. The present data show that opioids have a biphasic effect on GIRK currents in morphine tolerant rats. Opioid activation of GIRK currents is initially potentiated in morphine ($EC_{50} = 281$ nM) compared to saline ($EC_{50} = 8.8\,\mu$M) pretreated rats as indicated by a leftward shift in the concentration–response curve for met-enkephalin (ME)-induced currents. These currents were inhibited by superfusion of the μ-opioid receptor antagonist β-funaltrexamine (β-FNA) suggesting that repeated morphine administration enhances agonist stimulation of μ-opioid receptor coupling to G-proteins. Although supersensitivity of μ-opioid receptors in the PAG is counterintuitive to the development of tolerance, peak GIRK currents from tolerant rats desensitized more than currents from saline pretreated rats (56% of peak current after 10 min compared to 15%, respectively). These data indicate that antinociceptive tolerance may be triggered by enhanced agonist potency resulting in increased desensitization of μ-opioid receptors.
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INTRODUCTION

Morphine and its derivatives are the most common and effective treatments for severe pain. Unfortunately, opiates are limited as long-term therapies due to the development of tolerance to their antinociceptive effects (McQuay, 1999). Recent evidence suggests that the periaqueductal gray (PAG) is particularly important in the development of tolerance to antinociception produced by systemic morphine administration. Repeated microinjection of morphine into the ventrolateral PAG is sufficient to produce tolerance (Tortorici *et al*, 1999; Morgan *et al*, 2005a) and cross-tolerance develops between systemic and direct PAG administration (Jacquet and Lajtha, 1976; Siuciak and Advokat, 1987; Morgan *et al*, 2006). Moreover, antinociceptive tolerance to systemic morphine administration is disrupted if the action of morphine is blocked in the ventrolateral PAG (Lane *et al*, 2005).

Continuous morphine pretreatment induces profound changes in ventrolateral PAG neurons, including decreased opioid activation of G-protein-mediated inwardly rectifying potassium (GIRK) and K_v channels (Chieng and Christie, 1996; Ingram *et al*, 1998; Hack *et al*, 2003; Bagley *et al*, 2005a, b). Given that opioid inhibition of GABA neurons and disinhibition of PAG output neurons is necessary for antinociception (Moreau and Fields, 1986; Depaulis *et al*, 1987; Osborne *et al*, 1996), decreasing opioid activation of potassium channels is consistent with antinociceptive tolerance. Many studies provide evidence that a key cellular mechanism underlying tolerance is desensitization of opioid receptors following continuous opiate administration (for reviews, Williams *et al*, 2001; Kieffer and Evans, 2002; Connor *et al*, 2004). The cellular adaptations in the PAG associated with short-term repeated administration of morphine have not been studied previously. The purpose of this study was to determine whether changes in opioid-sensitive PAG neurons contribute to antinociceptive tolerance to morphine induced with short-term repeated subcutaneous (s.c.) injections of morphine.

METHODS

Subjects

Male Sprague–Dawley rats (180 ± 6 g; range = 72–295 g;) were housed two per cage throughout the experiment. Food and water were available *ad libitum*. Lights were maintained on a reverse 12 : 12 cycle (on at 1900 hours) so

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testing could be conducted during the active dark phase. Experiments were conducted in accordance with the animal care and use guidelines of the International Association for the Study of Pain and approved by the Animal Care and Use Committee at Washington State University.

Rats were injected with saline or morphine (5 mg/kg, s.c.) twice a day for 2 days to induce tolerance. Rats were returned to their cages immediately following each injection. Nociception was assessed using the hot plate test (Trials 1 and 5) that consisted of placing the rat on a hot plate (52°C) and measuring the latency to lick a hind paw or attempt to jump out of the apparatus. The rat was removed after 40 s if no response occurred. All rats were injected with morphine on Trial 5 with a cumulative dosing procedure to determine whether tolerance had developed to the antinociceptive effect of morphine (Morgan *et al*, 2006). This procedure consisted of a baseline hot plate test followed by five successive injections of morphine at 20 min intervals resulting in quarter log doses (1.8, 3.2, 5.6, 10, and 18 mg/kg, s.c.). Rats were tested on the hot plate 15 min after each injection.

Tolerance to Continuous Morphine Exposure

Rats were briefly anesthetized with Halothane and implanted with two saline or morphine pellets (75 mg) wrapped in nylon under the skin of the upper back. All rats were also briefly anesthetized after the 72-h hot plate test so the pellets could be removed. The rats were injected with cumulative doses of morphine 6 h after removal of pellets. A baseline hot plate test was followed by five successive injections of morphine at 20 min intervals resulting in quarter log doses (1.8, 3.2, 5.6, 10, and 18 mg/kg, s.c.). Rats were tested on the hot plate 15 min after each injection. Brains were removed for brain slice recordings at least 24 h later.

Naloxone-Precipitated Withdrawal

In order to assess the development of dependence, naloxone (1 mg/kg, s.c.) was administered to rats pretreated with morphine pellets for 72 h prior to removal of pellets (pellets IN), 24 h after pellet removal (pellets OUT) or 24 h after the last morphine injection. The incidence of withdrawal behaviors including wet-dog shakes, tearing, diarrhea, vocalization, tachypnea, defecation, and self-stimulation were assessed at 10, 20, and 30 min following the injection of naloxone. The total number of behaviors was used for data analysis.

Electrophysiological Recordings

The objective of the electrophysiological recordings was to determine whether tolerance to intermittent administration of morphine induced changes in the response of PAG neurons to opioids. A total of 1–4 days after the last injection, rats were anesthetized with Halothane and decapitated. Brains were removed quickly and immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl; 21.4 $NaHCO_3$; 11.1 dextrose; 2.5 KCl; 2.4 $CaCl_2$; 1.2 $MgCl_2$; 1.2 NaH_2PO_4, pH7.35, and equilibrated with 95% O_2/5% CO_2. Brain slices containing the ventral PAG were cut with a vibratome (250–300 μm thick) and placed in a holding chamber with oxygenated ACSF maintained at approximately 32°C until needed for recording (30–360 min).

Brain slices were placed into a recording chamber mounted on an Olympus BX51 upright microscope and superfused with 32°C ACSF at 2 ml/min. Cells were viewed with a water immersion ×40 objective (Olympus) and Nomarski infrared optics. Recordings were made with electrodes pulled to 2–4 MΩ resistance with internal solutions consisting of (in mM): 138 potassium methyl sulfate; 10 HEPES; 10 KCl; 1 $MgCl_2$; 1 EGTA; 0.3 $CaCl_2$; 4 MgATP; 3 NaGTP, pH 7.4. Junction potentials were corrected at the beginning of the experiments. Capacitance and series resistance compensation (>70–80%) were corrected and access resistance was monitored throughout the experiments. Data were collected with a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA) at 5–10 kHz and low-pass filtered at 2–5 kHz. Currents were digitized with Digidata 1322A, collected via Axograph Data Acquisition software and analyzed using Axograph (Axograph Scientific, Sydney, Australia). Experiments were monitored with Chart software (MacLab; ADInstruments, Castle Hill, NSW, Australia). Neurons were voltage-clamped at −60 or −70 mV and current–voltage relationships were determined with 10 mV voltage steps from −120 to −20 mV (duration of step = 1 s). Concentration–response curves were generated by superfusion of 2–4 successive increasing doses of ME per neuron. Each neuron was tested with a maximal concentration of ME (10 or 20 μM) to ensure that the neuron responded to opioids. The ME-induced current was defined as the peak current induced by ME minus the average of the current before the first ME concentration and after wash out or antagonist reversal of the final ME concentration.

Drugs

Morphine sulfate was a gift from the National Institute on Drug Abuse and was diluted in saline for injections. ME, baclofen, deltorphin II, NO-711, BaCl, and β-funaltrexamine (β-FNA) were obtained from Sigma (St Louis, MO). ICI-174864 and CGP-55845 were obtained from Tocris Biochemicals (Ellisville, MO) and r-teriaptinQ was obtained from Alamone Labs (Jerusalem, Israel). All drugs were diluted in appropriate buffers as concentrated stocks for further dilution in ACSF solution.

Statistical Analyses

All data are expressed as mean ± SEM, unless otherwise noted. In behavioral experiments, differences between groups were determined with ANOVA analysis of dose–response curves. The half-maximal antinociceptive effect (D_{50}) was estimated with nonlinear regression (GraphPad Prism, San Diego, CA) using the mean hot plate latency at each dose of morphine. In electrophysiological experiments, differences between groups were assessed using Student's *t*-test and ANOVA when appropriate.

RESULTS

Intermittent Morphine Administration Induces Tolerance to Morphine

Repeated administration of morphine (5 mg/kg, s.c., twice a day for 2 days) caused a significant shift in the dose–response curve for morphine ($D_{50} = 7.8$ mg/kg; 95% CI $= 6.4$–9.1 mg/kg; $n = 33$) compared to saline pretreated rats ($D_{50} = 4.6$ mg/kg; 3.4–5.4 mg/kg; $n = 20$; $F(1, 312) = 14.04$; $p < 0.0002$) as would be expected in morphine tolerant rats (Figure 1).

Morphine Pretreatment Increases Opioid-Mediated GIRK Currents

The repeated intermittent injection procedure used in our experiments produces tolerance that lasts up to 1 week after the last injection of morphine (Morgan *et al*, 2005b) suggesting that long-term cellular changes have been induced in morphine pretreated rats. In order to look at these long-term changes, brain slices for *in vitro* electrophysiological recordings were prepared 1–4 days after the last behavioral test. Whole cell patch clamp recordings were made from a subpopulation of opioid-sensitive ventrolateral PAG neurons. As expected, the opioid agonist met-enkephalin (ME) dose-dependently stimulated outward currents in saline and morphine pretreated rats (Figure 2). These ME-induced currents were inhibited in the presence of naloxone (1–10 μM; Figure 2c) in both saline ($n = 10$) and morphine pretreated rats ($n = 5$). In addition, the μ-receptor selective antagonist β-FNA (10 μM; Figure 2c) reversed the ME-induced currents in saline ($n = 4$) and morphine ($n = 4$) pretreated rats. Although *continuous* administration of morphine has been shown to upregulate δ-opioid receptor expression in the mouse PAG (Hack *et al*, 2005), there was no evidence for currents induced by the δ-opioid receptor agonist, deltorphin II (1 μM; $n = 6$) in morphine pretreated rats, even in cells with a ME-induced current ($n = 3$). The selective δ-opioid receptor antagonist, ICI-174864 (1 μM), also had no effect on the ME-induced currents ($n = 5$).

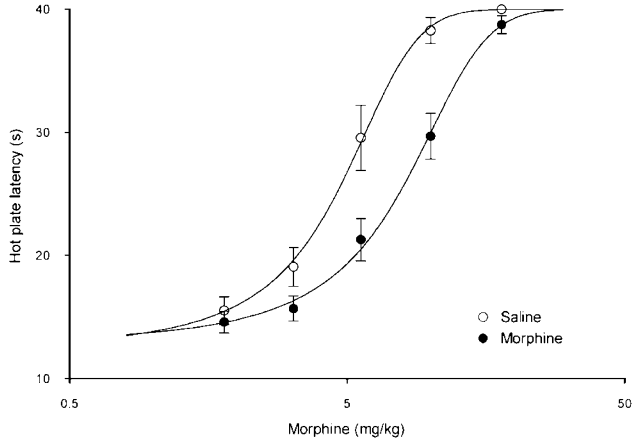

Figure 1 Repeated intermittent administration of morphine induces antinociceptive tolerance. Cumulative dose (s.c.)–response relationships were determined on day 3 using the hot plate test in saline ($n = 20$) and morphine ($n = 33$) pretreated rats. Rats pretreated with morphine were tolerant as indicated by a rightward shift in the dose–response curve.

Surprisingly, ME induced larger currents in morphine compared to saline pretreated rats (Figure 2a and b). The potentiation of ME-induced currents at −70 mV was evident in a significant leftward shift in the concentration–response relationships for morphine pretreated rats ($EC_{50} = 281$ nM; 95% CI $= 110$ nM–715 nM) compared to saline pretreated rats ($EC_{50} = 8.8$ μM; 5–16 μM; $F(1, 89) = 45.03$; $p < 0.0001$; Figure 2d). The proportion of neurons that responded to opioids was approximately the same in saline (34/98; 35%) and morphine (46/117; 39%) pretreated rats, similar to other studies in the ventrolateral PAG (Chieng and Christie, 1994b). The GIRK channel inhibitor, BaCl (1 mM) inhibited the outward current elicited by ME in both morphine ($n = 5$) and saline pretreated rats ($n = 3$). In addition, another GIRK inhibitor r-teriaptinQ (10 nM) inhibited ME-induced currents ($n = 7$) confirming that ME-induced outward currents are due to activation of GIRK channels.

Similar supersensitivity was observed with morphine-induced currents (Figure 3). Morphine (5 μM) elicited peak currents at −70 mV of 5 ± 2 pA ($n = 8$) and 18 ± 4 pA ($n = 5$) in saline and morphine pretreated rats, respectively. A higher morphine (20 μM) concentration elicited responses of 24 ± 6 pA ($n = 6$) and 32 ± 8 pA ($n = 4$) in saline and morphine pretreated rats, respectively. The morphine-induced currents were significantly larger in morphine pretreated rats (two-way ANOVA; $F(1, 19) = 5.42$, $p = 0.0311$) suggesting that agonists produce more potent activation of opioid receptors in morphine compared to saline pretreated rats.

Previous studies have shown that *continuous* administration of morphine induces an additional opioid-sensitive current in the PAG that is associated with the GABA transporter (GAT) (Bagley *et al*, 2005b). This cation current is inhibited by opioids and contributes to ME-induced responses by augmenting the outward current elicited by ME and shifting the reversal potential to more hyperpolarized potentials than the expected value for a potassium current (ie GIRK). Our data also show a shift in the reversal potentials of ME (10 μM)-induced currents in morphine pretreated rats ($E_{rev} = -111 \pm 2$ mV; $n = 7$) compared to controls ($E_{rev} = -94 \pm 4$ mV; $n = 7$; one-way ANOVA, $F(2, 18) = 9.112$, $p = 0.002$; Figure 4). ME-induced currents reversed near the equilibrium potential for potassium (-88 ± 5 mV; $n = 5$) in the presence of the GAT inhibitor, NO-711 (10 μM) indicating that the more negative reversal potentials in morphine pretreated rats were caused by opioid inhibition of inward current mediated by GAT. However, intermittent morphine does not appear to induce GAT currents as large as those observed by Bagley *et al* (2005b) because ME-induced currents reversed at -111 ± 3 mV, whereas the reversal potentials for ME-induced currents were generally more negative than −120 mV in rats pretreated with continuous morphine administration. In order to determine the amplitude of GAT currents in rats pretreated with morphine injections, the GAT inhibitor NO-711 (10 μM) was superfused in the presence of the $GABA_B$ antagonist CGP-55845 (10 μM). CGP-55845 was necessary to block outward GIRK currents induced by activation of the $GABA_B$ receptor through accumulation of GABA. Superfusion of NO-711 (10 μM)

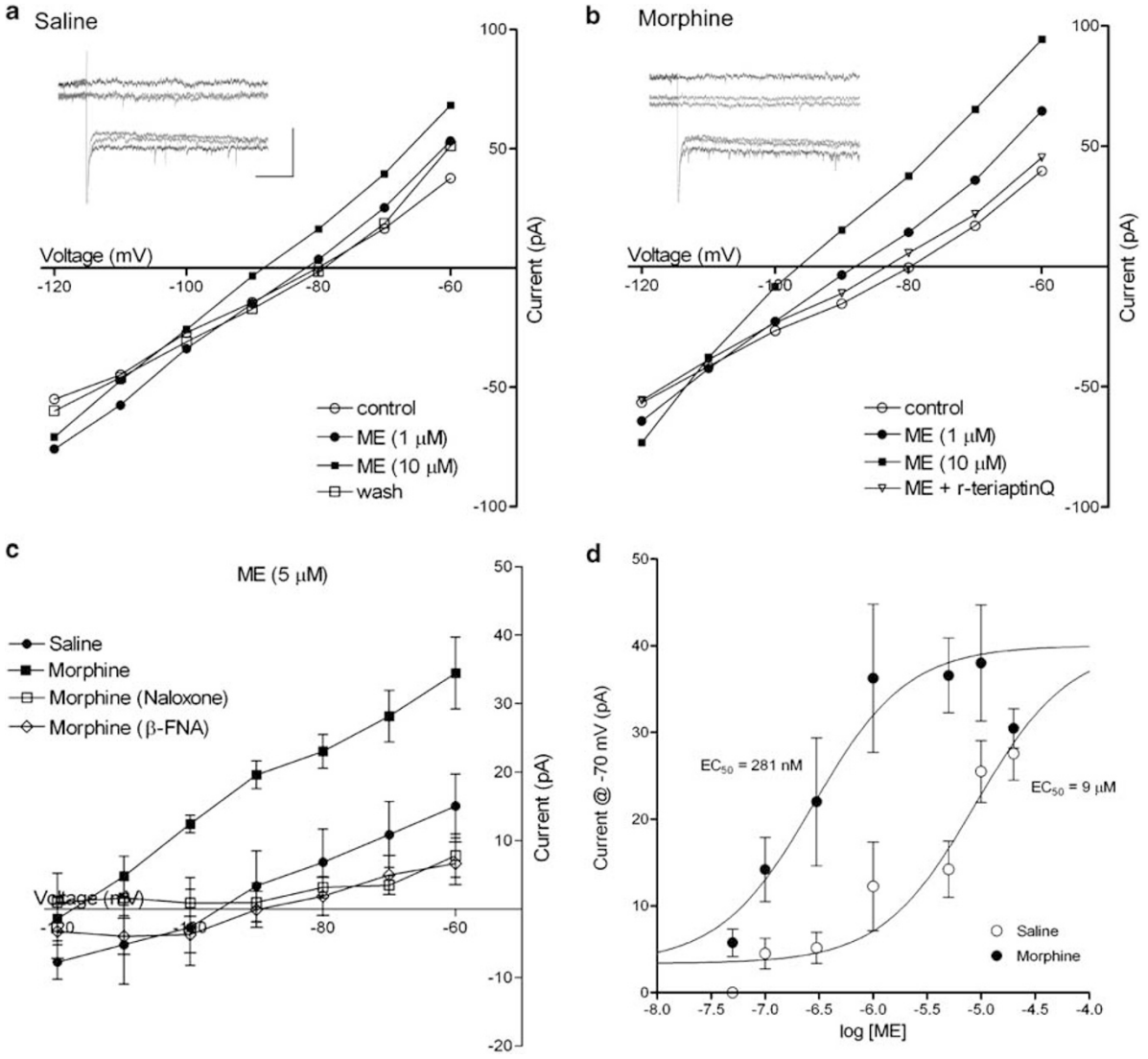

Figure 2 Repeated intermittent administration of morphine induces larger met-enkephalin (ME)-induced currents. (a) Representative current–voltage relationships to different concentrations of ME (1 and 10 μM) in a slice from a saline pretreated rat. The neuron was voltage-clamped to −70 mV. The inset shows currents elicited in steps to −120 mV and at the baseline (−70 mV) in control/wash (light traces) and ME (10 μM; dark traces). Scale bar is the same for both sets of traces (50 pA per 250 ms). (b) Representative current–voltage relationships from a morphine pretreated rat. The neuron was voltage-clamped to −70 mV. ME induced outward currents were inhibited by the GIRK blocker, r-teriaptinQ (10 nM). The inset shows currents elicited in steps to −120 mV and at the baseline (−70 mV) in control/r-teriaptinQ (light traces) and ME (10 μM; dark traces). (c) Averaged current–voltage relationships for ME (5 μM)—control for saline ($n=10$) and morphine ($n=7$) pretreated rats. Naloxone (1–10 μM; $n=5$) and β-funaltrexamine (β-FNA) (10 μM; $n=4$) reversed the effects of ME. (d) Concentration–response relationship for ME-induced outward currents in saline and morphine pretreated slices. Morphine pretreatment induces an increase in potency of ME. Number of cells for saline/morphine pretreated groups were 50 nM (4/4); 100 nM (5/6); 300 nM (3/6); 1 μM (4/4); 5 μM (7/9); 10 μM (4/10); 20 μM (13/14).

revealed that the GAT-mediated currents were small after repeated morphine administration and only contributed 2 ± 1 pA ($n=8$) to the ME-induced GIRK currents at −70 mV. Thus, upregulation of GAT does not account for the potentiated currents observed in morphine pretreated rats.

Desensitization of ME-Induced Responses

During dose–response experiments, we noticed that responses to longer applications of ME began to desensitize and that the desensitization was more prominent in morphine pretreated rats (see Figure 2d). In order to look at desensitization more carefully, responses to longer applications of ME (20 μM) were recorded. In Figure 5, current–voltage relationships were determined at the peak ME response and after 10 min of continuous ME superfusion. In saline pretreated rats, superfusion of a maximal concentration of ME (20 μM) produced peak currents of 35 ± 4 pA that decreased slightly to 30 ± 3 pA ($n=12$) after 10 min (Figure 5a and c). In morphine pretreated rats (Figure 5b and c), there was a marked desensitization of the currents from a peak of 32 ± 4 pA ($n=14$) to a plateau within 10 min of 14 ± 3 pA ($n=14$; Repeated measures ANOVA $F(1,24)=24.22$; $p<0.0001$). These results indicate that μ-opioid receptors desensitize with agonist application

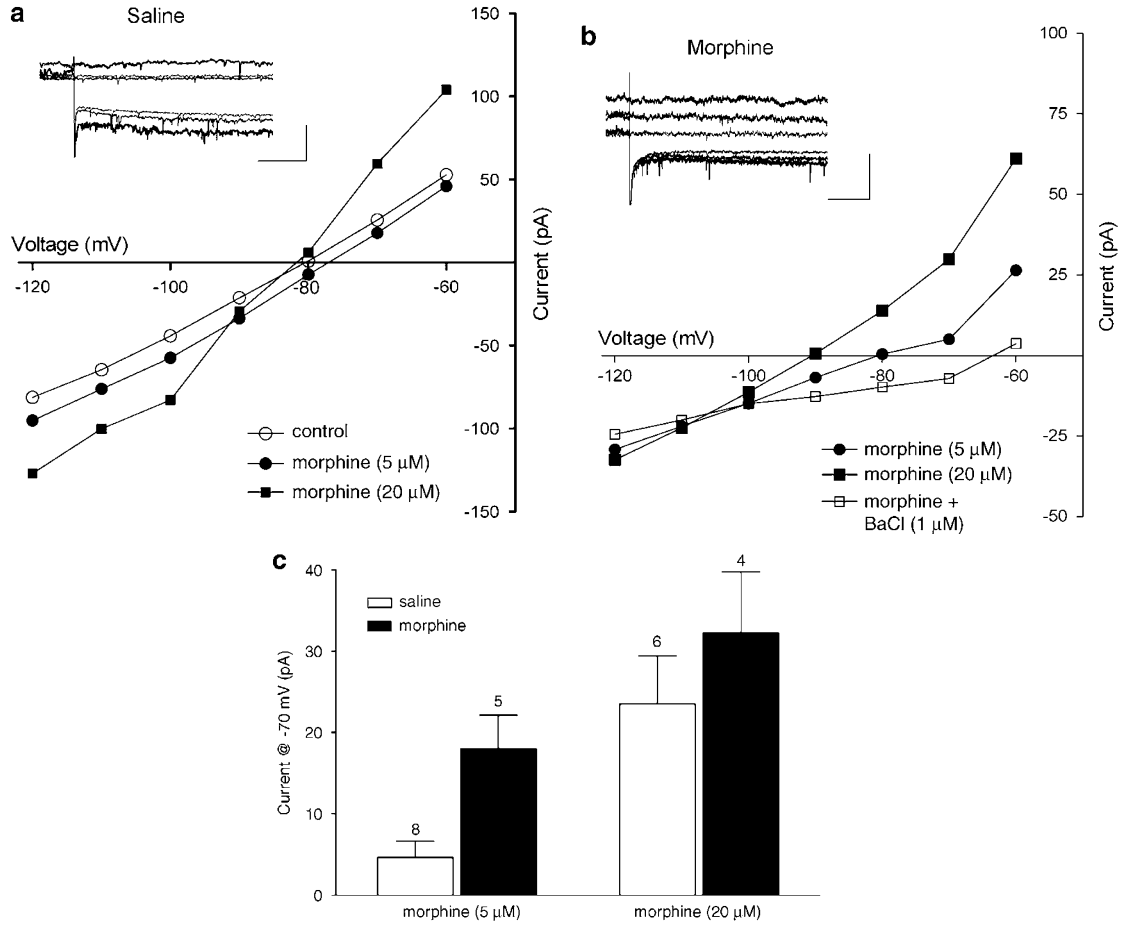

Figure 3 Repeated intermittent administration of morphine induces larger morphine-induced currents. (a) Representative current–voltage relationships to different concentrations of morphine (5 and 20 μM) in a slice from a saline pretreated rat. The neuron was voltage-clamped to −70 mV. The inset shows currents elicited in steps to −120 mV and at the baseline (−70 mV) in control (lightest traces) and morphine (5 μM (medium traces); 20 μM (dark traces)). Scale bar (50 pA per 250 ms). (b) Representative current–voltage relationships from a morphine pretreated rat. The neuron was voltage-clamped to −70 mV. Morphine-induced outward currents were inhibited with the GIRK blocker BaCl (1 mM). The inset shows currents elicited in steps to −120 mV and at the baseline (−70 mV) in BaCl (1 mM; light traces) and morphine (5 μM (medium traces); 20 μM (dark traces)). Scale bar (50 pA per 250 ms). (c) Bar graph showing the amplitudes of morphine-induced currents at −70 mV in saline and morphine pretreated rats. Currents are significantly larger in morphine pretreated rats (two-way ANOVA; $F(1, 19) = 5.424$; $p = 0.0311$).

in PAG neurons and that this desensitization is greater after morphine pretreatment.

Morphine Pretreatment Does Not Affect $GABA_B$ Coupling to GIRK Channels

μ-Opioid and $GABA_B$ receptors couple to the same population of GIRK channels in ventrolateral PAG neurons (Chieng and Christie, 1996). In order to control for the possibility that morphine pretreatment affects the number of GIRK channels in ventrolateral PAG neurons, $GABA_B$ receptor activation of GIRK channels was assessed using the $GABA_B$ agonist, baclofen (Figure 6). Currents elicited by a submaximal concentration of baclofen (10 μM) were similar for both morphine (24 ± 3 pA; $n = 16$) and saline (26 ± 4 pA; $n = 8$) pretreated rats. The baclofen-induced currents were inhibited with BaCl (1 mM; $n = 6$). In addition, there was no evidence for increased desensitization of these currents in morphine pretreated rats (see Figure 6b), similar to previous studies in the PAG (Bagley *et al*, 2005a; Chieng and Christie, 1996). These results indicate that morphine pretreatment did not alter the sensitivity of GIRK channels.

GIRK-Mediated Currents Are Desensitized in Rats Pretreated with Continuous Morphine

Previous studies using *continuous* morphine administration have demonstrated a profound uncoupling of μ-opioid receptors from GIRK channels when recording 1–8 h after the brain is removed from the source of morphine (Chieng and Christie, 1996; Bagley *et al*, 2005a, b). One difference between our repeated administration paradigm and earlier studies using *continuous* morphine administration is that morphine is absent from the rat for at least 24–72 h prior to electrophysiological recordings. This increased delay between morphine pretreatment and recordings was used because we hypothesized that GIRK currents are desensitized by circulating morphine at the time brain slices are cut in rats pretreated with morphine pellets. In order to directly test this hypothesis, rats were pretreated with morphine or placebo pellets for 72 h and then the pellets were removed.

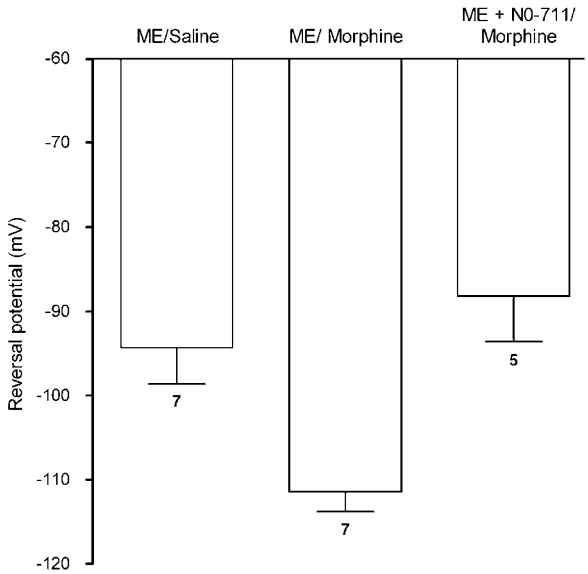

Figure 4 Repeated administration of morphine induces a current associated with the GABA transporter (GAT). Bar graph showing the reversal potentials of met-enkephalin (ME)-induced currents in saline (ME/saline) and morphine (ME/morphine) pretreated rats. The reversal potentials were significantly hyperpolarized in the morphine pretreated rats (one-way ANOVA; $F(2, 18) = 9.112$; $p = 0.0023$). Superfusion of the GAT inhibitor NO-711 (10 μM) in morphine pretreated rats reversed the shift.

Rats were tested on the hot plate 6 h after pellets were removed to allow circulating morphine to be cleared from the brain. Tolerance to the antinociceptive effect of morphine was evident on the hot plate test at this time point (Figure 7a) where strong desensitization of GIRK currents is observed in electrophysiological experiments (Chieng and Christie, 1996; Bagley *et al*, 2005a, b). The dose–response curve for morphine antinociception was significantly shifted to the right in morphine pretreated rats ($D_{50} = 11.8$ mg/kg; 95% CI = 7.0–16.6 mg/kg; $n = 3$) compared to placebo pretreated rats ($D_{50} = 2.7$ mg/kg; 95% CI = 2.1–3.4 mg/kg; $n = 4$; $F(2, 38) = 20.02$; $p < 0.0001$; Figure 7a).

In order to determine the extent of development of opioid dependence, a series of rats were tested with injections of naloxone (1 mg/kg, s.c.; Figure 7b). Rats were assessed for withdrawal behaviors for a total of 3 min within 30 min of the injection of naloxone. The total number of withdrawal behaviors was significantly greater in the Pellets IN group of rats (one-way ANOVA; $F(3, 28) = 40.62$; $p = 0.001$). There were almost no withdrawal behaviors in rats pretreated with morphine injections 24 h after the last morphine injection.

Given that only 30–40% of ventrolateral PAG neurons respond to opioids, it is difficult to assess whether ME induces GIRK currents in slices prepared from rats with pellets that remain implanted at the time of slicing. However, ME currents are easily observed in slices prepared greater than 24 h after removing the morphine pellets. In fact, a maximal concentration of ME (20 μM) induced similar peak currents in rats treated with placebo (29 ± 7 pA; $n = 4$) or morphine pellets (27 ± 5 pA; $n = 4$), but the currents in morphine pretreated rats desensitized significantly more than placebo pretreated rats following 10 min of ME superfusion (two-way repeated measures ANOVA; interaction $F(1, 6) = 62.71$, $p = 0.0002$; Figure 7c). These results suggest that uncoupling of opioid receptors from GIRK channels induced by *continuous* morphine administration reverses during the 24 h period in the absence of morphine but that μ-opioid receptor coupling to GIRK channels is more susceptible to agonist-induced desensitization 24 h later than in control rats. Therefore, cellular changes important for increasing desensitization to agonist contribute to antinociceptive tolerance whether rats are pretreated with morphine pellets or repeated injections of morphine.

DISCUSSION

These data indicate that antinociceptive tolerance induced by repeated intermittent administration of morphine is associated with both an increase in the potency of opioids acting on μ-opioid receptors coupled to GIRK channels in the PAG and a subsequent enhanced desensitization. Although acute morphine does not readily promote desensitization in the PAG and many other cell types, repeated morphine administration induces cellular changes that enhance agonist-induced desensitization necessary for antinociceptive tolerance. The results also indicate that the mechanism for tolerance in ventrolateral PAG neurons is similar whether induced with repeated injections or continuous administration of morphine.

Morphine-induced antinociception in the PAG is produced by disinhibition of PAG output neurons that project to the rostral ventromedial medulla (Moreau and Fields, 1986; Depaulis *et al*, 1987; Osborne *et al*, 1996). Previous studies have shown that the rat PAG predominately expresses the μ-receptor subtype (Chieng and Christie, 1994b; Vaughan and Christie, 1997) and that GIRK currents are elicited by μ-opioid receptors in the PAG (Chieng and Christie, 1994b; Bagley *et al*, 2005a). In the present study, ME-induced currents in both saline and morphine pretreated rats were blocked by the μ-selective inhibitor β-FNA. μ-Opioid receptors are localized on GABAergic neurons that tonically inhibit ventrolateral PAG output neurons. These receptors activate postsynaptic GIRK channels (Chieng and Christie, 1994b; Osborne *et al*, 1996; Ingram *et al*, 2007), inhibit Ca^{2+} channels (Connor and Christie, 1998) and inhibit GABA release from presynaptic terminals via activation of K_v channels (Chieng and Christie, 1994a; Vaughan and Christie, 1997; Vaughan *et al*, 1997). Previous studies have shown that *continuous* morphine administration alters the signaling through each of these pathways. μ-Opioid receptor coupling to GIRK and K_v potassium channels and voltage-gated Ca^{2+} channels in the ventrolateral PAG is decreased after 5 days of *continuous* morphine administration (Christie *et al*, 1987; Chieng and Christie, 1996; Bagley *et al*, 2005a, b). Uncoupling of the μ-opioid receptors from these effectors is consistent with decreased antinociception, or tolerance to opiates. In contrast, μ-opioid receptor inhibition of GABA release is enhanced via a cAMP-dependent mechanism (Ingram *et al*, 1998; Hack *et al*, 2003) suggesting that *continuous* morphine administration also induces supersensitivity of μ-opioid receptor coupling to adenylyl cyclase.

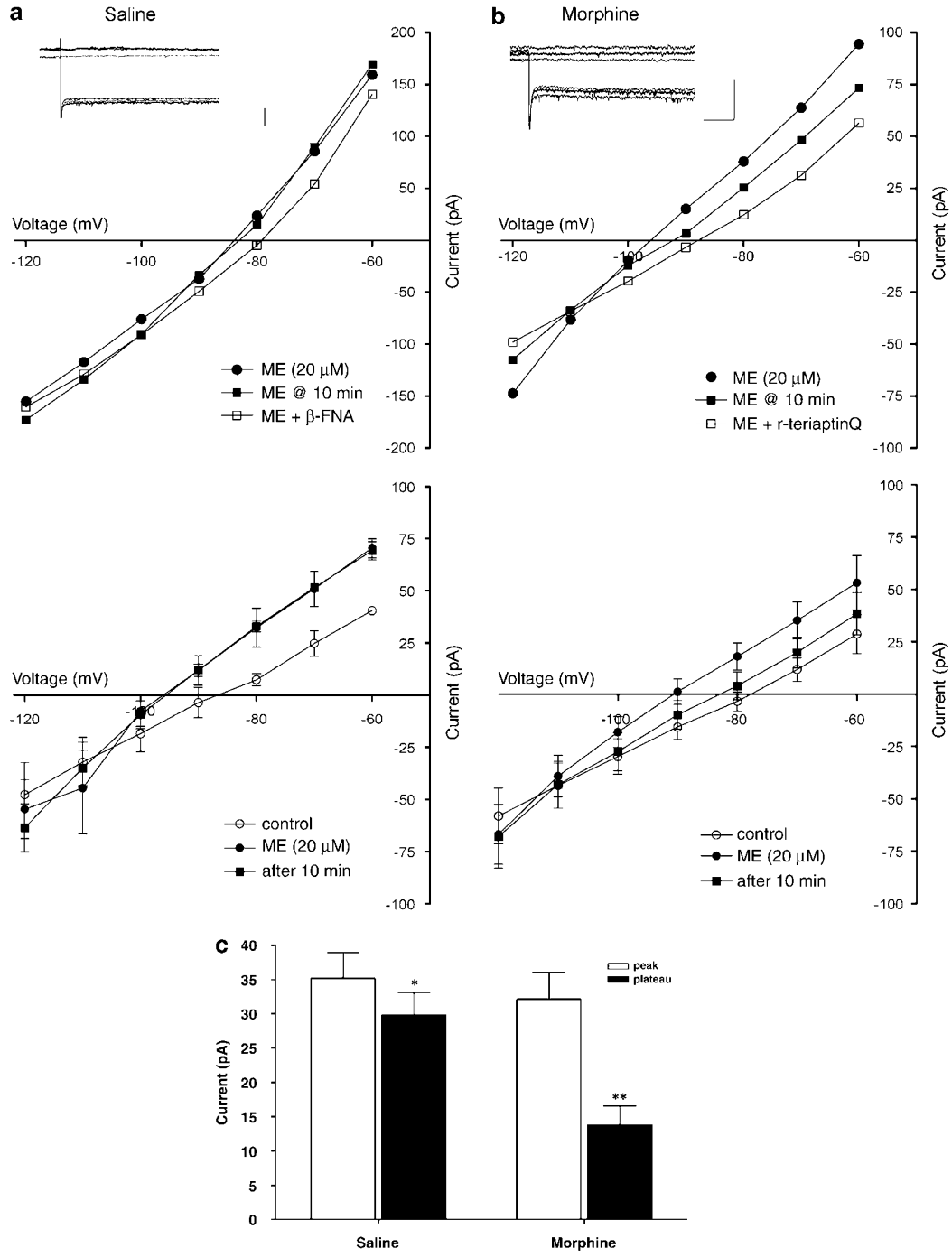

Figure 5 Repeated intermittent administration of morphine increases desensitization of met-enkephalin (ME)-induced responses from morphine tolerant rats. (a) Top left panel: representative current–voltage relationships for a saline pretreated rat voltage-clamped at −70 mV at the peak ME (20 μM) response and after 10 min of superfusion. The inset shows currents elicited in steps to −120 mV and at the baseline (−70 mV) by ME (20 μM) at the peak response (medium traces) and after 10 min superfusion (dark traces). The remaining current was blocked by the selective μ-opioid receptor inhibitor β-funaltrexamine (β-FNA) (10 μM; light traces). Scale bar (100 pA per 250 ms). Middle left panel: averaged current–voltage relationships for saline pretreated rats voltage-clamped at −70 mV at the peak ME (20 μM) response and after 10 min of superfusion ($n = 3$). (b) Top right panel: representative current–voltage relationships for a morphine pretreated rat voltage-clamped at −70 mV at the peak ME (20 μM) response and after 10 min of superfusion. The inset shows currents elicited in steps to −120 mV and at the baseline (−70 mV) by ME (20 μM) at the peak response (medium traces) and after 10 min superfusion (dark traces). The remaining current was blocked by the selective GIRK blocker r-teriaptinQ (10 nM; light traces). Scale bar (100 pA per 250 ms). Middle right panel: averaged current–voltage relationships for morphine pretreated rats voltage-clamped at −70 mV at peak ME (20 μM) and after 10 min of superfusion ($n = 7$). (c) Bar graph showing amount of desensitization of peak outward ME-induced currents after 10 min in saline ($n = 11$) and morphine ($n = 14$) pretreated rats. The higher n in bar graph is the addition of experiments where current–voltage relationships were not run during the experiment. ME-induced currents significantly desensitize within 10 min (*$p < 0.05$, **$p < 0.001$).

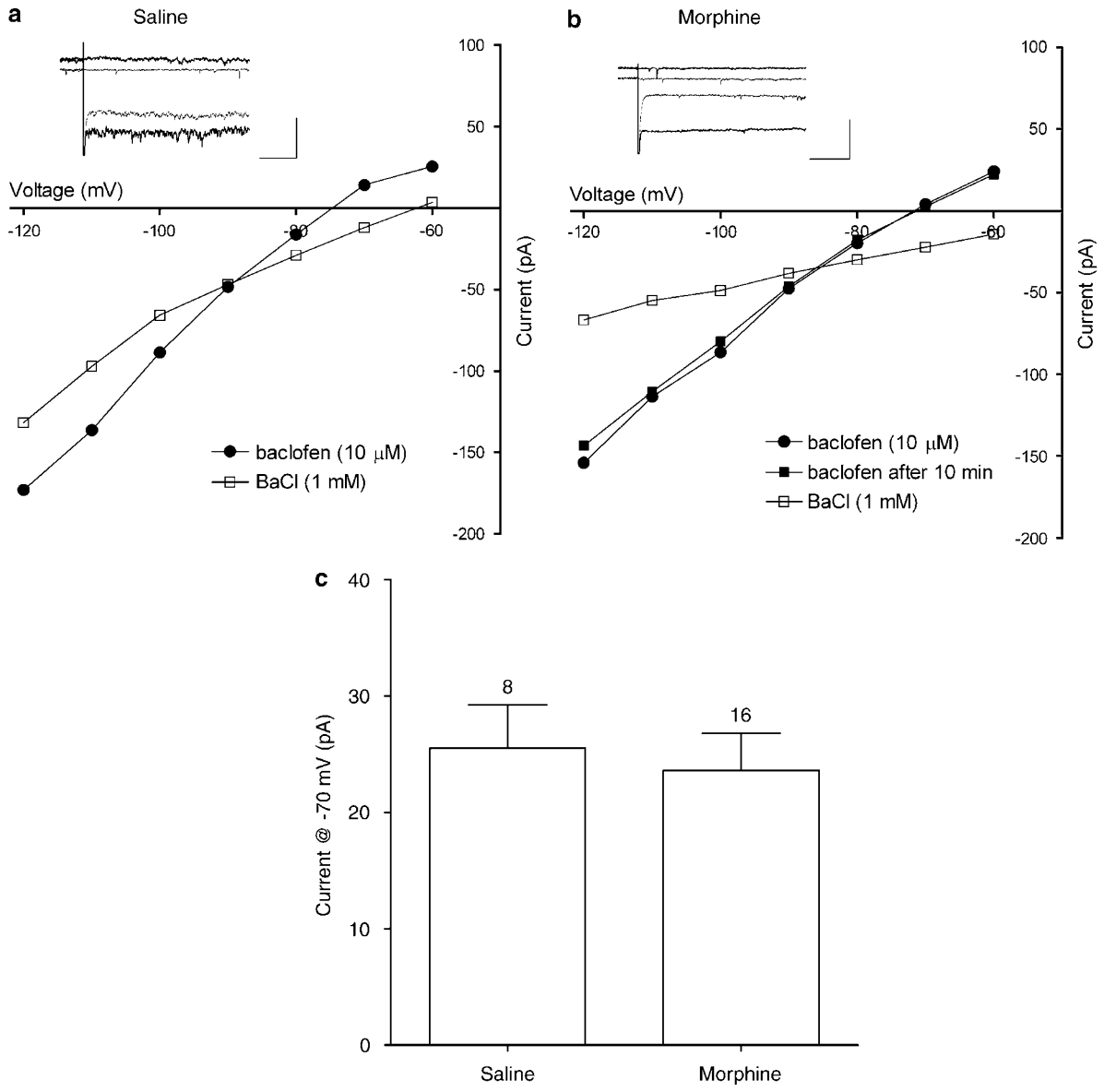

Figure 6 Baclofen-induced currents are not altered with morphine pretreatment. (a) Representative current–voltage relationships to a submaximal concentration of baclofen (10 μM) in a slice from a saline pretreated rat. The neuron was voltage-clamped to −70 mV. The inset shows currents elicited in steps to −120 mV and at the baseline (−70 mV) in baclofen (dark traces) and after blockade with BaCl (1 mM; light traces). Scale bar (100 pA per 200 ms). (b) Representative current–voltage relationships to a submaximal concentration of baclofen (10 μM) in a slice from a morphine pretreated rat. The neuron was voltage-clamped to −70 mV. Note that the current–voltage relationship does not change with 10 min baclofen superfusion. The inset shows currents elicited in steps to −120 mV and at the baseline (−70 mV) in baclofen (dark traces) and after blockade with BaCl (1 mM; light traces). Scale bar (100 pA per 200 ms). (c) Bar graph showing the compiled baclofen-induced currents in saline and morphine pretreated rats.

It has been difficult to understand how agonist sensitivity at the same receptor can be simultaneously increased and decreased. The present data indicate that enhanced agonist sensitivity is also observed in μ-opioid receptor coupling to GIRK channels after intermittent morphine administration but that uncoupling of the receptors and GIRK channels occurs rapidly. These results suggest that the differences in observed agonist sensitivity are not due to differences in morphine administration but are related to specific interactions between μ-opioid receptors and effectors that determine the extent and/or rate of uncoupling.

The intermittent repeated morphine injection paradigm is advantageous in several ways. First, tolerance is long-lasting and is maintained up to 1 week after the last injection (Morgan *et al*, 2005b). Second, tolerance is largely dissociated from opioid dependence because rats do not display as intense naloxone-precipitated withdrawal behaviors in contrast to rats implanted with morphine pellets (Cerletti *et al*, 1976). The primary advantage of repeated injections is that there is no morphine in the brain for at least 12 h prior to the behavior studies or electrophysiological recordings, removing potential confounds due to receptor desensitization during the recordings. Although it can be argued that the rat is undergoing withdrawal during this period, there are no obvious physical signs of withdrawal such as wet dog shakes, jumping, or diarrhea

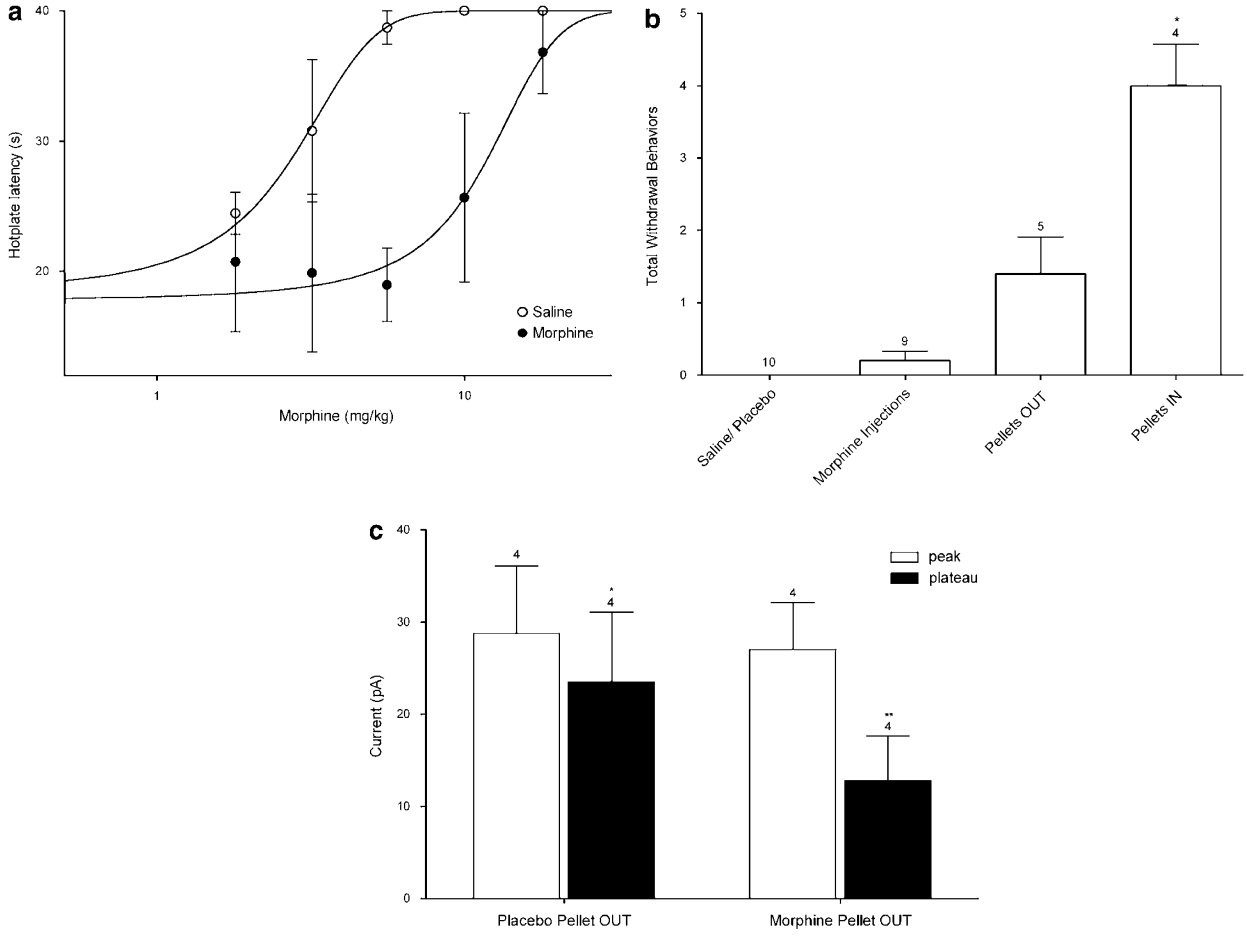

Figure 7 GIRK-mediated currents are desensitized in rats treated with continuous morphine. (a) Cumulative dose (s.c.)–response relationships using the hot plate test were determined on day 3 in rats pretreated with placebo ($n = 4$) and morphine ($n = 3$) pellets. Rats pretreated with morphine were tolerant as indicated by a rightward shift in the dose–response curve. (b) Bar graph showing the number of total withdrawal behaviors precipitated with an injection of naloxone (1 mg/kg, s.c.). The naloxone injection was given 24 h following the last injections in rats pretreated with repeated saline or morphine injections (s.c.), 24 h after removal of the morphine pellets in the Pellets OUT group and prior to removal of pellets in the Pellets IN group. Only the Pellets IN group elicited significant naloxone-precipitated withdrawal behaviors. (c) Bar graph comparing ME (20 μM)-induced currents from rats treated with either placebo or morphine pellets for 3 days and removed at least 24 h prior to recordings. There is no evidence for decreased peak ME-induced currents under these conditions suggesting that the μ-opioid receptors have recovered from desensitization but morphine pretreated rats desensitize more within 10 min ($^{*}p < 0.01$, $^{**}p < 0.001$).

in these rats. In addition, naloxone superfusion has no effect in the absence of an opioid agonist in whole-cell patch-clamp recordings from these rats.

ME-induced GIRK currents are larger and the concentration–response relationship is shifted to the left in rats pretreated with morphine compared to saline. Although supersensitivity of μ-opioid receptor coupling to adenylyl cyclase in the PAG has been reported previously (Ingram *et al*, 1998), it is the first time that supersensitivity has been observed for μ-opioid receptor coupling to membrane delimited potassium channels (ie GIRK channels). The increase in GIRK activation is probably evident because morphine is not present. In the case of *continuous* morphine pretreatment, morphine is generally present until brain slices are cut and in some cases is added to the incubation solution (Christie *et al*, 1987; Chieng and Christie, 1996; Bagley *et al*, 2005a, b). Therefore, it is likely that μ-opioid receptor coupling to GIRK channels is fully desensitized due to the presence of agonist during the recordings. Consistent with this idea is the observation that recovery from desensitization is slow after chronic morphine treatment (Dang and Williams, 2004). The present data are also consistent with previous studies showing profound GIRK desensitization with chronic morphine pretreatment (Christie *et al*, 1987; Chieng and Christie, 1996; Bagley *et al*, 2005a, b). The important distinction between repeated and continuous morphine administration is that the absence of agonist, in the case of repeated intermittent administration, allows one to observe the supersensitive peak agonist effect and the development of increased desensitization of the response with continued application of agonist.

The cellular mechanism underlying supersensitivity of μ-opioid receptor coupling to GIRK channels is not known. Several possible mechanisms may contribute to supersensitivity of these responses, including downregulation of endopeptidases with chronic morphine treatment, decreased desensitization or internalization of μ-opioid receptors, changes in coupling to G-proteins and alterations in agonist-receptor binding. The increase in both ME and

morphine-induced GIRK currents in morphine pretreated rats suggests that downregulation of endopeptidases is not a significant factor in these responses. In fact, studies have generally found either no significant change or increased peptidase activity induced by morphine (Malfroy *et al*, 1978; Irazusta *et al*, 2003). The supersensitivity is not caused by decreased desensitization because we provide evidence that desensitization is enhanced in morphine pretreated rats. Increased desensitization also has been observed in locus coeruleus neurons after chronic morphine pretreatment (Dang and Williams, 2005).

Furthermore, there is strong evidence that the cellular mechanisms of desensitization are required for tolerance. Recruitment of β-arrestin2 is necessary for desensitization of G-protein-coupled receptors (Gainetdinov *et al*, 2004) and mice lacking β-arrestin2 display an increased sensitivity to morphine and decreased tolerance on the hot plate test compared to wild-type controls (Bohn *et al*, 1999, 2000). Mice lacking β-arrestin2 also display increased GTPγS binding in PAG membranes without a change in μ-opioid receptor density (Bohn *et al*, 1999, 2000). Although acute postsynaptic sensitivity of μ-opioid receptor activation of GIRK channels has been reported to be the same in β-arrestin2 knock-out and wild-type mice (Bradaia *et al*, 2005), μ-opioid receptor supersensitivity may only occur after prolonged, or repeated morphine treatment and not with acute administration.

The fact that opioid activation of GIRK channels and inhibition of adenylyl cyclase is potentiated after either repeated or *continuous* morphine administration, respectively, suggests that there is a change in the agonist-receptor interaction that increases G-protein activation or that the effector system responds more efficiently to G-proteins. Although chronic morphine induces increased sensitivity of adenylyl cyclase to $G_{\beta\gamma}$ activation in the guinea pig (Chakrabarti *et al*, 1998), it is unlikely that an increase in sensitivity of GIRK channels occurs with the morphine treatment paradigm used in this study. There were no differences in the ability of $GABA_B$ receptors to stimulate GIRK currents in saline or morphine pretreated rats. However, there are several potential mechanisms that may increase signaling through G-proteins, including alterations in G-proteins that couple to the μ-opioid receptor. Increased agonist potency may simply increase the number of $\beta\gamma$ subunits after activation of the G_i-protein. In addition, several studies have also provided evidence that the μ-opioid receptor couples to the $G_{\alpha s}$ subtype after chronic morphine pretreatment (Chakrabarti *et al*, 1998, 2005; Wang and Burns, 2006). GIRK channels are directly activated by $\beta\gamma$ subunits (Huang *et al*, 1995), so an increase in $\beta\gamma$ release after increased excitatory $G_{\alpha s}$ signaling mediated by μ-opioid receptors could also increase activation of GIRK channels. Interestingly, μ-opioid receptor inhibition of GABA release is potentiated via a $G_{\alpha i}$-adenylyl cyclase-PKA signaling pathway in rats pretreated with continuous morphine (Ingram *et al*, 1998) indicating that this μ-opioid receptor coupling is not diminished by desensitization. Therefore, it is likely that μ-opioid receptors become uncoupled from some effectors (ie membrane delimited potassium channels) more readily than other effectors, (ie adenylyl cyclase) after chronic morphine administration.

In conclusion, the results of this paper show electrophysiological and behavioral evidence that an increase in opioid agonist potency and desensitization of μ-opioid receptors in the PAG are correlated with the development of tolerance to intermittent systemic morphine administration. In addition, the results indicate that similar cellular changes are induced with both repeated and continuous administration of morphine. Further studies are necessary to understand the cellular mechanisms that produce an increase in agonist potency at μ-opioid receptors. One exciting possibility is that the cellular pathways mediating supersensitivity and desensitization are different such that agonist potency and analgesia may be potentiated while blocking the changes associated with desensitization or tolerance. Further research on these cellular mechanisms may lead to the development of new pain medications that are resistant to tolerance.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that, except for income received from primary employers, no financial support or compensation has been received over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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