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Withdrawal from acute morphine dependence is accompanied by increased anxiety-like behavior in the elevated plus maze

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Abstract

Pretreatment with a single moderate dose of morphine (e.g. 5.6-10 mg/kg) 4-24 hr prior to challenge with an opioid antagonist such as naloxone results in reliable expression of behaviors that resemble aversive or emotional consequences of withdrawal from chronic opioid exposure, including suppression of operant responding, elevations in brain reward thresholds, and conditioned place aversion. Repeated daily or weekly treatment with these same morphine doses results in a progressive increase in naloxone potency to elicit these withdrawal signs. The current study sought to determine whether increased anxiety-like behavior during withdrawal from chronic opioid dependence is also seen after acute morphine exposure, and progresses with repeated intermittent treatment. Male Wistar rats were handled and injected with either vehicle or morphine for 4 consecutive days. Three injection regimens were employed: Morphine Naive (4 vehicle injections), Acute Morphine (3 vehicle injections, 4th injection 5.6 or 10 mg/kg morphine), or Repeat Morphine (all 4 injections with 5.6 or 10 mg/kg morphine). Acute pretreatment with 5.6 mg/kg or 10 mg/kg morphine resulted in time-dependent increases in exploration of the open arms of the plus maze in naloxone-naive rats when tested at 2, 4 or 8 hr after the final pretreatment injection, with the effects at the higher dose appearing later (4 hr) than after the lower dose (2 hr). This pattern of results, in combination with a separate study which confirmed a significant anxiolytic-like effect of a low dose of morphine (0.56 mg/kg) administered 15 min prior to test, suggested that low residual morphine levels remaining in plasma at 2-4 hr after 5.6 and 10 mg/kg morphine may be sufficient to elicit anxiolytic-like effects. Repeat treatment with either dose of morphine resulted in a further increase in the magnitude and duration of this anxiolytic-like effect. These effects had dissipated by 8 hr post-morphine, and therefore precipitation of withdrawal by one of several doses of naloxone (0.10-3.3 mg/kg) was assessed in separate cohorts of rats 8 hr after the final pretreatment under Morphine Naïve, Acute Morphine, or Repeat Morphine conditions. Naloxone resulted in a significant dose-dependent expression of anxiety-like behavior with no effects on general activity after Acute Morphine pretreatment at either 5.6 or 10 mg/kg morphine. A further significant shift in naloxone potency was observed after Repeat Morphine pretreatment at the 10 mg/kg but not the 5.6 mg/kg dose. Thus, anxiety-like behavior is a prominent feature of the negative emotional consequences of naloxone-precipitated withdrawal from acute opioid dependence.
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1. Introduction

Drug abstinence in human opioid addicts can result in a number of well-characterized autonomic and somatic symptoms of opioid withdrawal (American-Psychiatric-Association, 2007; Koob and Le Moal, 2005a; Lindesmith, 1968; O'Brien, 1996), but also includes emotional or affective symptoms such as depressed mood/dysphoria, restlessness, hyperirritability, and anxiety (American-Psychiatric-Association, 2007; Haertzen and Hooks, 1969; Henningfield, 1987; Jasinski et al., 1985; Koob and Le Moal, 2005a; Schulteis and Koob, 1996). These negative emotional states resulting from withdrawal may contribute to escalation to compulsive use, maintenance of use, and relapse after periods of abstinence (Aston-Jones and Harris, 2004; Koob and Le Moal, 2005b; Schulteis and Koob, 1996; Self and Nestler, 1998).

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A number of animal models have been developed to permit the study of neuroanatomical and neurochemical substrates mediating negative emotional signs of withdrawal from opioids and other drugs of abuse (reviewed in Aston-Jones and Harris, 2004; Harris and Gewirtz, 2005; Higgins and Sellers, 1994; Koob and Le Moal, 2005a). For opioid withdrawal anxiety, studies have shown that both spontaneous and antagonist-precipitated opioid withdrawal result in significant signs of anxiety-like behavior. For example, spontaneous opioid withdrawal results in time-dependent emergence and resolution of anxiety-like behavior in the defensive probe burying paradigm and elevated plus maze (Bhattacharya et al., 1995; Grasing et al., 1996; Harris and Aston-Jones, 1993; Schulteis et al., 1998). In addition, during spontaneous morphine withdrawal rats trained to discriminate the stimulus effects of the anxiogenic agent pentylenetetrazol (PTZ) show generalization to the PTZ stimulus (Emmett-Oglesby et al., 1984). These same paradigms have also reliably measured anxiety-like behavior resulting from antagonist-precipitated opioid withdrawal (Emmett-Oglesby et al., 1984; Higgins and Sellers, 1994; Schulteis et al., 1998).

Recently there is renewed interest in the phenomenon of acute opioid dependence, defined as "a state in which abstinence [withdrawal] can be demonstrated or *precipitated* following either a single dose or a short-term infusion of [an opioid]" (Bickel et al., 1988; Martin and Eades, 1964). Signs of withdrawal that closely parallel those observed during withdrawal from chronic opioid exposure can be precipitated by an opioid antagonist administered 2-24 hr after a single bolus dose of an opioid agonist such as morphine in humans (e.g. Azorlosa et al., 1994; Jones, 1980) and animals (e.g. Azar et al., 2003; Liu and Schulteis, 2004; Parker and Joshi, 1998; Schulteis et al., 1997) with no prior history of opioid exposure. Notably, aversive emotional states measured by elevations in brain reward thresholds (Easterling et al., 2000; Liu and Schulteis, 2004) and conditioned place aversion (CPA; Azar et al., 2003; Parker and Joshi, 1998) are more readily elicited by the opioid antagonist naloxone than are most somatic signs of withdrawal (Schulteis et al., 1999, 1997) after acute pretreatment with morphine.

The strategy of quantifying shifts to the left in the opioid antagonist dose-effect function elicited by opioid agonist exposure has long been used for the characterization of neuroadaptive changes associated with opioid dependence (Villereal and Castro, 1979; Way et al., 1969), with the magnitude of shift in the antagonist dose-effect function presumed to serve as a valid index of the magnitude of the underlying state of dependence. This strategy has proven particularly valuable in the study of acute opioid dependence, and a number of studies indicate that repeated treatment with morphine at daily or weekly intervals results in progressive left-ward shifts in the potency of naloxone or naltrexone to precipitate withdrawal-like signs, as one might expect if acute dependence reflects the early stages in the development of a full state of opioid dependence (Adams and Holtzman, 1990; Azar et al., 2003; Azorlosa et al., 1994; Easterling et al., 2000; Liu and Schulteis, 2004; Schulteis et al., 1999; Schulteis et al., 2004).

To date the study of anxiety-like behavior during withdrawal from acute opioid dependence has received limited attention. Several studies using the acoustic startle reflex indicate that naloxone or naltrexone administered 2-6 hr after acute pretreatment with morphine can increase magnitude of the basal startle response (Harris and Gewirtz, 2004; Kalinichev et al., 2004), and that repeated morphine exposure results in a further increase in startle magnitude (Harris et al., 2004). These data have been interpreted as evidence of increased anxiety-like behavior during acute withdrawal from morphine (Harris and Gewirtz, 2005, 2004; Harris et al., 2004). However, basal startle reflex is reported to be either suppressed (Kalinichev and Holtzman, 2003; Mansbach et al., 1992) or unaffected (Fendt and Mucha, 2001) by withdrawal from chronic morphine treatment, yielding an apparent dissociation between the behavioral profile exhibited in this paradigm during withdrawal from acute versus chronic opioid dependence. This contrasts sharply with other measures of aversive emotional states of opioid withdrawal such as brain reward threshold elevations and CPA that are similarly elicited during withdrawal from acute or chronic opioid exposure (Azar et al., 2003; Bruijnzeel et al., 2006; Delfs et al., 2000; Easterling et al., 2000; Liu and Schulteis, 2004; Parker and Joshi, 1998; Schaefer and Michael, 1986; Schulteis et al., 1994; Stinus et al., 1990). In addition, recent work in our laboratory (Schulteis et al., submitted) suggests that interpretation of data on basal startle reflex during the hours immediately following acute pretreatment with morphine may be complicated by significant effects of the morphine metabolite morphine-3-glucuronide on startle.

The purpose of the present study was to characterize naloxone-precipitated withdrawal from acute and repeated intermittent morphine treatment using the elevated plus maze as an animal model of withdrawal-related anxiety that develops during the initiation and early progression of opioid dependence. The elevated plus maze was utilized in the present study because it was previously shown to be a sensitive index of anxiety-like behavior accompanying antagonist-precipitated withdrawal from chronic opioids (Higgins and Sellers, 1994; Schulteis et al., 1998). In addition, this model has been shown to produce consistent anxiety-like behavioral profiles during withdrawal from both chronic and acute ethanol exposure (Baldwin et al., 1991; Doremus et al., 2003; File, 1994; Valdez et al., 2004; Zhang et al., 2007). Although the elevated plus maze is a novelty-based task that requires a between-subjects approach, a within-subjects approach is not optimal when seeking to study the withdrawal response to a single acute opioid treatment. Moreover, our previous work has shown that conditioned withdrawal responses may contribute to the magnitude of naloxone-precipitated withdrawal if animals are repeatedly tested under both acute and repeated intermittent morphine conditions, making a between-subjects approach desirable (Schulteis et al., 2003, 2004, 2005). Our goals herein were to demonstrate 1) a significant increase in naloxone potency to elicit anxiety-like behavior following acute morphine pretreatment when compared to the antagonist's effects in opioid-naive controls; 2) a further potentiation of naloxone potency upon repeated exposure to morphine; and 3) plus maze behavioral profiles after acute and repeated intermittent morphine exposure that directly parallel effects observed in rats exposed chronically to morphine.

2. Materials and methods

2.1. Animal subjects

Male Wistar rats (n=443, Harlan Labs, Indianapolis, IN) weighing 275-375 g at the time of testing served as subjects for these studies. All rats were group housed (2-3/cage) in a temperature- and humidity-controlled room with a 12 hour light/12 hour dark cycle (lights ON at 6:00 AM). Rats had *ad libitum* access to food and water at all times. All testing took place from 9:00 AM to 4:00 PM. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the VA San Diego Healthcare System, an AAALAC-accredited facility, and are in strict accordance with the "Guide for the Care and Use of Laboratory Animals" (revised 1996).

2.2. Drugs

Morphine sulfate was generously provided by the Research Resources Drug Supply System of the National Institute on Drug Abuse (Bethesda, MD, USA). Naloxone HCl was purchased from Sigma (St. Louis, MO, USA). Both drugs were prepared for injection in physiological saline (0.9%), and all injections were made subcutaneously (SC) in a volume of 0.1 ml/100 g body weight. Doses of both drugs are expressed as the salt. Morphine was administered at doses ranging from 0.56 mg/kg to 10 mg/kg, and naloxone was administered at doses ranging from 0.10 mg/kg to 3.3 mg/kg. All doses were based upon prior work on acute opioid dependence in rats (Adams and Holtzman, 1990; Azar et al., 2003; Easterling et al., 2000; Harris and Gewirtz, 2004; Kalinechev and Holtzman, 2003; Schulteis et al., 1997, 1999, 2004).

2.3. Elevated plus maze apparatus and procedure

The elevated plus maze apparatus used in these studies was an automated system acquired from Kinder Scientific (Poway CA). The maze consisted of two opposing open arms (Length: 50 cm, Width 10.8 cm), bounded by 4-mm-high ledges on the sides and at the end of the arms, and two opposing closed arms of equal length and width but with 33.5 cm high walls on all sides except the 10.8 cm-wide entrance to the center of the maze. The center of the maze was a 10.8×10.8 cm square area from which each of the four arms was connected at 90° relative to the adjacent arms. The maze floor was elevated 85 cm from the floor of the testing room. All sides and floor surfaces of the open and closed arms were constructed from black Plexiglas. Position of the rat in the maze was continuously tracked by photo beam arrays embedded along the entire length of the base of each closed arm, the entry point to all arms, and in clear Plexiglas tubes that extended from the distal end of each closed arm and ran parallel to the open arms.

Testing was conducted in a quiet room with a white noise generator providing approximately 65 dB background noise. The testing room was illuminated only by two 25-W light bulbs in clip-on fixtures that were attached to the legs of the enclosed arms and positioned to direct light against the walls of the testing room behind each closed arm. To begin a test session, rats were placed in the center of the maze facing towards one of the enclosed arms. Between each trial, the maze was cleaned with a damp sponge and dried with paper towels.

Data were collected and analyzed by a Windows-XP-driven computer using MotorMonitor Software (Kinder Scientific, Poway CA). From the computer-recorded data, the following measures were computed for each rat: 1) time spent in the open arms as a percentage of the total time spent exploring both the open and closed arms (Percent Time); 2) number of entries into the open arms as a percentage of the total number of entries into both open and closed arms (Percent Entries); and 3) the total number of entries into the closed arms (Closed Entries). Our primary anxiety-like measures of interest were Percent Time and Percent Entries into the open arms, as they consistently have been shown in factor analyses to have the highest loading on the factor representing the "anxiety" dimension (Cruz et al., 1994; Fernandes and File, 1996; Ohl et al., 2001; Rodgers and Dalvi, 1997; Wall and Messier, 2001), and have proven to be among the most reliable indices of anxiogenic-like effects of drug withdrawal, including opioid withdrawal (Baldwin et al., 1991; Doremus et al., 2003; File, 1994; Kliethermes, 2005; Rassnick et al., 1993; Schulteis et al., 1998; Valdez et al., 2002; Valdez et al., 2004; Valdez et al., 2003). Our measure of general activity was Closed Entries, a reliable and validated index of locomotor activity in the maze (e.g. Fernandes and File, 1996; File, 1994).

2.4. Experimental design

Six to 9 days after arrival in the colony, rats were transported to a testing suite that consisted of a front "holding" room where rats could be handled, weighed, injected, and housed temporarily before and after testing, and a back "testing" room where the rats were exposed to the maze. The illumination and background noise in the holding room matched that described above for the testing room. Thirty to 60 min after initial transport to the holding room, each rat was gently handled for 5 min, weighed, injected with vehicle or morphine, then returned to their home cage and kept in the room for approximately four hours to acclimate to the ambient lighting and noise conditions before being returned to the vivarium room. This procedure was repeated on each of three consecutive days. On the fourth day when testing took place, all rats were again moved to the holding room, weighed, injected with vehicle or morphine, and placed back into their own home cages with food and water freely available. Separate cohorts of rats received a) vehicle injection on all 4 days (Morphine Naive); b) vehicle on the first 3 days and morphine (5.6 or 10 mg/kg) on the 4th day (Acute Morphine); or c) morphine (5.6 or 10 mg/kg) on all 4 days (Repeat Morphine). The interval between each of the four injections was held constant at approximately 24 hr.

At a specific time interval (see below) after the fourth and final injection, rats received an additional SC injection that consisted of either vehicle or a dose of naloxone (0.10 - 3.3 mg/kg), and this injection was followed 10 min later by free exploration of the maze for 5 min. All maze testing took place between 9 AM and 4

PM, with injection times on the test day varying to produce the appropriate interval between morphine injection and test. Each rat was tested only once. The same investigator performed all habituation, injection and testing procedures with a given group of rats.

Previously reported preliminary work with the elevated plus maze (Schulteis and Zhang, 2006) was based upon extensive prior work with other behavioral indices of opioid withdrawal (suppression of operant responding, brain stimulation reward thresholds, CPA) in which naloxone administered 4 hr after acute or repeated intermittent pretreatment with a 5.6 mg/kg dose of morphine elicited reliable signs of precipitated withdrawal with little or no measurable residual response to morphine itself (Adams and Holtzman, 1990; Azar et al., 2003; Young, 1986; Easterling and Holtzman, 1997; Easterling et al., 2000; Liu and Schulteis, 2004; Schulteis et al., 1997, 1999, 2004). Thus, initial cohorts of Morphine Naïve, Acute Morphine (5.6 mg/kg) or Repeat Morphine (5.6 mg/kg) subjects received vehicle or naloxone (1.0, 3.3 mg/kg) 4 hr post-morphine and 10 min prior to testing in the elevated plus maze. Although naloxone elicited significant anxiety-like effects at 4 hr post-morphine under Acute Morphine treatment, naloxone potency was not potentiated significantly by Repeat Morphine treatment in these preliminary studies. Unexpected findings of significant anxiolytic-like effects 4 hr after Repeat Morphine alone in a group injected with vehicle instead of naloxone prior to test appeared to confound effects of naloxone-precipitated withdrawal. (Schulteis and Zhang, 2006). These ambiguous results led to the expanded series of studies reported herein, designed to characterize the time course of anxiolytic-like effects of morphine itself to permit selection of a time point for evaluation of naloxone-precipitated withdrawal that would not be confounded by residual morphine effects. Specifically, the expanded set of studies, the data for which are fully reported herein, included:

1) A time course of plus maze behavior in response to morphine alone: Morphine Naive, Acute Morphine, and Repeat Morphine groups (both 5.6 and 10 mg/kg morphine) were tested following vehicle injection at 2, 4, or 8 hr post-treatment.
2) The effects of low doses of morphine (0.1 -1.0 mg/kg) alone on elevated plus maze behavior: This study was conducted to examine the possibility that residual low doses of morphine present at 2-4 hr post-treatment with 5.6-10 mg/kg doses could account for the unexpected anxiolytic-like effects at these time points, since prior studies had indicated anxiolytic-like effects of low doses of morphine (Anseloni et al., 1999; Koks et al., 1999; Koks et al., 1998; Motta and Brandao, 1993; Nobre et al., 2000).
3) Examination of the naloxone dose-effect function at 8 hr after the final pretreatment injection: Morphine Naive, Acute Morphine, and Repeat Morphine groups were tested after injection of naloxone. At least 2 doses of naloxone were tested under each condition, with an initial dose of 0.33 or 1.0 mg/kg followed by tests of higher and/or lower dose(s) based upon magnitude of effect (or lack thereof) at the preceding dose; in this fashion, it was possible to minimize animal subject requirements while establishing a minimum effective dose of naloxone under all conditions of morphine treatment (up to the maximum tested dose of 3.3 mg/kg). The full range of naloxone doses (0.10, 0.33, 1.0, 3.3 mg/kg) tested under all Acute and Repeat Morphine conditions (both 5.6 and 10 mg/kg doses) were examined in cohorts of Morphine Naive rats to permit direct comparisons to control conditions.

2.5. Data analysis

Data for all behavioral measures of plus maze activity (Percent Time, Percent Entries, Closed Entries) were analyzed by two-way (Figs. 1 and 3, Tables 1 and 2) or one-way (Fig. 2) ANOVA as dictated by the experimental design. To avoid cluttering of the results section with multiple ANOVA values for Percent Time, Percent Entries, and Closed Entries, most overall ANOVA results are presented in accompanying tables. Follow-up comparisons using a Bonferroni correction to maintain a constant overall familywise error rate of $p<0.05$ were conducted as appropriate given the outcome of the overall ANOVA.

A full three-factor analysis of morphine history (Morphine Naive, Acute Morphine, Repeat Morphine), morphine dose (5.6, 10 mg/kg), and either time post-treatment (Fig. 1 and Table 1) or naloxone dose (Fig. 3, Table 2) as the third factor would require identical time points or ranges of naloxone dose be evaluated under all conditions, and also require a separate Morphine Naive control group for both the 5.6 and 10 mg/kg doses of morphine. To conserve animal subject requirements where possible in a study that was already quite large, the decision was made to test a single cohort of Morphine Naive controls at each time point or naloxone dose concurrently with the corresponding Acute and Repeat Morphine groups treated with 5.6 and 10 mg/kg morphine. In addition, as described above slightly different naloxone dose ranges were sometimes tested in Acute versus Repeat Morphine groups to establish minimum effective doses under each condition while again conserving animal subjects where possible. As a result, all studies required separate analyses of data for the 5.6 and 10 mg/kg doses of morphine, and some studies additionally required separate analyses of naloxone effects under Acute and Repeat Morphine conditions (in each case compared to the single Morphine Naïve control condition).

3. Results

3.1. Acute and repeat morphine time course

As shown in Fig. 1, pretreatment with 5.6 mg/kg (panels A-C) or 10 mg/kg (panels D-F) morphine resulted in behavioral changes in the elevated plus maze that varied in a dose- and time-specific fashion. An initial one-factor ANOVA comparing all Morphine Naive control groups over time (2, 4, and 8 hr post-injection) revealed no significant differences on any of the

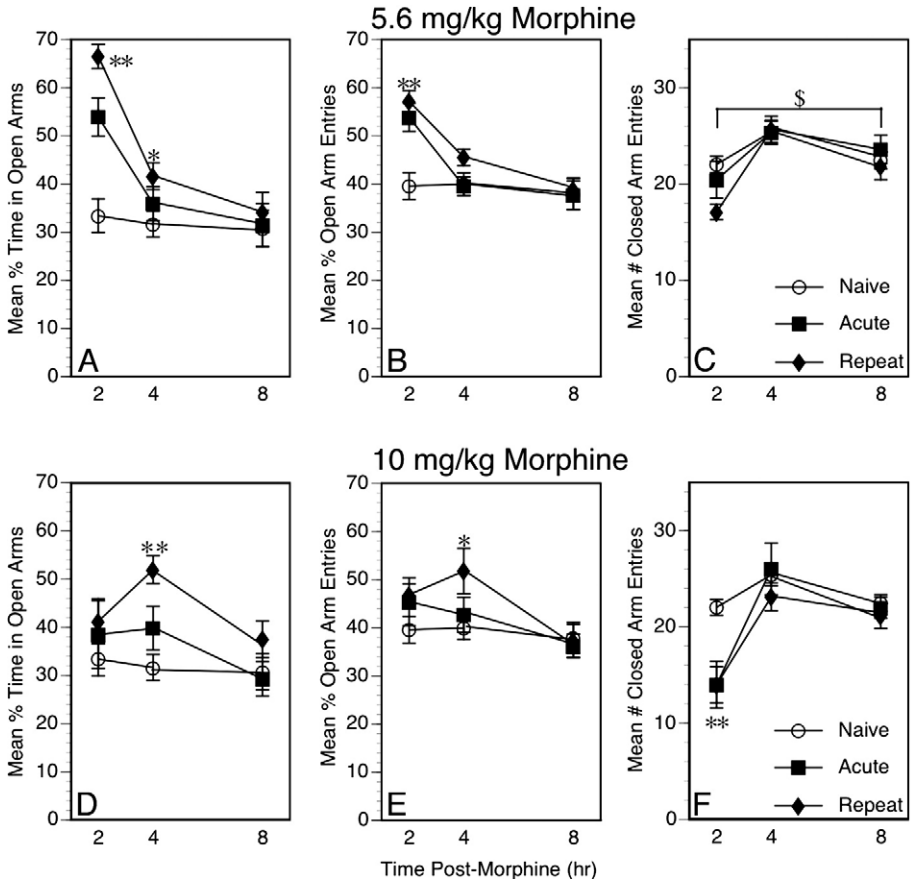

Fig. 1. Pretreatment with Acute (■) or Repeat (♦) Morphine produces dose- and time-dependent effects on all 3 measures of plus maze behavior relative to Morphine Naive (○) controls. Data represent mean±SEM; sample size=11-15/group. Note that Morphine Naive control groups were tested concurrently with both 5.6 mg/kg and 10 mg/kg morphine-treated cohorts, therefore the same Morphine Naïve control data are presented in both the upper and lower panels for ease of comparison to both morphine doses. See Table 1 for all overall ANOVA results to support the follow-up comparisons reported herein. Panels A-C, 5.6 mg/mg morphine: Acute Morphine treatment at the 5.6 mg/kg dose increases Percent Time (A) and Percent Entries (B) at 2 hr post-morphine; Repeat Morphine elicits similar effects at 2 hr post-morphine, and additionally increases Percent Time on the open arms at 4 hr post-morphine. All treatment groups (Morphine Naïve, Acute Morphine, Repeat Morphine) showed a modest reduction in activity at the 2 hr time point relative to 4 and 8 hr (C), but there were no differences across treatment conditions. Panels E-G, 10 mg/kg morphine: Acute Morphine treatment at the 10 mg/kg dose increases Percent Time (E) and Percent Entries (F) at 4 hr post-morphine, but effects have declined to Morphine Naïve baseline levels by 8 hr post-morphine. Repeat Morphine produces a further increase in Percent Time (E) on the open arms at 4 hr post-morphine, but again the effect returned to baseline by 8 hr post-morphine. The effects on Percent Time and Percent Entries at 4 hr were not accompanied by changes in Closed Arm Entries (F), although there was a significant reduction in activity at the earlier 2 hr time point under both Acute and Repeat Morphine conditions. **$p<0.05$, both Acute and Repeat conditions differ from Morphine Naïve controls via Bonferroni-corrected individual means comparisons; *$p<0.05$, Repeat Morphine condition only differs from Morphine Naïve controls via Bonferroni-corrected individual means comparisons; \$$p<0.05$, main effect of time in the absence of a significant main effect of morphine condition or significant interaction, no further follow-up comparisons conducted.

3 measures of plus maze behavior (Percent Time, Percent Entries, Closed Entries, all F's [2,45]<2.01, p's>0.10).

Morphine 5.6 mg/kg: Two-factor ANOVAs on all plus maze measures were conducted comparing the Morphine Naive, Acute Morphine 5.6 mg/kg, and Repeat Morphine 5.6 mg/kg groups over time (2, 4, and 8 hr after 4th and final pre-treatment). As shown in Table 1 and Fig. 1 A-C, both Acute and Repeat Morphine 5.6 mg/kg elicited time-dependent elevations in Percent Time and Percent Entries into the open arms, as well as time-dependent decreases in Closed Entries, relative to Morphine Naive controls. Significant interaction of time with morphine history (Morphine Naive, Acute Morphine, Repeat Morphine) for both the Percent Time (Fig. 1A) and Percent Entries (Fig. 1B) measures was attributable to a selective increase in open arm exploration at early but not late time points. The effect was significant for both the Acute Morphine and Repeat Morphine condition at 2 hr post-treatment relative to Morphine Naive controls ($p<0.05$, Bonferroni-corrected individual means comparisons, Fig. 1A), and had declined to baseline by 4 hr post-morphine for the Acute but not the Repeat Morphine condition (the latter had declined to Morphine Naïve levels by 8 hr post-treatment). Analysis of the primary activity measure, Closed Entries, revealed only a significant main effect of time post-treatment, attributable to a modest reduction in activity at 2 hr post-morphine relative to later time points for all conditions.

Morphine 10 mg/kg: Two-factor ANOVA on the Percent Time and Percent Entries measures revealed a significant main effect of morphine history and main effect of time post-morphine (Table 1). As shown in Fig. 1D and E and confirmed

Table 1
Summary of ANOVA results for acute and repeat morphine time course

Plus maze measure	Two-factor ANOVA	Morphine 5.6 mg/kg (Acute vs Repeat vs Naive)	Morphine 10 mg/kg (Acute vs Repeat vs Naive)
Percent Time	Main Effect Morphine History[a]	F[2,116]=15.45, p<0.0001	F[2,117]=6.37, p<0.005
	Main Effect Time[b]	F[2,116]=23.04, p<0.0001	F[2,117]=3.28, p<0.05
	Interaction	F[4,116]=5.16, p<0.001	F[4,117]=1.03, N.S.
Percent Entries	Main Effect Morphine History[a]	F[2,116]=8.67, p<0.001	F[2,117]=3.18, p<0.05
	Main Effect Time[b]	F[2,116]=16.97, p<0.0001	F[2,117]=5.43, p<0.01
	Interaction	F[4,116]=3.50, p<0.01	F[2,117]=0.76, N.S.
Closed Entries	Main Effect Morphine History[a]	F[2,116]=2.06, N.S.	F[2,117]=4.99, p<0.01
	Main Effect Time[b]	F[2,116]=17.08, p<0.0001	F[2,117]=21.59, p<0.0001
	Interaction	F{4,116]=1.29, N.S.	F[4,117]=3.15, p<0.05

Abbreviations: N.S.=not significant.
[a]Morphine History refers to treatment regimen on 4 days leading up to plus maze testing: Morphine Naive (vehicle×4), Acute Morphine (vehicle×3, morphine×1), or Repeat Morphine (morphine×4).
[b]All treatment groups included separate cohorts tested at 2, 4, and 8 hr after the 4th and final injection of their given treatment regimen.

by follow-up comparisons across morphine history at individual time points, a time-dependent increase in open arm exploration was significant under Acute and Repeat Morphine conditions only at 4 hr post-treatment ($p<0.05$, Bonferroni-corrected); no difference from Morphine Naïve controls was observed after either Acute or Repeat Morphine treatment at 2 or 8 hr post-treatment. For Closed Arm Entries (Fig. 1F), a significant interaction of the time and morphine history factors, followed by post-hoc individual means comparisons across morphine conditions at individual time points revealed a transient reduction in Closed Entries at 2 hr post-treatment in both Acute and Repeat Morphine groups as compared to Morphine Naive controls ($p<0.05$, Bonferroni-corrected).

3.2. Effects of acute low doses of morphine tested 15 min post-injection

As shown in Fig. 2, morphine in a low dose range injected 15 min prior to plus maze testing produced an increase in Percent Time and Percent Entries into the open arms of the maze in a non-linear dose-dependent fashion (inverted U-shape). One-way ANOVA with morphine dose as a between subjects factor revealed a significant effect for the Percent Time ($F[3,49]=2.89$, $p<0.05$) and Percent Entries ($F[3,49]=2.82$, $p<0.05$) measures, with no significant effect on Closed Entries, the measure of general activity ($F[3,49]=0.146$, N.S.). Post-hoc comparisons of each morphine dose to vehicle controls revealed a significant

Table 2
Summary of ANOVA results for naloxone administered 8 hr post-morphine

Plus maze measure	Two-factor ANOVA	Morphine 5.6 mg/kg Naïve vs Acute vs Repeat (0.33, 1.0, 3.3 mg/kg NAL)	Morphine 10 mg/kg Acute vs. Morphine Naïve (0.33, 1.0, 3.3 mg/kg NAL)[a]	Morphine 10 mg/kg Repeat vs Morphine Naïve (0.10, 0.33, 1.0 mg/kg NAL)[a]
Percent Time	Main Effect Morphine History[b]	F[2,97]=7.95, p<0.001	F[1,66]=17.92, p<0.0001	F[1,68]=44.27, p<0.0001
	Main Effect Naloxone Dose	F[2,97]=0.66, N.S.	F[2,66]=1.20, N.S.	F[2,68]=3.19, p<0.05
	Interaction	F[3,97]=1.45, N.S.	F[2,66]=5.64, p<0.01	F[2,68]=4.42, p<0.05
Percent Entries	Main Effect Morphine History[b]	F[2,97]=4.85, p<0.01	F[1,66]=10.81, p<0.005	F[1,68]=15.40, p<0.0005
	Main Effect Naloxone Dose	F[2,97]=0.86, N.S.	F[2,66]=1.68, N.S.	F[2,68]=1.54, N.S.
	Interaction	F[3,97]=0.60, N.S.	F[2,66]=3.16, p<0.05	F[2,68]=3.22, p<0.05
Closed Entries	Main Effect Morphine History[b]	F[2,97]=1.77, N.S.	F[1,66]=0.18, N.S.	F[1,68]=6.25, p<0.05
	Main Effect Naloxone Dose	F[2,97]=0.62, N.S.	F[2,66]=0.27, N.S.	F[2,68]=1.07, N.S.
	Interaction	F[3,97]=1.11, N.S.	F[2,66]=0.53, N.S.	F[2,68]=0.38, N.S.

Abbreviations: NAL=naloxone, N.S.=not significant.
[a]To establish dose-dependence and minimum effective doses of naloxone while minimizing animal subject requirements wherever possible, different doses of naloxone were tested under Acute and Repeat Morphine 10 mg/kg conditions, and consequently these groups were compared to Morphine Naive controls in separate two-factor ANOVAs.
[b]Morphine History refers to treatment regimen on 4 days leading up to plus maze testing: Morphine Naive (vehicle×4), Acute Morphine (vehicle×3, morphine×1), or Repeat Morphine (morphine×4).

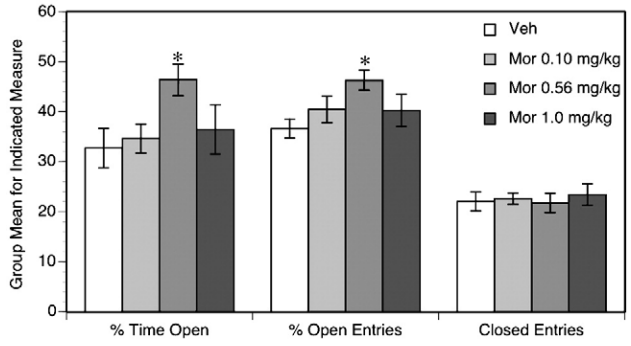

Fig. 2. Acute treatment with morphine (15 min prior to test) in a low dose range (0.10-1.0 mg/kg) produces an inverted U-shaped relationship between morphine dose and Percent Time on and Percent Entries into the open arms of the elevated plus maze. The 0.56 mg/kg dose of morphine produced a significant anxiolytic-like effect as measured by both Percent Time and Percent Entries measures (*$p<0.05$ vs. vehicle (Veh)-treated controls, with a higher (1.0) and lower (0.10 mg/kg) dose being without effect. There was no effect on general activity in the maze (Closed Entries) at any dose of morphine tested. Data represent mean (±SEM) for each measure; sample size=12-15/group.

effect only at the intermediate dose tested for both Percent Time and Percent Entries measures (0.56 mg/kg, $p<0.05$ Bonferroni-corrected).

3.3. Naloxone-precipitated withdrawal 8 hr post-morphine

Initial one-factor ANOVA comparing all doses of naloxone to vehicle in Morphine Naive rats at the 8 hr testing interval revealed no significant effects on any of the 3 measures of plus maze behavior examined (all F's[4,58]<1.69, p's>0.15).

Morphine 5.6 mg/kg: Comparison of the Acute and Repeat Morphine conditions to Morphine Naive controls on the Percent Time (Fig. 3A) and Percent Entries (Fig. 3B) measures revealed only significant main effects of morphine history (see Table 2), with follow-up simple main effects revealing that both the Acute and Repeat Morphine groups differed significantly from the Morphine Naïve controls ($p<0.05$, Bonferroni-corrected), but not from each other. The Closed Entries measure yielded no significant effects whatsoever for Acute or Repeat Morphine (Fig. 3C).

Morphine 10 mg/kg: As shown in Fig. 3D-F and Table 2, the data for naloxone-induced withdrawal at 8 hr after administration of acute or repeated intermittent morphine at the 10 mg/kg dose yielded dose-dependent effects of naloxone after Acute Morphine pretreatment, with a further significant shift in naloxone potency upon Repeat Morphine experience, consistent with our prior findings with other measures of opioid withdrawal (Azar et al., 2003; Schulteis et al., 1997, 2004, Liu and Schulteis, 2006).

As summarized in Table 2, comparison of either the Acute Morphine or Repeat Morphine 10 mg/kg groups to Morphine Naive controls yielded significant main effects of morphine history and a history x naloxone dose interaction for both the Percent Time and Percent Entries measures (Fig. 3D-E). Post-hoc individual means comparisons of Acute and Repeat Morphine groups to Morphine Naïve controls at individual doses of naloxone revealed minimum effective doses of 1.0 mg/kg and 0.33 mg/kg naloxone after Acute and Repeat Morphine pretreatment, respectively ($p<0.05$, Bonferroni-corrected). As shown in Fig. 3F and confirmed by a significant main effect of morphine history in the Repeat Morphine vs. Morphine Naïve comparison, naloxone produced a modest reduction in Closed Entries that was not dose-dependent in the Repeat Morphine condition only.

4. Discussion

The present study achieved all three of our goals for development of an animal model of withdrawal-associated anxiety-like behavior in the initial onset and early progression of opioid dependence, as outlined at the end of the Introduction:

1) Anxiety-like behavior was clearly observed during naloxone-precipitated withdrawal from a single pretreatment with morphine (Fig. 3). Naloxone administered 8 hr after Acute Morphine treatment (5.6 and 10 mg/kg) elicited dose-dependent decreases in Percent Time on and Percent Entries into the open arms of the elevated plus maze, with no significant effects on general activity in the maze as measured by Closed Arm Entries. Qualitatively these results are similar to prior reports of suppression of operant responding, brain reward threshold deficits, and conditioned place aversion precipitated by naloxone or naltrexone after acute morphine (Adams and Holtzman, 1990; Azar et al., 2003; Easterling and Holtzman, 1997; Easterling et al., 2000; Liu and Schulteis, 2004; Parker and Joshi, 1998; Schulteis et al., 1999, 1997; Schulteis et al., 2004).
2) Potentiation of naloxone potency upon Repeat Morphine treatment. With 4 daily pretreatments of 10 mg/kg morphine, the minimum dose of naloxone to elicit anxiety-like behavior decreased from 1.0 mg/kg to 0.33 mg/kg. Although there was a very modest effect of naloxone on the measure of general activity (Closed Entries) after Repeat Morphine 10 mg/kg, this activity effect was not naloxone dose-dependent, whereas the anxiogenic-like effects were clearly dose-dependent.
3) Unlike the sometimes dichotomous effects on basal acoustic startle reflex in animals undergoing withdrawal from acute versus chronic morphine (Fendt and Mucha, 2001; Harris and Gewirtz, 2005, 2004; Harris et al., 2004; Kalinichev and Holtzman, 2003; Mansbach et al., 1992), directionality of effect in the elevated plus maze is similar during naloxone-precipitated withdrawal from acute, repeated intermittent, and chronic morphine exposure. Moreover, in our prior report of anxiety-like behavior precipitated by naloxone in rats chronically dependent on morphine through implantation of morphine pellets (Schulteis et al., 1998), the minimum effective dose of naloxone to precipitate anxiety-like behavior was 0.01 mg/kg, 30-fold lower than the minimum dose required to precipitate withdrawal after 4 daily treatments with 10 mg/kg morphine in the present study (0.33 mg/kg, see Fig. 3). This ratio of relative naloxone potency after chronic versus repeated intermittent (4x) morphine is similar to the 10-30 fold potency

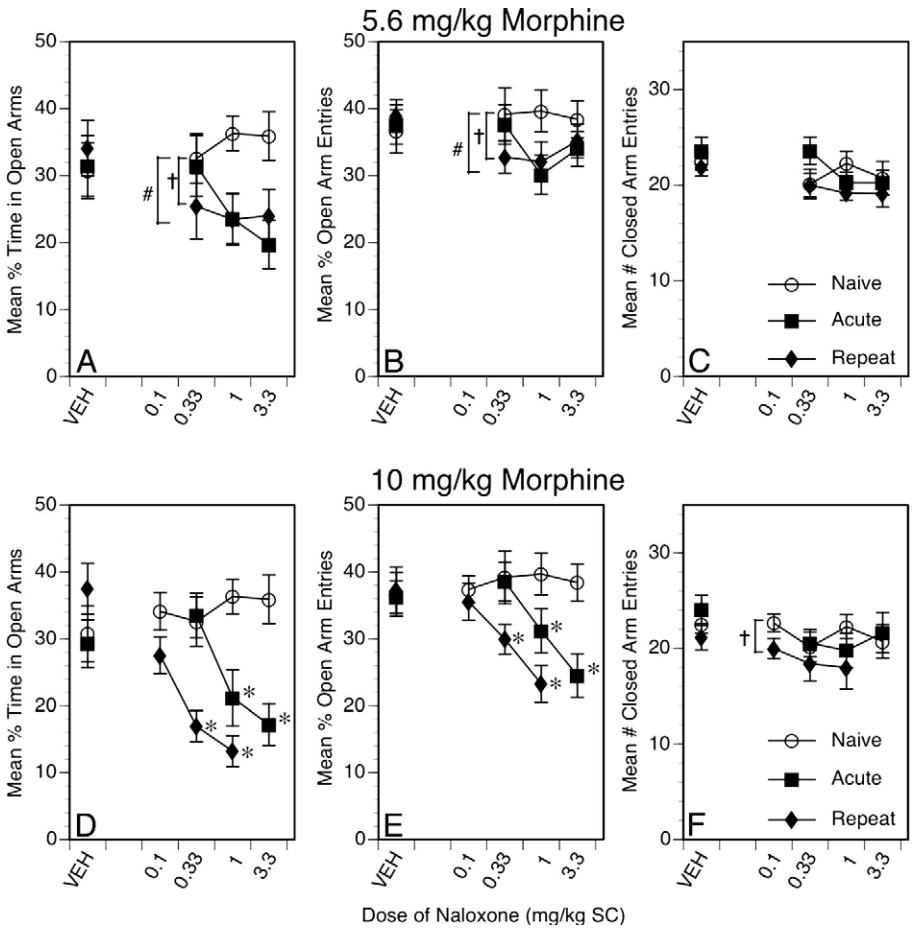

Fig. 3. Relative to its effects in Morphine Naive controls (○), naloxone elicits clear dose-dependent anxiogenic-like effects when administered 8 hr after pretreatment with Acute (■) 10 mg/kg morphine that is potentiated by 4 daily repeated treatments with this same dose of morphine (Repeat, ♦) (Panels D-F). Treatment with Acute or Repeat Morphine at the 5.6 mg/kg dose also yielded significant anxiogenic-like effects, but naloxone dose-dependence was not as clear, and Repeat treatment with this dose of morphine did not consistently increase naloxone potency when administered 8 hr after the final morphine pretreatment (Panels A-C). Data represent mean (±SEM); sample size = 11-15/group. Note that Morphine Naive control groups were tested concurrently with cohorts of animals treated with 5.6 or 10 mg/kg morphine, therefore the same control data are presented in both the upper and lower panels. Data from groups treated with Vehicle (VEH) 8 hr after pretreatment with either vehicle (Morphine Naive) or the appropriate dose of morphine (Acute, Repeat, 5.6 or 10 mg/kg) are reproduced here from Fig. 1 to facilitate visual comparison to the groups treated with naloxone, but are not included in the statistical analysis of naloxone effects. See Table 2 for all overall ANOVA results to support the follow-up comparisons reported herein. Panels A-C, 5.6 mg/mg morphine: Naloxone administered 8 hr post-morphine elicited significant reductions in (A) Percent Time spent on and (B) Percent Entries into the open arms of the maze following both Acute and Repeat 5.6 mg/kg morphine, but the effects were not dependent on naloxone dose, and there was no incremental effect of Repeat Morphine treatment beyond the effects produced by Acute treatment (# $p < 0.05$, Simple Main Effect of Morphine History, Acute vs. Naive; † $p < 0.05$, Simple Main Effect of Morphine History, Repeat vs. Naive). Naloxone was without significant effect on Closed Arm Entries (C) after either Acute or Repeat 5.6 mg/kg morphine. Panels E-G, 10 mg/kg morphine: Significant interactions of morphine history and naloxone dose were found when comparing either Acute or Repeat 10 mg/kg morphine to Morphine Naive controls for Percent Time (E) and Percent Entries (F), indicating naloxone dose-dependent reductions in both measures of anxiety-like behavior. Naloxone was effective at a lower dose (0.33 mg/kg) after Repeat Morphine 10 mg/kg than after Acute Morphine 10 mg/kg (* $p < 0.05$ vs. Morphine Naive controls at that dose of naloxone, Bonferroni-corrected). For the activity measure of Closed Arm Entries (F), a significant main effect of naloxone was observed only when comparing Repeat Morphine 10 mg/kg to Morphine Naive controls, attributable to a modest but significant reduction in closed arm entries that was not dependent on naloxone dose († $p < 0.05$, Simple Main Effect of Morphine History, Repeat vs. Naive).

differences for naloxone precipitation of brain reward deficits (Liu and Schulteis, 2004; Schulteis et al., 1994) and suppression of operant responding (Schulteis et al., 1994, 2004) after chronic versus repeated intermittent morphine. Similar potency differences exist for naloxone precipitation of somatic signs of withdrawal following chronic (Schulteis et al., 1994) versus repeated intermittent morphine (Schulteis et al., 1999), with relatively mild (e.g. teeth chattering, swallowing movements, eye blinks, paw shakes) but not more severe (e.g. weight loss, diarrhea, wet dog shakes, jumping behavior) somatic signs observed at minimal doses naloxone required to elicit negative emotional signs such as brain reward deficits or anxiety-like behavior (Adams and Holtzman, 1990; Kalinichev and Holtzman, 2003; Schulteis et al., 1994, 1999).

Although our preliminary work with the elevated plus maze (Schulteis and Zhang, 2006) focused initially on parameters of morphine dose (5.6 mg/kg) and interval between morphine and naloxone (4 hr) that were well-established in our prior work with suppression of operant responding, CPA, and brain stimulation reward, it quickly became evident that several minor

deviations in experimental design would be necessary to produce a model of opioid withdrawal anxiety-like behavior that encompassed the three primary characteristics highlighted above. Our pilot data (Schulteis and Zhang, 2006) indicated that naloxone-induced anxiety-like behavior, although detectable at 4 hr after acute treatment with our standard dose of 5.6 mg/kg morphine, was not potentiated by repeated morphine pretreatment, and it was postulated that this was due at least in part to an unexpected anxiolytic-like profile observed at 4 hr post-morphine under Repeat Morphine conditions. In work with suppression of operant responding, brain stimulation reward, and place aversion, we had not observed such residual agonist effects at 4 hr post-morphine (Azar et al., 2003; Liu and Schulteis, 2004; Schulteis et al., 1997, 1999, 2004).

Therefore, the first step in the present study was to characterize the time course of morphine-induced changes in plus maze behavior to identify time points that would be free of residual morphine effects that might "compete" with expression of naloxone-precipitated withdrawal. As shown in Fig. 1, both 5.6 and 10 mg/kg morphine produced time-dependent anxiolytic-like effects after acute treatment, with peak effects at 2 hr following 5.6 mg/kg morphine and at 4 hr following 10 mg/kg morphine. One possible interpretation of the delay in onset of anxiolytic-like effect is that it is mediated by a build-up of morphine metabolites, which in the rat consist primarily of morphine-3-glucuronide (M3G; Christrup, 1997; Shang et al., 2006; Zheng et al., 1998). However, several pieces of evidence argue against this interpretation. First, M3G levels in plasma and CSF are higher at 2 hr after injection of 10-15 mg/kg of morphine (Barjavel et al., 1995; Shang et al., 2006) than they are at 4 hr post-injection, yet the anxiolytic-like effects observed after acute administration of 10 mg/kg morphine peaked at 4 hr, and were absent at 2 hr. Second, the lower dose of morphine (5.6 mg/kg) produces anxiolytic-like effects at 2 hr post-treatment where the higher dose of 10 mg/kg is without effect, yet plasma levels of M3G at 2 hr post-injection are significantly higher with 10 mg/kg than 5.6 mg/kg of morphine (Barjavel et al., 1995; Zheng et al., 1998). Finally, we are unaware of any reports in the literature of M3G producing anxiolytic-like effects, but there are a number of reports that central administration of this morphine metabolite elicits an excitatory reaction characterized by explosive motor behavior, touch-evoked agitation, myoclonic jerks, and wet-dog shakes, behaviors that the rat is most likely to experience as aversive rather than anxiolytic (Bartlett et al., 1994; Hemstapat et al., 2003; Labella et al., 1979; Smith and Smith, 1998). In support of this latter assertion, Bartlett et al. (1994) reported that some excitatory effects of M3G are attenuated by pretreatment with an anxiolytic agent (midazolam).

An additional possibility that we therefore considered is that residual levels of unconjugated morphine may account for the delayed anxiolytic-like effects observed in the present study. Morphine-induced anxiolytic-like effects as measured by increases in Percent Time on and Percent Entries into the open arms of the elevated plus-maze are most often observed at 15-30 min after injection of low doses of morphine (e.g. 0.3-1.0 mg/kg), with a non-linear inverted-U-shaped dose-effect function resulting in loss of effect at higher doses of morphine (Anseloni et al., 1999; Koks et al., 1999; Koks et al., 1998; Motta and Brandao, 1993; Nobre et al., 2000; Patti et al., 2006). The inverted U-shaped nature of the dose-effect function may be attributable to significant reductions in general activity elicited by higher doses of morphine (Koks et al., 1999). An additional possibility supported by site-directed infusion studies is that lower doses of morphine increase exploration of the open arms of the maze through activation of μ opioid receptors in the nucleus accumbens and/or dorsal periaqueductal gray (DPAG), whereas higher doses elicit opposing effects through activation of κ opioid receptors in the DPAG (Anseloni et al., 1999; Motta and Brandao, 1993; Motta et al., 1995; Nobre et al., 2000).

The current study verified the inverted U-shaped dose effect function of low dose morphine-induced anxiolytic-like effects under the same testing conditions where we observed delayed emergence of such effects following higher morphine doses (5.6 - 10 mg/kg). Significant increases in Percent Time on and Percent Entries into the open arms were observed at a dose of 0.56 mg/kg morphine, and these effects were lost at a higher dose of 1.0 mg/kg (see Fig. 2). Thus, a dose 10 or 18-fold lower than the 5.6 and 10 mg/kg pretreatment doses, respectively, produced an anxiolytic-like effect within 15 min of administration. It is noteworthy that plasma levels of intact morphine measured 4 hr after acute SC administration of the 10 mg/kg dose and 2 hr after administration of 5.6 mg/kg dose, times when peak anxiolytic-like effects are observed for each respective dose (see Fig. 1), are in a similar range (140-200 ng/ml; Schulteis and Zhang, 2006; Schulteis et al., submitted), and these residual plasma morphine levels represent approximately 10-15% of peak levels achieved after acute injection of 5.6 and 10 mg/kg morphine. Taken together, these findings are consistent with the interpretation that residual low levels of intact morphine produce anxiolytic-like effects at 2 and 4 hr after administration of 5.6 and 10 mg/kg morphine, respectively.

As shown in Fig. 1, repeated daily treatment with morphine at either 5.6 or 10 mg/kg produced a further increase in the magnitude and duration of anxiolytic-like effects beyond what was observed after a single treatment. These effects are not readily attributable to pharmacokinetic mechanisms, as previous work has shown that daily treatment with 10 mg/kg morphine for 7 days does not alter the time course of morphine concentrations in plasma or brain (Kalivas and Duffy, 1987). However, the data are consistent with the possibility that repeated daily morphine treatment produced sensitization to the anxiolytic-like effects of low residual levels of morphine. It is known that 10 mg/kg morphine can induce sensitization to opioid- or psychostimulant-induced locomotor behavior when administered 1-5 times at intervals of 24-48 hr between treatments (Cunningham et al., 1997; McDaid et al., 2006; Vanderschuren et al., 2001). Similar to the results observed in the present study, such sensitized behavior in response to a challenge dose of morphine can manifest as both increased peak magnitude and increased duration of effect. Notably behavioral sensitization in these studies is accompanied by sensitization of neurochemical responses to morphine in the nucleus accumbens (McDaid et al., 2006; Vanderschuren et al., 2001), or can be elicited by direct microinfusion of morphine into the nucleus accumbens (Cunningham et al., 1997), and acute microinfusion of morphine into the nucleus accumbens also elicits anxiolytic-like effects (Anseloni et al., 1999). We postulate

therefore that the increased magnitude and duration of anxiolytic-like effect observed in the present study upon repeated treatment with 5.6-10 mg/kg morphine may reflect a sensitization process, possibly in the nucleus accumbens. Although no apparent sensitization of the Closed Entries measure of activity was detectable in the present study, this may have been obscured by the large increase in exploration of the open arms of the maze. Verification of our postulate will require further studies that extend beyond the scope of the present work, since our primary focus herein was to establish a reliable model of anxiogenic-like effects of withdrawal from acute and repeated intermittent morphine. Nonetheless, the present work highlights the necessity to understand clearly all possible factors contributing to behavioral responses when seeking to establish a particular paradigm as a reliable animal model of the emotional consequences of antagonist-precipitated withdrawal from acute opioid dependence, including factors such as spontaneous withdrawal effects, possible contributions of morphine metabolites, and potential effects of residual low doses of morphine.

In summary, negative emotional states accompanying withdrawal from acute morphine include anxiety-like behavior as measured in the elevated plus maze (present study) in addition to previously reported elevated brain reward thresholds (Easterling et al., 2000; Liu and Schulteis, 2004), suppression of operant responding (Adams and Holtzman, 1990; Schulteis et al., 1997, 1999, 2004), and CPA (Azar et al., 2003; Parker and Joshi, 1998). We previously argued (Criner et al., 2007; Liu and Schulteis, 2004) that suppression of operant responding and CPA, as general measures of the aversive stimulus effects of opioid withdrawal, could reflect the elicitation of one or more negative emotional components of withdrawal such as anxiety or dysphoria. In rats exposed chronically to morphine, these aversive states are produced by low doses of opioid antagonists that fail to elicit significant somatic signs of withdrawal (e.g. escape jumps, wet dog shakes, abdominal constrictions, diarrhea, body weight loss, profuse salivation; Higgins and Sellers, 1994; Schulteis et al., 1994). In contrast, naloxone elevates brain reward thresholds and produces anxiogenic-like effects at doses comparable to those that produce suppression of operant responding and CPA after chronic (Fendt and Mucha, 2001; Higgins and Sellers, 1994; Schulteis et al., 1994; Schulteis et al., 1998) and acute morphine (present study and Azar et al., 2003; Liu and Schulteis, 2004; Schulteis et al., 1997, 1999, 2004). The nucleus accumbens, bed nucleus of the stria terminalis, and central amygdala have been identified as critical loci where opioid receptor blockade elicits suppression of operant responding and CPA during withdrawal from chronic (Koob et al., 1989; Stinus et al., 1990) or acute (Criner et al., 2007) morphine. These same structures are prominently implicated in expression of negative emotional states, including anxiety-like behavior (Davis, 1998; Nair et al., 2005). Future work to identify the brain sites most sensitive to precipitation of brain reward deficits and anxiogenic-like effects therefore should provide a direct test of the hypothesis that these emotional signs of withdrawal contribute to the aversive stimulus that engenders suppression of operant responding and CPA.

It is noteworthy that brain reward deficits and anxiety-like behavior during withdrawal from acute morphine closely parallel negative emotional consequences of withdrawal from acute ethanol (Liu and Schulteis, 2004; Schulteis and Liu, 2006; Zhang et al., 2007). Throughout these studies it has been clearly demonstrated that a single exposure to morphine or alcohol is sufficient to induce a state of acute dependence as measured by negative emotional indices of withdrawal, and severity of said negative emotional withdrawal is further potentiated with several repeated intermittent daily or weekly exposure to opioids or ethanol, suggesting rapid induction and progression of neuroadaptation within brain emotional and reward circuitry. Our animal models of these negative affective consequences of withdrawal from acute and repeated intermittent opioid and ethanol intoxication should prove useful in delineating the specific neuroanatomical and neurochemical substrates within brain reward, emotional, and stress circuitry (e.g. CRF systems in the CNS) that are critical to initial onset and early progression of neuroadaptation, not only to opioids but also to other drugs of abuse such as alcohol. In this regard, it should be emphasized that although physical or somatic signs of withdrawal may vary widely across classes of abused drugs from opioids to sedatives to stimulants, negative emotional states including dysphoria and elevated anxiety are prominent features of the withdrawal syndrome to multiple classes of drugs, including opioids, sedatives such as alcohol and benzodiazepines, and stimulants such as cocaine, amphetamines and nicotine (Koob and Le Moal, 2005a,b). Recent theories of dependence and addiction have therefore accorded these negative emotional states a more prominent role than somatic withdrawal signs in motivating maintenance of compulsive drug use and relapse after periods of abstinence (Aston-Jones and Harris, 2004; Koob and Le Moal, 2005b; Schulteis and Koob, 1996; Self and Nestler, 1998), and our models of dysphoria-like and anxiety-like behavior resulting from acute opioid dependence suggest they may also play a prominent role in the transition from casual to compulsive use.

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References

Adams JU, Holtzman SG. Pharmacologic characterization of the sensitization to the rate-decreasing effects of naltrexone induced by acute opioid pretreatment in rats. J Pharmacol Exp Ther 1990;253:483–9.

American-Psychiatric-Association. DSM-IV-TR, Diagnostic and Statistical Manual of Mental Disorders. Arlington, VA: American Psychiatric Publishing, Inc; 2007.

Anseloni VC, Coimbra NC, Morato S, Brandao ML. A comparative study of the effects of morphine in the dorsal periaqueductal gray and nucleus accumbens of rats submitted to the elevated plus-maze test. Exp Brain Res 1999;129:260–8.

Aston-Jones G, Harris GC. Brain substrates for increased drug seeking during protracted withdrawal. Neuropharmacology 2004;47(Suppl 1):167–79.

Azar MR, Jones BC, Schulteis G. Conditioned place aversion is a highly sensitive index of acute opioid dependence and withdrawal. Psychopharmacology (Berl) 2003;170:42–50.

Azorlosa JL, Stitzer ML, Greenwald MK. Opioid physical dependence development: effect of single versus repeated morphine pretreatments and of subjects' opioid exposure history. Psychopharmacology (Berl) 1994;114:71–80.

Baldwin HA, Rassnick S, Rivier J, Koob GF, Britton KT. CRF antagonist reverses the "anxiogenic" response to ethanol withdrawal in the rat. Psychopharmacology (Berl) 1991;103:227–32.

Barjavel MJ, Scherrmann JM, Bhargava HN. Relationship between morphine analgesia and cortical extracellular fluid levels of morphine and its metabolites in the rat: a microdialysis study. Br J Pharmacol 1995;116:3205–10.

Bartlett SE, Cramond T, Smith MT. The excitatory effects of morphine-3-glucuronide are attenuated by LY274614, a competitive NMDA receptor antagonist, and by midazolam, an agonist at the benzodiazepine site on the GABAA receptor complex. Life Sci 1994;54:687–94.

Bhattacharya SK, Chakrabarti A, Sandler M, Glover V. Rat brain monoamine oxidase A and B inhibitory (tribulin) activity during drug withdrawal anxiety. Neurosci Lett 1995;199:103–6.

Bickel WK, Stitzer ML, Liebson IA, Bigelow GE. Acute physical dependence in man: effects of naloxone after brief morphine exposure. J Pharmacol Exp Ther 1988;244:126–32.

Bruijnzeel AW, Lewis B, Bajpai LK, Morey TE, Dennis DM, Gold M. Severe deficit in brain reward function associated with fentanyl withdrawal in rats. Biol Psychiatry 2006;59:477–80.

Christrup LL. Morphine metabolites. Acta Anaesthesiol Scand 1997;41:116–22.

Criner SH, Liu J, Schulteis G. Rapid neuroadaptation in the nucleus accumbens and bed nucleus of the stria terminalis mediates suppression of operant responding during withdrawal from acute opioid dependence. Neuroscience 2007;144:1436–46.

Cruz AP, Frei F, Graeff FG. Ethopharmacological analysis of rat behavior on the elevated plus-maze. Pharmacol Biochem Behav 1994;49:171–6.

Cunningham ST, Finn M, Kelley AE. Sensitization of the locomotor response to psychostimulants after repeated opiate exposure: role of the nucleus accumbens. Neuropsychopharmacology 1997;16:147–55.

Davis M. Are different parts of the extended amygdala involved in fear versus anxiety? Biol Psychiatry 1998;44:1239–47.

Delfs JM, Zhu Y, Druhan JP, Aston-Jones G. Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. Nature 2000;403:430–4.

Doremus TL, Brunell SC, Varlinskaya EI, Spear LP. Anxiogenic effects during withdrawal from acute ethanol in adolescent and adult rats. Pharmacol Biochem Behav 2003;75:411–8.

Easterling KW, Holtzman SG. Intracranial self-stimulation in rats: sensitization to an opioid antagonist following acute or chronic treatment with mu opioid agonists. J Pharmacol Exp Ther 1997;281:188–99.

Easterling KW, Plovnick RM, Holtzman SG. Acute opioid but not benzodiazepine dependence in rats responding for intracranial self-stimulation. Psychopharmacology (Berl) 2000;148:263–71.

Emmett-Oglesby MW, Harris CM, Lane JD, Lal H. Withdrawal from morphine generalizes to a pentylenetetrazol stimulus. Neuropeptides 1984;5:37–40.

Fendt M, Mucha RF. Anxiogenic-like effects of opiate withdrawal seen in the fear-potentiated startle test, an interdisciplinary probe for drug-related motivational states. Psychopharmacology (Berl) 2001;155:242–50.

Fernandes C, File SE. The influence of open arm ledges and maze experience in the elevated plus-maze. Pharmacol Biochem Behav 1996;54:31–40.

File SE. Chronic exposure to noise modifies the anxiogenic response, but not the hypoactivity, detected on withdrawal from chronic ethanol treatment. Psychopharmacology (Berl) 1994;116:369–72.

Grasing K, Wang A, Schlussman S. Behavioral measures of anxiety during opiate withdrawal. Behav Brain Res 1996;80:195–201.

Haertzen CA, Hooks Jr NT. Changes in personality and subjective experience associated with the chronic administration and withdrawal of opiates. J Nerv Ment Dis 1969;148:606–14.

Harris AC, Gewirtz JC. Acute opioid dependence: characterizing the early adaptations underlying drug withdrawal. Psychopharmacology (Berl) 2005;178:353–66.

Harris AC, Gewirtz JC. Elevated startle during withdrawal from acute morphine: a model of opiate withdrawal and anxiety. Psychopharmacology (Berl) 2004;171:140–7.

Harris AC, Hanes SL, Gewirtz JC. Potentiated startle and hyperalgesia during withdrawal from acute morphine: effects of multiple opiate exposures. Psychopharmacology (Berl) 2004;176:266–73.

Harris GC, Aston-Jones G. Beta-adrenergic antagonists attenuate withdrawal anxiety in cocaine- and morphine-dependent rats. Psychopharmacology (Berl) 1993;113:131–6.

Hemstapat K, Monteith GR, Smith D, Smith MT. Morphine-3-glucuronide's neuro-excitatory effects are mediated via indirect activation of N-methyl-D-aspartic acid receptors: mechanistic studies in embryonic cultured hippocampal neurones. Anesth Analg 2003;97:494–505.

Henningfield J, Johnson RE, Jasinski DR. Clinical procedures for the assessment of abuse potential. In: Bozarth M, editor. Methods of assessing the reinforcing properties of abused drugs. New York: Springer-Verlag; 1987. p. 573–90.

Higgins GA, Sellers EM. Antagonist-precipitated opioid withdrawal in rats: evidence for dissociations between physical and motivational signs. Pharmacol Biochem Behav 1994;48:1–8.

Jasinski DR, Johnson RE, Kocher TR. Clonidine in morphine withdrawal. Differential effects on signs and symptoms. Arch Gen Psychiatry 1985;42:1063–6.

Jones R. Dependence in non-addict humans after a single dose of morphine. In: Way E, editor. Endogenous and exogenous opiate agonists and antagonists. New York: Pergamon Press; 1980. p. 557–60.

Kalinichev M, Holtzman SG. Changes in urination/defecation, auditory startle response, and startle-induced ultrasonic vocalizations in rats undergoing morphine withdrawal: similarities and differences between acute and chronic dependence. J Pharmacol Exp Ther 2003;304:603–9.

Kalinichev M, White DA, Holtzman SG. Individual differences in locomotor reactivity to a novel environment and sensitivity to opioid drugs in the rat. I. Expression of morphine-induced locomotor sensitization. Psychopharmacology (Berl) 2004;177:61–7.

Kalivas PW, Duffy P. Sensitization to repeated morphine injection in the rat: possible involvement of A10 dopamine neurons. J Pharmacol Exp Ther 1987;241:204–12.

Kliethermes CL. Anxiety-like behaviors following chronic ethanol exposure. Neurosci Biobehav Rev 2005;28:837–50.

Koks S, Soosaar A, Voikar V, Bourin M, Vasar E. BOC-CCK-4, CCK(B) receptor agonist, antagonizes anxiolytic-like action of morphine in elevated plus-maze. Neuropeptides 1999;33:63–9.

Koks S, Soosaar A, Voikar V, Volke V, Ustav M, Mannisto PT, et al. Opioid antagonist naloxone potentiates anxiogenic-like action of cholecystokinin agonists in elevated plus-maze. Neuropeptides 1998;32:235–40.

Koob GF, Le Moal M. Neurobiology of Addiction. San Diego: Academic Press; 2005a.

Koob GF, Le Moal M. Plasticity of reward neurocircuitry and the 'dark side' of drug addiction. Nat Neurosci 2005b;8:1442–4.

Koob GF, Wall TL, Bloom FE. Nucleus accumbens as a substrate for the aversive stimulus effects of opiate withdrawal. Psychopharmacology (Berl) 1989;98:530–4.

Labella FS, Pinsky C, Havlicek V. Morphine derivatives with diminished opiate receptor potency show enhanced central excitatory activity. Brain Res 1979;174:263–71.

Lindesmith AR. Addiction and opiates. Chicago, IL: Aldine; 1968.

Liu J, Schulteis G. Brain reward deficits accompany naloxone-precipitated withdrawal from acute opioid dependence. Pharmacol Biochem Behav 2004;79:101–8.

Mansbach RS, Gold LH, Harris LS. The acoustic startle response as a measure of behavioral dependence in rats. Psychopharmacology (Berl) 1992;108:40–6.

Martin WR, Eades CG. A Comparison between Acute and Chronic Physical Dependence in the Chronic Spinal Dog. J Pharmacol Exp Ther 1964;146:385–94.

McDaid J, Dallimore JE, Mackie AR, Napier TC. Changes in accumbal and pallidal pCREB and deltaFosB in morphine-sensitized rats: correlations with receptor-evoked electrophysiological measures in the ventral pallidum. Neuropsychopharmacology 2006;31:1212–26.

Motta V, Brandao ML. Aversive and antiaversive effects of morphine in the dorsal periaqueductal gray of rats submitted to the elevated plus-maze test. Pharmacol Biochem Behav 1993;44:119–25.

Motta V, Penha K, Brandao ML. Effects of microinjections of mu and kappa receptor agonists into the dorsal periaqueductal gray of rats submitted to the plus maze test. Psychopharmacology (Berl) 1995;120:470–4.

Nair HP, Gutman AR, Davis M, Young LJ. Central oxytocin, vasopressin, and corticotropin-releasing factor receptor densities in the basal forebrain predict isolation potentiated startle in rats. J Neurosci 2005;25:11479–88.

Nobre MJ, Ribeiro dos Santos N, Aguiar MS, Brandao ML. Blockade of mu- and activation of kappa-opioid receptors in the dorsal periaqueductal gray matter produce defensive behavior in rats tested in the elevated plus-maze. Eur J Pharmacol 2000;404:145–51.

O'Brien CP. In: Hardman J, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG, editors. Drug addiction and drug abuse. McGraw-Hill; 1996. p. 557–77.

Ohl F, Toschi N, Wigger A, Henniger MS, Landgraf R. Dimensions of emotionality in a rat model of innate anxiety. Behav Neurosci 2001;115:429–36.

Parker LA, Joshi A. Naloxone-precipitated morphine withdrawal induced place aversions: effect of naloxone at 24 hours postmorphine. Pharmacol Biochem Behav 1998;61:331–3.

Patti CL, Kameda SR, Carvalho RC, Takatsu-Coleman AL, Lopez GB, Niigaki ST, et al. Effects of morphine on the plus-maze discriminative avoidance task: role of state-dependent learning. Psychopharmacology (Berl) 2006;184:1–12.

Rassnick S, Heinrichs SC, Britton KT, Koob GF. Microinjection of a corticotropin-releasing factor antagonist into the central nucleus of the amygdala reverses anxiogenic-like effects of ethanol withdrawal. Brain Res 1993;605:25–32.

Rodgers RJ, Dalvi A. Anxiety, defence and the elevated plus-maze. Neurosci Biobehav Rev 1997;21:801–10.

Schaefer GJ, Michael RP. Changes in response rates and reinforcement thresholds for intracranial self-stimulation during morphine withdrawal. Pharmacol Biochem Behav 1986;25:1263–9.

Schulteis G, Heyser CJ, Koob GF. Differential expression of response-disruptive and somatic indices of opiate withdrawal during the initiation and development of opiate dependence. Behav Pharmacol 1999;10:235–42.

Schulteis G, Heyser CJ, Koob GF. Opiate withdrawal signs precipitated by naloxone following a single exposure to morphine: potentiation with a second morphine exposure. Psychopharmacology (Berl) 1997;129:56–65.

Schulteis G, Koob GF. Reinforcement processes in opiate addiction: a homeostatic model. Neurochem Res 1996;21:1437–54.

Schulteis G, Liu J. Brain reward deficits accompany withdrawal (hangover) from acute ethanol in rats. Alcohol 2006;39:21–8.

Schulteis G, Liu J, Amitai N, Tzeng S. Context- and cue-conditioned potentiation of acute morphine dependence and withdrawal. Pharmacol Biochem Behav 2005;82:82–9.

Schulteis G, Markou A, Gold LH, Stinus L, Koob GF. Relative sensitivity to naloxone of multiple indices of opiate withdrawal: a quantitative dose-response analysis. J Pharmacol Exp Ther 1994;271:1391–8.

Schulteis G, Morse AC, Liu J. Repeated experience with naloxone facilitates acute morphine withdrawal: Potential role for conditioning processes in acute opioid dependence. Pharmacol Biochem Behav 2003;76:493–503.

Schulteis G, Morse AC, Liu J. Conditioning processes contribute to severity of naloxone-precipitated withdrawal from acute opioid dependence. Psychopharmacology (Berl) 2004;175:463–72.

Schulteis G, Yackey M, Risbrough V, Koob GF. Anxiogenic-like effects of spontaneous and naloxone-precipitated opiate withdrawal in the elevated plus-maze. Pharmacol Biochem Behav 1998;60:727–31.

Schulteis G, Zhang Z. Increased anxiety-like behavior during naloxone-precipitated withdrawal from acute opioid dependence. 2006 Meeting of the College on Problems of Drug Dependence. Scottsdale, AZ.; 2006.

Self DW, Nestler EJ. Relapse to drug-seeking: neural and molecular mechanisms. Drug Alcohol Depend 1998;51:49–60.

Shang GW, Liu DN, Yan LH, Cui XY, Zhang KP, Qi C, et al. Nociceptive stimulus modality-related difference in pharmacokinetic-pharmacodynamic modeling of morphine in the rat. Pharmacol Biochem Behav 2006;85:464–73.

Smith GD, Smith MT. The excitatory behavioral and antianalgesic pharmacology of normorphine-3-glucuronide after intracerebroventricular administration to rats. J Pharmacol Exp Ther 1998;285:1157–62.

Stinus L, Le Moal M, Koob GF. Nucleus accumbens and amygdala are possible substrates for the aversive stimulus effects of opiate withdrawal. Neuroscience 1990;37:767–73.

Valdez GR, Roberts AJ, Chan K, Davis H, Brennan M, Zorrilla EP, et al. Increased ethanol self-administration and anxiety-like behavior during acute ethanol withdrawal and protracted abstinence: regulation by corticotropin-releasing factor. Alcohol Clin Exp Res 2002;26:1494–501.

Valdez GR, Sabino V, Koob GF. Increased anxiety-like behavior and ethanol self-administration in dependent rats: reversal via corticotropin-releasing factor-2 receptor activation. Alcohol Clin Exp Res 2004;28:865–72.

Valdez GR, Zorrilla EP, Roberts AJ, Koob GF. Antagonism of corticotropin-releasing factor attenuates the enhanced responsiveness to stress observed during protracted ethanol abstinence. Alcohol 2003;29:55–60.

Vanderschuren LJ, De Vries TJ, Wardeh G, Hogenboom FA, Schoffelmeer AN. A single exposure to morphine induces long-lasting behavioural and neurochemical sensitization in rats. Eur J Neurosci 2001;14:1533–8.

Villereal J, Castro A. A reformulation of the dual-action model of opioid dependence: Opioid-specific neuronal kindling. In: Beers RF, Bassett EG, editors. Mechanisms of Pain and Analgesic Compounds. New York: Raven Press; 1979. p. 407–28.

Wall PM, Messier C. Methodological and conceptual issues in the use of the elevated plus-maze as a psychological measurement instrument of animal anxiety-like behavior. Neurosci Biobehav Rev 2001;25:275–86.

Way EL, Loh HH, Shen FH. Simultaneous quantitative assessment of morphine tolerance and physical dependence. J Pharmacol Exp Ther 1969;167:1–8.

Zhang Z, Morse AC, Koob GF, Schulteis G. Dose- and time-dependent expression of anxiety-like behavior in the elevated plus-maze during withdrawal from acute and repeated intermittent ethanol intoxication in rats. Alcohol Clin Exp Res 2007;31:1811–9.

Zheng M, McErlane KM, Ong MC. High-performance liquid chromatography-mass spectrometry-mass spectrometry analysis of morphine and morphine metabolites and its application to a pharmacokinetic study in male Sprague-Dawley rats. J Pharm Biomed Anal 1998;16:971–80.