

## ENKEPHALIN RELEASE PROMOTES HOMEOSTATIC INCREASES IN CONSTITUTIVELY ACTIVE MU OPIOID RECEPTORS DURING MORPHINE WITHDRAWAL

J. R. SHOBLOCK\* AND N. T. MAIDMENT

Department of Psychiatry and Biobehavioral Sciences, Semel Institute for Neuroscience & Human Behavior, 760 Westwood Plaza, UCLA, Los Angeles, CA 90024, USA

**Abstract**—We previously demonstrated that naloxone administration produces a robust conditioned place aversion (CPA) in opiate-naive rodents by blocking the action of enkephalins at  $\mu$  opioid receptors (MORs). The aversive response to naloxone is potentiated by prior exposure to morphine. Morphine-induced MOR constitutive activity is hypothesized to underlie this enhanced effect of naloxone, an inverse agonist at the MOR. We sought additional evidence for the role of constitutively active MORs in this morphine-induced enhancement using the pro-enkephalin knockout (pENK<sup>-/-</sup>) mouse, which is devoid of naloxone CPA in the morphine-naive state. Naloxone, but not the neutral antagonist, 6- $\beta$ -naloxol, produced CPA and physical withdrawal signs in pENK<sup>-/-</sup> mice when administered 2 h, but not 20 h, after morphine administration. Naloxone-precipitated physical withdrawal signs were attenuated in the pENK<sup>-/-</sup> mice relative to wild-type (WT) animals. In both WT and pENK<sup>-/-</sup> mice, naloxone-precipitated withdrawal jumping was greatest when naloxone was administered 2 h after morphine treatment and diminished at 3 h, in agreement with previous estimates of the time course for morphine-induced MOR constitutive activity *in vitro*. However, naloxone regained an ability to precipitate physical withdrawal in the WT, but not the pENK<sup>-/-</sup> mice when administered 4.5 h after morphine administration. Taken together, the data suggest that a compensatory increase in enkephalin release during spontaneous morphine withdrawal promotes a second period of MOR constitutive activity in WT mice that is responsible for the enhanced naloxone aversion observed in such animals even when naloxone is administered 20 h after morphine. The endogenous enkephalin system and MOR constitutive activity may therefore play vital roles in hedonic homeostatic dysregulation following chronic opiate administration. © 2007 Published by Elsevier Ltd on behalf of IBRO.

**Key words:** naloxone, conditioned place aversion, dependence, knockout mice, opiate addiction, inverse agonist.

G-protein-coupled receptors, including the  $\mu$  opioid receptor (MOR), are believed to exist in a spontaneous equilibrium between the active and inactive states, such that a

\*Corresponding author. Present address: Johnson & Johnson, Pharmaceutical Research & Development, LLC, 3210 Merryfield Row, San Diego CA 92121, USA. Tel: +1-858-320-3483; fax: +1-858-450-2040. E-mail address: jshobloc@prdus.jnj.com (J. R. Shoblock).

**Abbreviations:** ANOVA, analysis of variance; CPA, conditioned place aversion; DOR, delta opioid receptor; MOR, mu opioid receptor; pENK<sup>-/-</sup>, pro-enkephalin knockout; SNK, Student–Newman–Keuls; WT, wild-type.

0306-4522/07/\$30.00+0.00 © 2007 Published by Elsevier Ltd on behalf of IBRO.  
doi:10.1016/j.neuroscience.2007.05.011

basal level of signaling occurs even in the absence of agonist binding (see Seifert and Wenzel-Seifert, 2002 for review). Exposure to agonist increases the number of receptors in the active state, either through selection or through induction (Hunyady et al., 2003). It has also been suggested that exposure to some agonists, such as morphine, can produce a long-lasting increase in constitutively active MORs (Wang et al., 2001; Liu and Prather, 2001) that outlasts exposure to the agonist and is most likely a result of a change in receptor phosphorylation (Wang et al., 1994; Cruz et al., 1996; Wang et al., 1996, 1999, 2001).

While most of the early studies on constitutively active MORs employed *in vitro* approaches (Wang et al., 1994, 2001; Cruz et al., 1996; Neilan et al., 1999; Burford et al., 2000; Liu and Prather, 2001), recently attempts were made to study these receptors *in vivo* to determine their physiological significance and potential as therapeutic targets (Selley et al., 2000; Wang et al., 2004; Raehal et al., 2005; Freye and Levy, 2005; Heinzen et al., 2005; Shoblock and Maidment, 2006). Several behavioral studies supported the hypothesis that constitutively active MORs are involved in physical dependence. Inverse MOR agonists produced greater withdrawal jumping compared with neutral antagonists (Wang et al., 1994, 2001, 2004; Raehal et al., 2005), a neutral antagonist attenuated the withdrawal jumping produced by the inverse agonist naloxone (Bilsky et al., 1996), and differences in time and dose dependency were demonstrated between neutral antagonists and inverse agonists (Wang et al., 2004; Walker and Sterious, 2005; Shoblock and Maidment, 2006).

Constitutively active MORs may also be involved in psychological dependence. Morphine pretreatment potentiates naloxone-conditioned place aversion (CPA) (Schulteis et al., 1994; Parker and Joshi, 1998; Blokhina et al., 2000; Parker et al., 2002; Azar et al., 2003), an effect that outlasts the presence of morphine (Parker et al., 2002). Such enhanced naloxone CPA following morphine treatment is thought to reflect the negative motivational and affective state associated with morphine withdrawal (Mucha and Walker, 1987; Gracy et al., 2001) and may be a product of the counteradaptive processes that lead to dysregulation of hedonic homeostasis and psychological dependence (Kreek and Koob, 1998). We recently provided evidence for the involvement of constitutively active MORs in this process by demonstrating that morphine pretreatment potentiates CPA to the inverse agonist, naloxone, but not to the neutral antagonists 6- $\alpha$ - or 6- $\beta$ -naloxol (Shoblock and Maidment, 2006).

We previously demonstrated that, in the morphine-naive state, naloxone produces aversion by blocking the action of endogenous pro-enkephalin-derived peptides at the MOR, because neither MOR knockout nor pro-enkephalin knockout (pENK<sup>-/-</sup>) mice display naloxone CPA (Skoubis et al., 2001, 2005). Furthermore, the delta opioid receptor (DOR) antagonist, naltrindole, is devoid of aversive effects (Skoubis et al., 2001; Shippenberg et al., 1987; Bals-Kubik et al., 1989; De Vries et al., 1995). Naloxone-precipitated physical withdrawal was also dependent on the presence of the MOR (Matthes et al., 1996). In the current study, we sought to determine whether, following morphine pretreatment, the induction of constitutively active MORs would render the presence of endogenous pro-enkephalin-derived peptides unnecessary for the expression of naloxone CPA and physical withdrawal symptoms by virtue of the inverse agonist property of the drug, thereby reinforcing the importance of MOR constitutive activity to morphine psychological and physical dependence.

## EXPERIMENTAL PROCEDURES

### Subjects

Adult male C57BL/6 wild-type (WT) and age-matched (8–10 weeks old at start of experiment) pENK<sup>-/-</sup> (Konig et al., 1996) mice were obtained from The Jackson Laboratories (Bar Harbor, ME, USA) or bred in-house from breeders obtained from The Jackson Laboratories. The pENK<sup>-/-</sup> congenic strain was previously backcrossed at least 10 generations onto a C57BL/6 background by The Jackson Laboratories. All experiments were carried out during the light phase of a 12-h light/dark cycle (0700–1900 h). Food and water were available *ad libitum*. All procedures complied with the NIH Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and any suffering.

### Drugs

Naloxone (10–100 mg/kg; Sigma, St. Louis, MO, USA), 6- $\beta$ -naloxol (10–50 mg/kg; National Institute on Drug Abuse [NIDA]), 6- $\alpha$ -naloxol (10 mg/kg; NIDA), and morphine (20 mg/kg) were dissolved in saline and administered s.c. in a volume of 10 ml/kg.

### CPA protocol

Details of the nonbiased conditioning apparatus were described previously (Shoblock and Maidment, 2006). Briefly, a square arena was divided into three chambers: a gray neutral start chamber and two black and white conditioning chambers that were discriminated based on different patterns (black and white checkers or black and white cow patterns) and different olfactory cues (almond or lemon scent, McCormick and Co., Hunt Valley, MD, USA). The two conditioning chambers were accessible via the neutral chamber through guillotine doors. Animals were habituated to the apparatus on the first day by being placed in the gray chamber and given free access to all three chambers for 15 min. Subsequently, animals were conditioned three times each to saline in one conditioning chamber and drug (naloxone 10 mg/kg, 6- $\beta$ -naloxol 10 mg/kg, or saline as a control, all s.c.) in the opposite conditioning chamber, starting with saline. The drug-paired chamber was chosen randomly. Immediately after injection, the animal was confined to the chamber for 30 min and observed for the presence or absence of jumping or diarrhea. Locomotor ac-

tivity was automatically recorded via photobeams (Coulbourn Instruments, Allentown, PA, USA). Following conditioning, animals were tested for CPA in a drug-free state. Animals were placed in the gray chamber with free access to all three chambers for 15 min and the time spent in each chamber and locomotor activity was recorded.

In the first experiment, pENK<sup>-/-</sup> animals received morphine (20 mg/kg, s.c.) to induce constitutively active MOR or saline (s.c.) 20 h before each saline and drug conditioning session and 20 h before the CPA test session, in the home cage, consistent with our previous study (Shoblock and Maidment, 2006), with the goal of conducting naloxone conditioning after clearance of morphine. Saline and drug conditioning sessions were therefore conducted on alternate days. In the second experiment, all animals received morphine (20 mg/kg, s.c.) 2 h before only the drug conditioning session, in the home cage, to test the conditioning effect of naloxone and 6- $\beta$ -naloxol when administered during the peak level of constitutive activity. Morphine was not given before saline conditioning because the high levels of morphine at 2 h would result in a place preference for the saline chamber. Because morphine was only given before drug conditioning, animals were conditioned to both saline and drug each day (days 2–4), 4 h apart, to avoid extended periods of morphine withdrawal. The third experiment was similar in design to the second experiment with groups of pENK<sup>-/-</sup> mice being administered morphine 2 h before each of three naloxone (but not saline) conditioning sessions. In this case, however, 6- $\beta$ -naloxol (10 or 50 mg/kg) or saline was administered just prior to naloxone (10 mg/kg) injection.

### Withdrawal jumping protocol

Quantitative analysis of withdrawal jumping in both WT and pENK<sup>-/-</sup> mice was assessed using additional sets of animals. In one experiment the dose–response curve for naloxone-precipitated withdrawal jumping following two daily high dose morphine injections was determined. Animals received an injection of morphine (100 mg/kg, s.c.) on day 1 and 3 h later were habituated to clear cylindrical chambers for 1 h. On day 2, 24 h after the first morphine injection, animals received a second injection of morphine (100 mg/kg, s.c.) and 3 h later were placed in the chambers again for a 1-h habituation. Animals then received an injection of naloxone (10–100 mg/kg, s.c.) and the number of jumps was recorded over a 30-min period.

The time course of naloxone-precipitated withdrawal jumping was determined in a separate experiment. Naloxone (10 mg/kg, s.c.) was administered 1, 2, 3, or 4.5 h after a single administration of a lower dose of morphine (20 mg/kg, s.c.), in separate groups of animals, and jumping was recorded over a 30-min period. As a comparison, the neutral antagonist 6- $\alpha$ -naloxol (10 mg/kg, s.c.), which has a higher affinity for the MOR than naloxone (Wang et al., 2001) and which is more potent than naloxone in morphine-naive animals, was also tested.

### Data analysis

Because the short morphine pretreatment time used in the second CPA experiment precluded the use of saline-conditioned controls (the animals would develop a place preference to morphine), all CPA data were analyzed for significant place aversion with a one-group *t* test. The one-group *t* test compared the time spent in the drug-paired chamber in each individual group with the theoretical mean of 300 s, which represents equal time spent in all chambers, or no aversion. The Bonferroni correction was used to control for the multiple comparisons within each experiment. Subsequently, significant differences between treatments were tested, where indicated, using analysis of variance (ANOVA) on the time spent in the drug-paired chamber. Locomotor activity during the conditioning sessions from the second CPA experiment was an-

alyzed as total distance traveled with a four-way ANOVA for naloxone/6- $\beta$ -naloxol treatment, dose, conditioning day, and conditioning session (first control conditioning vs. second drug conditioning), with two levels of repeated measures for day and session. The presence of diarrhea or jumping during the drug conditioning sessions was recorded in a binary manner (present or not present) and, when analyzed, Fisher's exact test comparing percentages of animals expressing the behavior was used. Because naloxone produced withdrawal jumping in all animals with the shorter morphine pretreatment, these data were instead analyzed as the total number of jumps. The number of jumps 4 h after 100 mg/kg morphine was analyzed using a two-way ANOVA genotype $\times$ dose. Jumping at 1–4.5 h after morphine administration was analyzed as total number of jumps with a two-way ANOVA for genotype and time or drug and time. All tests were two-tailed, except for tests of aversion. Because only the hypothesized effect of aversion, and not preference, was of concern (Shoblock and Maidment, 2006), one-tailed tests were used for the analyses. ANOVAs were followed by a Student–Newman–Keuls (SNK) post hoc test when appropriate. Analyses were carried out using Statistica software (Tulsa, OK, USA).

## RESULTS

### Naloxone did not produce CPA or jumping in pENK<sup>-/-</sup> mice when administered 20 h after morphine

There was no significant CPA produced by 10 mg/kg naloxone in pENK<sup>-/-</sup> mice, regardless of whether the animals were pretreated with chronic morphine or chronic saline (Fig. 1;  $P > 0.05$ , one-group  $t$  tests with Bonferroni correction). In our previous study (Shoblock and Maidment, 2006), this dose of naloxone produced CPA in WT mice, an effect that was potentiated by prior morphine treatment.

Also contrasting with our previous findings in WT mice was the degree to which naloxone-precipitated physical withdrawal signs developed in the pENK<sup>-/-</sup> mice. While naloxone-precipitated withdrawal jumping was apparent in

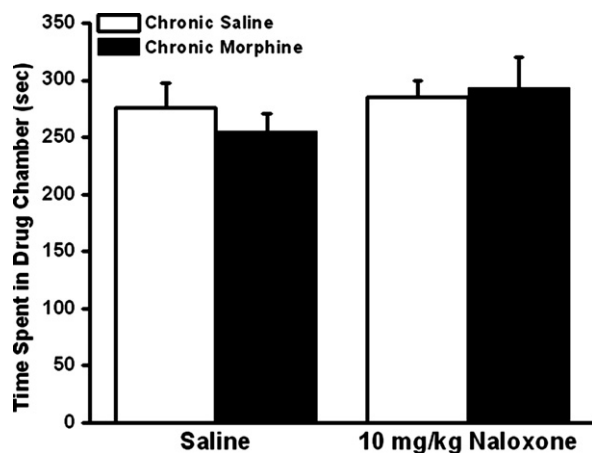


Fig. 1. Naloxone fails to induce CPA in pENK<sup>-/-</sup> mice. No CPA to naloxone was observed in pENK<sup>-/-</sup> mice irrespective of morphine or saline pretreatment 20 h prior to naloxone conditioning. Such a morphine pretreatment regime was previously shown to potentiate naloxone CPA in WT mice (Shoblock and Maidment, 2006).

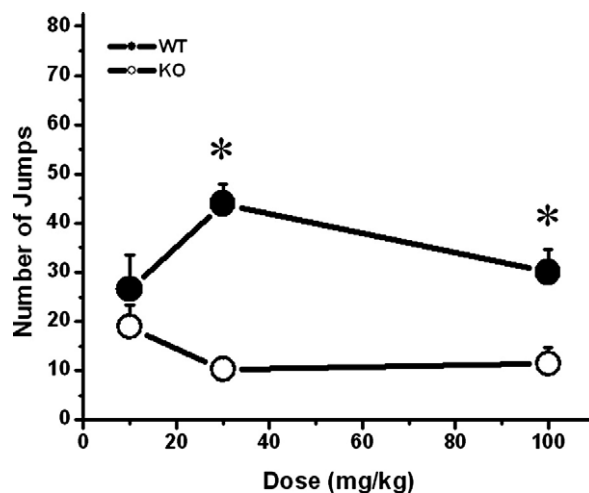


Fig. 2. Withdrawal jumping 4 h after morphine administration in pENK<sup>-/-</sup> and WT mice. In a separate group of animals, naloxone was less effective at producing withdrawal jumping 4 h after the second of two daily injections of morphine (100 mg/kg) in pENK<sup>-/-</sup> mice compared with WT animals (\* $P < 0.0002$ , from WT vs. pENK<sup>-/-</sup>, SNK post hoc).

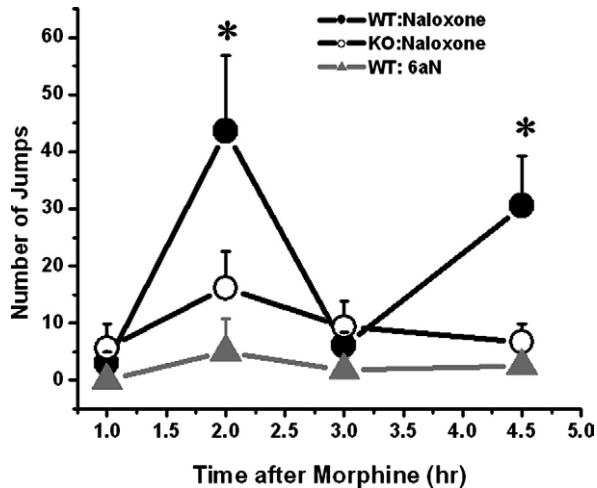
100% of the WT mice by the third conditioning session in our previous study (Shoblock and Maidment, 2006), naloxone failed to precipitate any withdrawal jumping in the pENK<sup>-/-</sup> mice (data not shown). Morphine-pretreated pENK<sup>-/-</sup> mice conditioned to saline also displayed low levels of spontaneous withdrawal, as measured by the presence of diarrhea (40% of animals) and jumping (0% of animals).

### pENK<sup>-/-</sup> mice display reduced naloxone-precipitated jumping 4 h after morphine

pENK<sup>-/-</sup> mice displayed less naloxone-precipitated withdrawal jumping than WT mice in a dose-dependent fashion when naloxone was administered 4 h after the second of two morphine (100 mg/kg, s.c.) injections (Fig. 2; effect of genotype $\times$ dose interaction,  $F_{2,30} = 4.62$ ,  $P < 0.02$ ,  $P < 0.0002$  SNK post hoc at 30 mg/kg).

### WT and pENK<sup>-/-</sup> mice display a different time course of naloxone-precipitated withdrawal jumping

When naloxone (10 mg/kg) was administered at different times after a single low dose of morphine (20 mg/kg) a triphasic pattern of withdrawal jumping was apparent in the WT but not the pENK<sup>-/-</sup> animals (Fig. 3;  $F_{3,42} = 3.77$ ,  $P < 0.02$ , genotype $\times$ time interaction). Naloxone precipitated less withdrawal jumping in the pENK<sup>-/-</sup> mice compared with WT mice when administered 2 h, but not 3 h, and again at 4.5 h after morphine administration ( $P < 0.01$  for 2 and 4.5 h SNK post hoc), as a result of naloxone reducing the ability to precipitate withdrawal in the WT at 4.5 h ( $P < 0.05$ , 4.5 h vs. 3 h in WT, SNK post hoc). 6- $\alpha$ -Naloxol did not produce any significant withdrawal jumping at any time point following 20 mg/kg morphine in the WT.



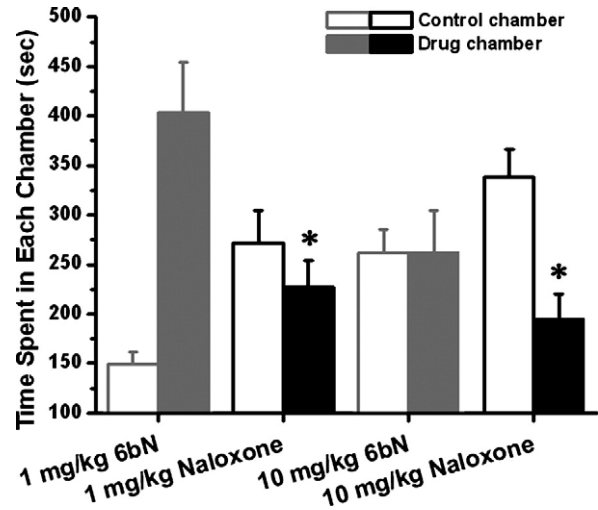
**Fig. 3.** Time course for naloxone-precipitated jumping in pENK<sup>-/-</sup> and WT mice. Naloxone-precipitated jumping first peaked at 2 h and declined at 3 h, suggesting that constitutively active mu receptors may quickly decay following induction by morphine. However, in the WT, naloxone regained its ability to precipitate jumping at 4.5 h, suggesting that enkephalin release during withdrawal contributes to persistent levels of constitutive activity ( $P < 0.05$ , comparing 4.5 and 3 h in the WT, SNK post hoc). The neutral antagonist, 6- $\alpha$ -naloxol (10 mg/kg), which is more potent than naloxone (Shoblock and Maidment, 2006) and has higher affinity for the  $\mu$  receptor than naloxone (Wang et al., 2001), did not produce any significant withdrawal jumping. Each time point represents a separate group of animals. (\*  $P < 0.01$ , naloxone WT vs. naloxone pENK<sup>-/-</sup>, SNK post hoc).

#### Naloxone, but not 6- $\beta$ -naloxol, produced CPA in pENK<sup>-/-</sup> mice when administered 2 h after morphine treatment

Naloxone, when administered 2 h after morphine, produced significant CPA in pENK<sup>-/-</sup> mice at both the 1 and the 10 mg/kg dose ( $P < 0.05$  and  $P < 0.02$ , respectively; one-group  $t$  test with Bonferroni correction), whereas 6- $\beta$ -naloxol was without any aversive effect (Fig. 4;  $P > 0.05$ ;  $n = 5-9$ ). (An apparent conditioned place preference at the 1 mg/kg dose reflects failure of the dose to block the effect of morphine pretreatment). There was an increase in locomotor activity during conditioning to 1 mg/kg 6- $\beta$ -naloxol, 2 h after morphine, compared with activity during saline conditioning (Fig. 5;  $F_{1,20} = 24.61$ ,  $P < 0.0001$ , treatment  $\times$  dose  $\times$  session;  $P < 0.0002$  comparing the saline session to the drug session for 1 mg/kg 6- $\beta$ -naloxol, SNK post hoc). There were no significant differences in locomotor activity between the saline conditioning session and the naloxone (1 or 10 mg/kg) or 6- $\beta$ -naloxol (10 mg/kg) conditioning sessions ( $P > 0.05$ , SNK post hoc). Only naloxone produced withdrawal jumping during conditioning (data not shown;  $P < 0.03$ , naloxone compared with 6- $\beta$ -naloxol, Fisher's exact test).

#### 6- $\beta$ -Naloxol attenuates naloxone CPA

Naloxone (10 mg/kg) conditioning, administered 2 h after morphine, produced CPA in all three groups similar to the previous experiment (Fig. 6;  $P < 0.05$ , one-group  $t$  test with Bonferroni correction;  $n = 4-8$ ). However, 6- $\beta$ -naloxol (10

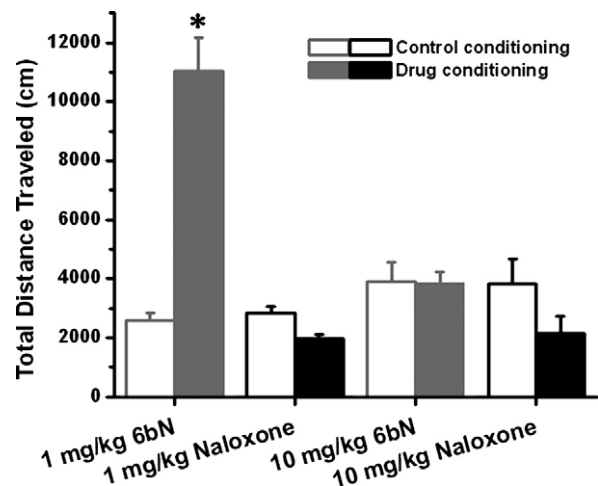


**Fig. 4.** CPA in pENK<sup>-/-</sup> mice 2 h after morphine treatment. Two hours after morphine (20 mg/kg) treatment, 1 and 10 mg/kg naloxone produced significant CPA in pENK<sup>-/-</sup> mice ( $P < 0.05$ , one-group  $t$  test with Bonferroni correction). The use of 10 mg/kg 6- $\beta$ -naloxol, which completely blocked the hyperlocomotor effect of morphine and is as effective as 1 and 10 mg/kg naloxone in inducing CPA in morphine-naive WT animals (Shoblock and Maidment, 2006), did not produce any aversion. Morphine was not effectively blocked with 1 mg/kg 6- $\beta$ -naloxol, resulting in an apparent place preference.

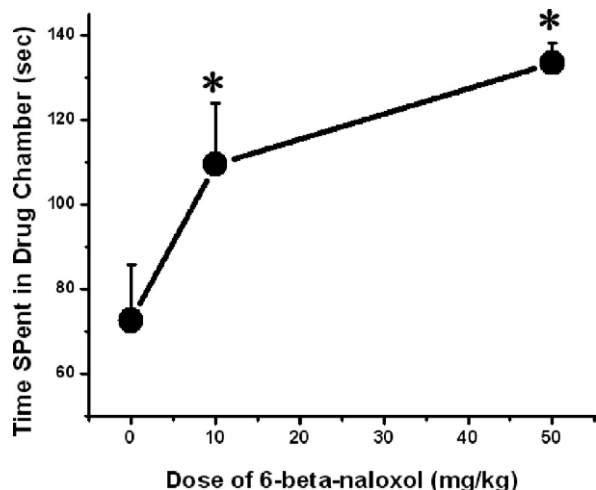
and 50 mg/kg) pretreatment significantly attenuated the CPA produced by naloxone ( $F_{2,17} = 4.38$ ,  $P < 0.01$ , main effect of dose;  $P < 0.03$  and  $P < 0.02$  comparing 10 and 50 mg/kg 6- $\beta$ -naloxol to saline, SNK post hoc).

## DISCUSSION

We previously demonstrated that naloxone fails to produce aversion in mice deficient in either the MOR or the pro-



**Fig. 5.** Locomotor activity in pENK<sup>-/-</sup> mice 2 h after morphine treatment. There was no increase in locomotor activity in the animals that received 10 mg/kg 6- $\beta$ -naloxol or 1–10 mg/kg naloxone 2 h after morphine (20 mg/kg), suggesting that these treatments effectively blocked morphine activation of the MOR. Morphine was not blocked by 1 mg/kg 6- $\beta$ -naloxol, as shown by elevated levels of locomotor activity ( $P < 0.0002$  comparing saline and drug conditioning sessions for 1 mg/kg 6- $\beta$ -naloxol, SNK post hoc).



**Fig. 6.** 6- $\beta$ -Naloxol attenuates naloxone CPA in pENK<sup>-/-</sup> mice 2 h after morphine treatment. Naloxone (10 mg/kg) conditioning, administered 2 h after morphine (20 mg/kg), produced a robust CPA in pENK<sup>-/-</sup> mice, similar to that of the previous experiment shown in Fig. 5 ( $P < 0.05$ , one-group  $t$  test with Bonferroni correction for each of the three groups). However, the effect was attenuated by 6- $\beta$ -naloxol (10 and 50 mg/kg) administered just prior to naloxone (\*  $P < 0.05$ , vs. the saline-naloxone group, SNK post hoc).

enkephalin, whereas it remains intact in  $\beta$ -endorphin-deficient mice (Skoubis et al., 2001, 2005). This, combined with the observation that the  $\delta$  receptor antagonist, naltrindole, does not induce CPA (Skoubis et al., 2001; Shippenberg et al., 1987; Bals-Kubik et al., 1989; De Vries et al., 1995) suggests that pro-enkephalin-derived peptide activation of the MOR, specifically, is responsible for an apparent endogenous opioid-mediated hedonic tone. Therefore, the endogenous pro-enkephalin system (not only Met and Leu enkephalin, but also, potentially, C-terminally extended Met-enkephalin peptides and the larger receptor-active peptides, BAM18, peptide E, and peptide F; Evans et al., 1986) may play a part in the disruptions to hedonic homeostasis that follow chronic exposure to morphine (Kreek and Koob, 1998). The dysregulation of hedonic homeostasis may be modeled by morphine potentiation of naloxone aversion, which is considered reflective of the negative affective state of “psychological withdrawal” (Azar et al., 2003). We recently provided evidence that such chronic morphine-induced potentiation of naloxone aversion in WT mice is a result of the production of constitutively active MORs (Shoblock and Maidment, 2006) by demonstrating that CPA to naloxone, an inverse agonist at the MOR, but not CPA to the neutral antagonists, 6- $\alpha$ - and 6- $\beta$ -naloxol, was enhanced following morphine treatment. Such upregulation of constitutively active MORs (Wang et al., 2001; Liu and Prather, 2001) may contribute to activation of downstream counteradaptive processes that ultimately produce dependence on those constitutively active receptors. The results of the present study in pENK<sup>-/-</sup> mice further consolidate the hypothesized role of MOR constitutive activity in both the negative motivational and the physical components of the morphine withdrawn state but indicate that enkephalin release may be a critical factor

in the formation and maintenance of such constitutively active receptors.

We hypothesized that naloxone would gain aversive properties in pENK<sup>-/-</sup> mice when administered following repeated morphine pretreatment by virtue of its inverse agonist property if such pretreatment does indeed induce constitutive activity of MORs. This proved not to be the case; naloxone remained ineffective at producing CPA in pENK<sup>-/-</sup> mice even when administered repeatedly 20 h after morphine injection, the same pretreatment regime that enhanced naloxone aversion in WT mice (Shoblock and Maidment, 2006). Perhaps the simplest conclusion that could be drawn from this result is that, contrary to our interpretation of earlier findings (Shoblock and Maidment, 2006), direct blockade of endogenous enkephalin action at the MOR rather than inverse agonist activity at that receptor remains the primary means by which naloxone produces its aversive effect, even when administered in conjunction with repeated spontaneous withdrawal from morphine. Indeed, it has been hypothesized that enkephalin release is increased during morphine withdrawal as a compensatory mechanism ameliorating the effects of withdrawal (Fukunaga and Kishioka, 2000).

However, if this were the case, one would predict greater spontaneous withdrawal in the pENK<sup>-/-</sup> mice, which was not observed in the current study. If anything, spontaneous withdrawal-induced diarrhea during conditioning was less in the pENK<sup>-/-</sup> mice compared with WT mice of our previous study (40% vs. 82%, unpublished results) and neither genotype displayed spontaneous withdrawal jumping. The observation conflicts with a previous report showing enhanced spontaneous withdrawal in pENK<sup>-/-</sup> mice (Nitsche et al., 2002), but this most likely reflects use of morphine pellets in that study rather than repeated injection (see below). Furthermore, none of the pENK<sup>-/-</sup> mice in the current study displayed naloxone-precipitated withdrawal jumping during conditioning after injection of a dose of naloxone that produced withdrawal jumping in 100% of WT animals in our previous study (Shoblock and Maidment, 2006). There is substantial evidence that naloxone-induced withdrawal jumping is an effect mediated by inverse agonist activity at constitutively active MORs (Wang et al., 1994, 2001, 2004; Bilsky et al., 1996; Raehal et al., 2005; Walker and Sterious, 2005; Shoblock and Maidment, 2006). This suggests that constitutively active receptors were present in the WT but not the pENK<sup>-/-</sup> mice 20 h after morphine administration, leading us to consider an alternative hypothesis; that pro-enkephalin-derived peptides are essential for the development or continued maintenance of MOR constitutive activity.

A theoretical basis for such a difference in morphine-induced MOR constitutive activity between the genotypes can be found from results of a previous study showing that the ability to induce constitutive activity of the MOR is not a property unique to morphine, but rather is a general property of MOR agonists, including enkephalins, in a manner positively correlated with intrinsic activity (Liu and Prather, 2001). Therefore, the morphine-induced MOR constitutive activity in WT animals should be considered a

synergistic effect in conjunction with endogenous enkephalins. This may be particularly important given that morphine releases enkephalins centrally (Olive et al., 1995). In the absence of enkephalins morphine would therefore be less potent in producing constitutively active receptors. In support of this concept, naloxone produced much less jumping in the pENK<sup>-/-</sup> mice compared with the WT even when administered 4 h after 100 mg/kg morphine treatment, when some morphine should still be present in the system. This again conflicts with the data of Nitsche et al. (2002), which demonstrated no difference between pENK<sup>-/-</sup> and WT animals in naloxone-precipitated withdrawal jumping. However, the use of morphine pellets in that study to induce dependence (rather than repeated morphine injection) and the consequent persistent high concentration of morphine would ensure that the receptors would be continuously saturated with agonist and the absence of enkephalins before removal of the pellet would therefore be predicted to have less effect. If enkephalins do produce constitutively active receptors as morphine does, then their absence would be felt during lower levels of morphine, much later after removal of the pellet or following metabolism of a bolus injection, such as 20 h after morphine treatment in the current study. As such, enkephalins could act to maintain the high levels of constitutively active MORs induced by morphine by sustaining kinase activity at a level sufficient to prolong their phosphorylated state.

To examine whether enkephalins are indeed involved in the maintenance of constitutively active MORs, the presence of such receptors at various time points after morphine administration was determined by the amount of withdrawal jumping precipitated by naloxone in both WT and pENK<sup>-/-</sup> mice. As a control, the withdrawal jumping produced by the neutral antagonist, 6- $\alpha$ -naloxol, which is more potent than naloxone (Shoblock and Maidment, 2006) and has higher affinity for the MOR than naloxone (Wang et al., 2001), was also measured. Because the neutral antagonist did not produce any significant withdrawal jumping in this test it is a reasonable assumption that the withdrawal jumping observed following naloxone was not due to a blockade of enkephalins or morphine, but a result of inverse agonist activity at constitutively active receptors. Naloxone-precipitated withdrawal jumping peaked 2 h after morphine injection in both WT and pENK<sup>-/-</sup> mice and was considerably diminished at 3 h. Interestingly, this is in agreement with both the time course for morphine-induced MOR phosphorylation *in vitro*, believed to be responsible for constitutive activity (Wang et al., 1996), and the measurements of MOR constitutive activity itself *in vitro* (Wang et al., 1994). However, naloxone was again able to precipitate withdrawal jumping when administered 4.5 h after morphine, at a time when animals were going through spontaneous withdrawal (rearing, sniffing, and wall climbing, but not jumping), but only in WT mice. This suggests that spontaneous withdrawal-induced enkephalin release may serve to promote a second wave of MOR constitutive activity during morphine withdrawal, explaining the apparent longevity of such constitutive ac-

tivity *in vivo* (in contrast with *in vitro*) following morphine administration (Wang et al., 2004).

Increases in enkephalin release during morphine withdrawal were implicated on the basis of measurements of peptide tissue content, pro-enkephalin mRNA levels, or microdialysis of extracellular enkephalins in several brain areas of potential importance to morphine physical and psychological dependence, such as the nucleus accumbens (Nylander et al., 1995), striatum (Pierce et al., 1992; Gudehithlu and Bhargava, 1995; Nylander et al., 1995), and periaqueductal gray (Fukunaga et al., 1996; Fukunaga et al., 1998; Nieto et al., 2002). Such secondary enkephalin-mediated induction of constitutively active receptors would be expected to induce further downstream counter-adaptive responses, perhaps themselves further enhancing enkephalin release in response to withdrawal from subsequent morphine administration, thereby furthering induction of constitutively active MORs in a spiraling fashion. To further test the hypothesis that the failure of naloxone to induce CPA in pENK<sup>-/-</sup> mice when administered 20 h after morphine was a result of the short duration of constitutive activity, we compared the CPA to naloxone with that of the neutral antagonist, 6- $\beta$ -naloxol when administered 2 h after morphine injection—the time of predicted peak MOR constitutive activity. Whereas both 1 and 10 mg/kg naloxone produced significant aversion and withdrawal jumping, 6- $\beta$ -naloxol was without effect in either regard. The ineffectiveness of the 1 mg/kg dose is explained by its inability to block morphine's action, as indicated by the elevated locomotor activity and an apparent place preference, consistent with our previous study in WT mice (Shoblock and Maidment, 2006). However, 10 mg/kg 6- $\beta$ -naloxol, which has an effectiveness between 1 and 10 mg/kg naloxone in morphine-naive animals (Shoblock and Maidment, 2006), completely blocked morphine-induced locomotor activity, indicating that it was effective as an antagonist. The difference between naloxone and 6- $\beta$ -naloxol, seen only 2 h after morphine and not in morphine-naive animals (Shoblock and Maidment, 2006), is consistent with the difference in negative intrinsic activity between the two drugs and suggests the neutral antagonist was rendered ineffective by high levels of MOR constitutive activity produced by morphine.

Further evidence that naloxone produces aversion following morphine by acting as an inverse agonist provided by the observation that 6- $\beta$ -naloxol significantly reduced the aversion produced by naloxone. Because a combination of two neutral antagonists of the same receptor would result in an increased effect (or no change if a maximal effect had been attained), such attenuation of naloxone aversion by 6- $\beta$ -naloxol implies that either naloxone acts as an inverse agonist, as proposed, or that 6- $\beta$ -naloxol is a partial agonist. It is unlikely that 6- $\beta$ -naloxol is a partial agonist because 10 mg/kg 6- $\beta$ -naloxol given alone after morphine resulted in complete neutrality, not slight place preference. In addition, structure–activity relationship studies show that  $\alpha$  substitution at C6 is vital to induce agonist activity, and 6- $\beta$  derivatives were devoid of partial agonist

effects (Chatterje and Inturrisi, 1975; Ronai et al., 1977; Koman et al., 1985).

Future demonstration of MOR functional status, by *ex vivo* measurement of guanosine 5'-O-(3-thiotriphosphate) binding, for instance, is required to confirm our hypothesis. However, such measurements in whole-brain tissue offer limited sensitivity for detection of what are likely to be small changes in isolated brain regions, with the previously measured differences in activity being quite subtle (Wang et al., 2004). In the absence of such data it is necessary to consider alternative explanations for our data. In addition to those highlighted above, it is possible that 6- $\alpha$ - and 6- $\beta$ -naloxol interact with receptor systems other than the MOR with greater potency or intrinsic activity than naloxone, that such interaction counters both CPA and withdrawal jumping, and that morphine pretreatment enhances this effect. The complete binding profiles of 6- $\alpha$ - and 6- $\beta$ -naloxol are not known but so far the only identified activities are at the MOR and DOR. Because their affinities for the DOR are similar to that of naloxone (Wang et al., 2001) and because the DOR is apparently not involved in the behavioral phenomena, activity at the DOR cannot explain the data. It is also necessary to recognize the possibility that developmental changes in other systems induced by pro-enkephalin deletion could potentially contribute differentially to the effects of naloxone versus 6- $\alpha$ - and 6- $\beta$ -naloxol in these behaviors. However, given that the only known difference between naloxone and its 6-hydroxyl derivatives is the difference in negative intrinsic activity, that naloxone acts as an inverse agonist to produce greater aversion and physical withdrawal is currently the best supported hypothesis.

## CONCLUSION

In summary, the data further support the hypothesis that repeated morphine administration enhances the CPA produced by naloxone by inducing constitutively active MORs (Shoblock and Maidment, 2006) but indicate that enkephalins, or other pro-enkephalin-derived peptides, released during morphine withdrawal may induce a second wave of constitutive activity, thereby prolonging the enhancement of naloxone's psychological and physical precipitated withdrawal effects. Indeed, the persistence of such constitutively active receptors, produced by enkephalin release, may be a homeostatic mechanism delaying or ameliorating the aversive effects of spontaneous opiate withdrawal. However, the induction of constitutively active MORs could lead to more dependence and greater subsequent withdrawal and therefore play a key role in the chronic opiate-induced "downward spiral" to hedonic homeostatic dysregulation, which is proposed to underlie the addicted state (Koob and Le Moal, 2001). Because neutral antagonists are effective at blocking morphine, but produce less physical and psychological withdrawal than inverse agonists in morphine-treated animals, they may offer a better alternative to naloxone or naltrexone in the treatment of opiate addiction.

*Acknowledgments*—This work was supported by the National Institutes of Health Grants DA 09359 (N.T.M.) and DA 05010

(N.T.M.), by a National Institutes of Health Training Grant DA 07272 (J.R.S.), and by a Hatos Fellowship (J.R.S.).

## REFERENCES

- Azar MR, Jones BC, Schulteis G (2003) Conditioned place aversion is a highly sensitive index of acute opioid dependence and withdrawal. *Psychopharmacology (Berl)* 170:42–50.
- Bals-Kubik R, Herz A, Shippenberg TS (1989) Evidence that the aversive effects of opioid antagonists and kappa-agonists are centrally mediated. *Psychopharmacology* 98:203–206.
- Bilsky EJ, Bernstein RN, Wang Z, Sadee W, Porreca F (1996) Effects of naloxone and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub> and the protein kinase inhibitors H7 and H8 on acute morphine dependence and antinociceptive tolerance in mice. *J Pharmacol Exp Ther* 277:484–490.
- Blokhina EA, Sukhotina IA, Beshpalov AY (2000) Pretreatment with morphine potentiates naloxone-conditioned place aversion in mice: effects of NMDA receptor antagonists. *Eur J Pharmacol* 406:227–232.
- Burford NT, Wang D, Sadee W (2000) G-protein coupling of mu-opioid receptors (OP3): elevated basal signalling activity. *Biochem J* 348 (Pt 3):531–537.
- Chatterje N, Inturrisi CE (1975) Stereospecific synthesis of the 6beta-hydroxy metabolites of naltrexone and naloxone. *J Med Chem* 18:490–492.
- Cruz SL, Villarreal JE, Volkow ND (1996) Further evidence that naloxone acts as an inverse opiate agonist: implications for drug dependence and withdrawal. *Life Sci* 58:L381–L389.
- De Vries TJ, Babovic-Vuksanovic D, Elmer G, Shippenberg TS (1995) Lack of involvement of delta-opioid receptors in mediating the rewarding effects of cocaine. *Psychopharmacology (Berl)* 120:442–448.
- Evans CJ, Erdelyi E, Barchas JD (1986) Candidate opioid peptides for interaction with the immune system. In: *Enkephalins and endorphins: stress and the immune system* (Plotnikoff NP, Faith RE, Murgio AJ, Good RA, eds), pp 3–15. New York: Plenum Press.
- Freye E, Levy J (2005) Constitutive opioid receptor activation: a prerequisite mechanism involved in acute opioid withdrawal. *Addict Biol* 10:131–137.
- Fukunaga Y, Kishioka S (2000) Enkephalineric neurons in the periaqueductal gray and morphine withdrawal. *Jpn J Pharmacol* 82:175–180.
- Fukunaga Y, Nishida S, Inoue N, Kishioka S, Yamamoto H (1996) Increase of preproenkephalin mRNA in the caudal part of periaqueductal gray by morphine withdrawal in rats: a quantitative *in situ* hybridization study. *Brain Res Mol Brain Res* 42:128–130.
- Fukunaga Y, Nishida S, Inoue N, Miyamoto M, Kishioka S, Yamamoto H (1998) Time course of morphine withdrawal and preproenkephalin gene expression in the periaqueductal gray of rats. *Brain Res Mol Brain Res* 55:221–231.
- Gracy KN, Dankiewicz LA, Koob GF (2001) Opiate withdrawal-induced fos immunoreactivity in the rat extended amygdala parallels the development of conditioned place aversion. *Neuropsychopharmacology* 24:152–160.
- Gudehithlu KP, Bhargava HN (1995) Modulation of preproenkephalin mRNA levels in brain regions and spinal cord of rats treated chronically with morphine. *Peptides* 16:415–419.
- Heinzen EL, Booth RG, Pollack GM (2005) Neuronal nitric oxide modulates morphine antinociceptive tolerance by enhancing constitutive activity of the mu-opioid receptor. *Biochem Pharmacol* 69:679–688.
- Hunyady L, Vauquelin G, Vanderheyden P (2003) Agonist induction and conformational selection during activation of a G-protein-coupled receptor. *Trends Pharmacol Sci* 24:81–86.
- Koman A, Einarsson M, Terenius L (1985) Agonist-antagonist properties of fluorescent opioid probes in the guinea-pig myenteric

- plexus-longitudinal muscle preparation. *Naunyn Schmiedebergs Arch Pharmacol* 331:355–358.
- Konig M, Zimmer AM, Steiner H, Holmes PV, Crawley JN, Brownstein MJ, Zimmer A (1996) Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. *Nature* 383:535–538.
- Koob GF, Le Moal M (2001) Drug abuse: hedonic homeostatic dysregulation. *Science* 278:52–58.
- Kreek MJ, Koob GF (1998) Drug dependence: stress and dysregulation of brain reward pathways. *Drug Alcohol Depend* 51:23–47.
- Liu JG, Prather PL (2001) Chronic exposure to mu-opioid agonists produces constitutive activation of mu-opioid receptors in direct proportion to the efficacy of the agonist used for pretreatment. *Mol Pharmacol* 60:53–62.
- Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dolle P, Tzavara E, Hanoune J, Roques BP, Kieffer BL (1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* 383:819–823.
- Mucha RF, Walker MJ (1987) Aversive property of opioid receptor blockade in drug-naive mice. *Psychopharmacology (Berl)* 93: 483–488.
- Neilan CL, Akil H, Woods JH, Traynor JR (1999) Constitutive activity of the delta-opioid receptor expressed in C6 glioma cells: identification of non-peptide delta-inverse agonists. *Br J Pharmacol* 128: 556–562.
- Nieto MM, Wilson J, Cupo A, Roques BP, Noble F (2002) Chronic morphine treatment modulates the extracellular levels of endogenous enkephalins in rat brain structures involved in opiate dependence: a microdialysis study. *J Neurosci* 22:1034–1041.
- Nitsche JF, Schuller AG, King MA, Zeng M, Pasternak GW, Pintar JE (2002) Genetic dissociation of opiate tolerance and physical dependence in delta-opioid receptor-1 and preproenkephalin knock-out mice. *J. Neurosci* 22:10906–10913.
- Nylander I, Vlaskovska M, Terenius L (1995) The effects of morphine treatment and morphine withdrawal on the dynorphin and enkephalin systems in Sprague-Dawley rats. *Psychopharmacology (Berl)* 118:391–400.
- Olive MF, Bertolucci M, Evans CJ, Maidment NT (1995) Microdialysis reveals a morphine-induced increase in pallidal opioid peptide release. *Neuroreport* 6:1093–1096.
- Parker LA, Cyr JA, Santi AN, Burton PD (2002) The aversive properties of acute morphine dependence persist 48 h after a single exposure to morphine. Evaluation by taste and place conditioning. *Pharmacol Biochem Behav* 72:87–92.
- Parker LA, Joshi A (1998) Naloxone-precipitated morphine withdrawal induced place aversions: effect of naloxone at 24 hours postmorphine. *Pharmacol Biochem Behav* 61:331–333.
- Pierce TL, Tiong GK, Olley JE (1992) Morphine and methadone dependence in the rat: withdrawal and brain met-enkephalin levels. *Pharmacol Biochem Behav* 42:91–96.
- Raehal KM, Lowery JJ, Bhamidipati CM, Paolino RM, Blair JR, Wang D, Sadee W, Bilsky EJ (2005) In vivo characterization of 6{beta}-naltrexol, an opioid ligand with less inverse agonist activity compared to naltrexone and naloxone in opioid dependent mice. *J Pharmacol Exp Ther* 313:1150–1162.
- Ronai AZ, Foldes FF, Hahn EF, Fishman J (1977) Orientation of the oxygen atom at C-6 as a determinant of agonistic activity in the oxymorphone series. *J Pharmacol Exp Ther* 200:496–500.
- Schulteis G, Markou A, Gold LH, Stinus L, Koob GF (1994) Relative sensitivity to naloxone of multiple indices of opiate withdrawal: a quantitative dose-response analysis. *J Pharmacol Exp Ther* 271: 1391–1398.
- Seifert R, Wenzel-Seifert K (2002) Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn Schmiedebergs Arch Pharmacol* 366:381–416.
- Selley DE, Cao CC, Liu Q, Childers SR (2000) Effects of sodium on agonist efficacy for G-protein activation in mu-opioid receptor-transfected CHO cells and rat thalamus. *Br J Pharmacol* 130: 987–996.
- Shippenberg TS, Bals-Kubik R, Herz A (1987) Motivational properties of opioids: evidence that an activation of delta-receptors mediates reinforcement processes. *Brain Res* 436: 234–239.
- Shoblock JR, Maidment NT (2006) Constitutively active micro opioid receptors mediate the enhanced conditioned aversive effect of naloxone in morphine-dependent mice. *Neuropsychopharmacology* 31:171–177.
- Skoubis PD, Lam HA, Shoblock J, Narayanan S, Maidment NT (2005) Endogenous enkephalins, not endorphins, modulate basal hedonic state in mice. *Eur J Neurosci* 21:1379–1384.
- Skoubis PD, Matthes HW, Walwyn WM, Kieffer BL, Maidment NT (2001) Naloxone fails to produce conditioned place aversion in mu-opioid receptor knock-out mice. *Neuroscience* 106:757–763.
- Walker EA, Sterious SN (2005) Opioid antagonists differ according to negative intrinsic efficacy in a mouse model of acute dependence. *Br J Pharmacol* 145:975–983.
- Wang D, Raehal KM, Bilsky EJ, Sadee W (2001) Inverse agonists and neutral antagonists at mu opioid receptor (MOR): possible role of basal receptor signaling in narcotic dependence. *J. Neurochem* 77:1590–1600.
- Wang D, Raehal KM, Lin ET, Lowery JJ, Kieffer BL, Bilsky EJ, Sadee W (2004) Basal signaling activity of mu opioid receptor in mouse brain: role in narcotic dependence. *J Pharmacol Exp Ther* 308:512–520.
- Wang Z, Arden J, Sadee W (1996) Basal phosphorylation of mu opioid receptor is agonist modulated and Ca<sup>2+</sup>-dependent. *FEBS Lett* 387:53–57.
- Wang Z, Bilsky EJ, Porreca F, Sadee W (1994) Constitutive mu opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. *Life Sci* 54:L339–L350.
- Wang Z, Bilsky EJ, Wang D, Porreca F, Sadee W (1999) 3-Isobutyl-1-methylxanthine inhibits basal mu-opioid receptor phosphorylation and reverses acute morphine tolerance and dependence in mice. *Eur J Pharmacol* 371:1–9.