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Repeated experience with naloxone facilitates acute morphine withdrawal: potential role for conditioning processes in acute opioid dependence

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Abstract

Single injections with morphine can induce a state of acute opioid dependence in humans and animals, typically measured as precipitated withdrawal when an antagonist such as naloxone is administered 4–24 h after morphine. Repeated treatment with morphine at 24-h intervals can result in a progressive shift in potency of naloxone to produce such acute withdrawal signs, including suppression of operant responding for food reward. The current study characterized fully both morphine and naloxone dose–effect functions in an effort to establish the relative contributions of repeated morphine vs. repeated naloxone (Nal) experience to these potency shifts. Rats trained on an FR15 schedule for food received four vehicle or morphine injections (0.56–5.6 mg/kg sc), spaced 24 h apart. Four hours after each morphine pretreatment (Repeat Nal), or 4 h after the fourth and final morphine pretreatment only (Single Nal), a cumulative dose–effect function for naloxone-induced suppression of responding was determined. Vehicle-pretreated (Morphine Naive) rats showed little change in the naloxone dose effect function even after four cumulative dose–effect determinations. By contrast, a progressive increase in naloxone potency was observed following successive pretreatments with morphine under Repeat Nal conditions, and the magnitude of naloxone potency shift was morphine dose dependent. At a morphine dose of 5.6 mg/kg, repeated naloxone experience in the presence of morphine was not an absolute requirement to produce an increase in naloxone potency across days, but repeated naloxone could potentiate the magnitude of the observed shift, indicating both experience-independent and experience-dependent processes at work. At lower doses of morphine (1.0 and 3.3 mg/kg) no shift in naloxone potency was observed across days of morphine treatment in the absence of repeated naloxone experience (Single Nal conditions), indicating an increasing contribution of naloxone experience-dependent processes as dose of morphine was decreased. It is argued that these experience-dependent processes in the progressive shift of naloxone potency observed in the current study may reflect an important role of conditioning in the early development of opioid dependence.

Keywords: Morphine; Dependence; Withdrawal; Abstinence; Addiction; Naloxone; Conditioning

1. Introduction

Acute opioid dependence, as measured by the precipitation of withdrawal-like signs by an opioid antagonist following a single exposure to morphine or other opioid agonist, is a well-described phenomenon in humans and a variety of animal species (Adams and Holtzman, 1990; Azar et al., in press; Azorlosa et al., 1994; Bickel et al., 1988; Cheney, 1971; Heishman et al., 1989a,b, 1990; Jacob and Michaud, 1974; Jones, 1980; Martin and Eades, 1964; Parker and Joshi, 1998; Schulteis et al., 1997, 1999; White-Gbadebo and Holtzman, 1993, 1994; Wiley and Downs, 1979; Young, 1986). It is important to note that this acute dependence phenomenon can be observed in opioid-naive humans (Jones, 1980; Azorlosa et al., 1994) and animals (Parker and Joshi, 1998; Schulteis et al., 1997, 1999), suggesting that even a single exposure to opioids can induce a mild dependence-like state measurable in the form of opioid withdrawal signs upon antagonist administration from 4 to 24 h postmorphine. The severity of antagonist-precipitated withdrawal following a

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single pretreatment with an opioid has been shown to depend on dose of opioid agonist (Adams and Holtzman, 1990; Azar et al., in press; Jones, 1980; Bickel et al., 1988), dose of antagonist (Azar et al., in press; Adams and Holtzman, 1990; Heishman et al., 1989a; Schulteis et al., 1997, 1999) and the interval between agonist pretreatment and naloxone administration (Heishman et al., 1989b; Young, 1986).

In addition, it is also well established that repeated treatments with morphine at daily or weekly intervals can increase the severity of withdrawal-like signs elicited upon antagonist administration (Adams and Holtzman, 1990; White-Gbadebo and Holtzman, 1993, 1994; Schulteis et al., 1997, 1999), suggesting a progressive development of dependence as one would expect if acute dependence reflected the early stages in the development of a full opioid dependence state. Earlier work has demonstrated that repeated experience with naloxone (i.e., repeated withdrawal) following each morphine pretreatment is necessary to observe this shift in antagonist potency under some (Schulteis et al., 1999) but not all conditions (Azorlosa et al., 1994; Schulteis et al., 1997). These observations are indicative of both naloxone-experience-dependent and -independent processes. The latter processes presumably reflect direct neuroadaptive responses to repeat administration of morphine itself, whereas the naloxone-experience-dependent processes have been suggested to reflect underlying conditioning mechanisms (Adams and Holtzman, 1990; Schulteis et al., 1999). For example, Adams and Holtzman (1990) reasoned that repeated exposure to a naltrexone cumulative-dosing regimen following pretreatments with morphine in their acute dependence model might have produced a conditioned "sensitization" of naltrexone-induced opioid withdrawal-like behaviors, with low doses of naltrexone serving as an interoceptive stimulus cue for higher doses that followed. In support of this interpretation, it has been shown that animals trained to detect the discriminative stimulus properties of opiate antagonists generalize to much lower doses of antagonists if pretreated with morphine (Easterling and Holtzman, 1999; France and Woods, 1985, 1987, 1988), an effect attributed to a "qualitatively unique [opioid] withdrawal stimulus" produced by the antagonist when following morphine exposure.

The current study sought to examine more fully the conditions under which naloxone-experience-dependent (conditioning) processes contribute to the progressive shift in naloxone potency produced by repeated pretreatment with morphine. Suppression of operant response rates for food reward is one of the most frequently used indices of withdrawal in acute opioid-dependence studies in animals (Adams and Holtzman, 1990; Schulteis et al., 1997, 1999; White-Gbadebo and Holtzman, 1993, 1994; Young, 1986), and in particular in those studies where effects of repeated antagonist experience have been noted and conditioning mechanisms have been inferred (Adams and Holtzman, 1990; Schulteis et al., 1999). Consequently, the operant response suppression model was chosen as the primary index of withdrawal-like behaviors in the current investigation. We also used a cumulative-dosing procedure for naloxone to minimize the number of experimental groups required to complete the study and to provide the potential extra cue (interoceptive stimulus cues of lower naloxone doses in the cumulative function) suggested by Adams and Holtzman (1990) to play a facilitative role in conditioned "sensitization" of antagonist-induced withdrawal behaviors. A range of morphine doses (0.56–5.6 mg/kg) was examined to determine whether the relative contributions of naloxone-experience-dependent and experience-independent processes varied as a function of morphine pretreatment dose. A critical feature of the current study was the use of separate groups of animals that received naloxone (NAL) either (1) after each of the four morphine pretreatments (Repeat Nal) or (2) after the fourth and final morphine pretreatment only (Single Nal; see Table 1 for details of design). In this way, the effects of prior naloxone experience could be examined directly.

2. Materials and methods

2.1. Animals

Male Wistar rats ($n = 80$, Charles River, Kingston, NY) weighing 300–400 g at the time of testing were used. All rats were group housed (two to three per cage) in a temperature- and humidity-controlled room with a 12-

Table 1
Summary of experimental design

Treatment group	Days 1, 2, and 3		Day 4	
	Pretreatment ($T = 0$)	Treatment (start $T = 4$ h)	Pretreatment ($T = 0$)	Treatment (start $T = 4$ h)
Morphine Naive	Vehicle	Nal CumDose (0.03–1.0 mg/kg)	Vehicle	Nal CumDose (0.03–1.0 mg/kg)
Repeat Nal	Morphine (0.56, 1, 3.3, 5.6 mg/kg)	Nal CumDose (0.03–1.0 mg/kg)	Morphine (same dose as Days 1–3)	Nal CumDose (0.03–1.0 mg/kg)
Single Nal	Morphine (1, 3.3, 5.6 mg/kg)	Vehicle (5 × at 15-min intervals)	Morphine (same dose as Days 1–3)	Nal CumDose (0.03–1.0 mg/kg)

Nal = naloxone; CumDose = cumulative dosing at 15-min increments.

h light/12-h dark cycle (lights on at 6:00 a.m.). Rats had ad-libitum access to food until the start of operant training, and had ad-libitum access to water at all times. Once operant training was begun, rats were maintained on 15 g of rat chow per day in addition to the food pellets earned in the operant boxes (total food intake was approximately 20–22 g/rat/day). All training and testing took place from 10:00 a.m. to 4:00 p.m. daily, Monday through Friday. On days when rats were not trained in the operant boxes (Saturday and Sunday), an additional 5 g of rat chow was provided to ensure that total food intake remained relatively constant, and all rats continued to gain weight at an average of 10–20 g/week throughout training and testing. All experimental procedures were approved by the Subcommittee on Animal Studies 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 purchased from King Pharmaceuticals (Bristol, TN), and naloxone HCl was purchased from Sigma (St. Louis, MO). Both drugs were prepared for injection in sterile physiological saline, and all injections were made subcutaneously 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 of 0.56, 1.0, 3.3, or 5.6 mg/kg, and naloxone was administered using a cumulative-dosing procedure at 1/2-log incremental doses from 0.03 to 1.0 mg/kg as described below (see Acute dependence and withdrawal testing regimen). Care was taken to prepare fresh solutions of drug every 3 days, to draw from the drug vials *only* with clean needles that had not been previously used on any animal, and to inject each animal with a separate syringe. In our experience over the past 5 years under these conditions, the cumulative-dosing procedure has not been associated with any incidence of scarring, tissue injury, signs of infection, or signs of distress (e.g., vocalization/struggling during injection) on the part of the subjects even after 5 days or more of multiple subcutaneous injections, nor have such signs been reported in other established reports with this procedure (e.g., Adams and Holtzman, 1990; White-Gbadebo and Holtzman, 1993, 1994; Young, 1986).

2.3. Operant training

Fourteen operant chambers (Coulbourn Instruments, Columbus, OH) served as the training and testing environments. Each chamber was equipped with a food hopper located 4 cm above a grid floor, a lever located to the right of the food hopper, and a cue light located above the lever. The cue light illuminated for 1 s as a food pellet (45 mg) was delivered each time a rat completed a fixed-ratio (FR) component. Rats were autoshaped to lever press for food pellets in 30-min sessions 5 days a week, beginning on an FR1 schedule and progressing to an FR15 schedule (1 s timeout [TO1]).

After 2–3 weeks on the FR15 TO1 schedule, daily testing was separated into five windows of lever availability (5 min opportunity to respond on FR15 TO1 schedule). Each window of lever availability was separated from the next one by a 10-min period in which the levers were retracted. This training regimen ultimately would permit the periodic injection of vehicle or naloxone under the cumulative-dosing procedure. Rats were tested in this manner for 5 days/week until responding stabilized.

2.4. Acute dependence and withdrawal testing regimen

Once baseline stability was achieved (defined as less than 10% variation from the mean of five consecutive test days), rats were acclimated to the injection procedure by receiving vehicle (saline) injections 10 min before each 5-min response window on Tuesday through Friday (Baseline Week). The experimental procedure was as follows: Rats were given an injection of vehicle and placed into the operant chamber; 10 min after being placed in the chamber, the levers were extended and the rats had a 5 min window to respond for food. Then the levers were retracted, the rats were removed from the operant chambers, and another vehicle injection was administered. The rats were immediately returned to their operant chambers, and 10 min later the levers again were extended. This cycle was repeated five times.

The data from Thursday and Friday of the Baseline Week were averaged and the averages used as the baseline response rates for each rat in the absence of any morphine or naloxone treatment. The following week (Experimental Week), rats were tested on Monday exactly as they had been during the Baseline Week. The data from the Monday test session was not entered into any analysis, because most animals typically show 10–20% higher response rates, particularly early in the operant sessions, following weekends without any operant testing. It is important to stress that rats received 5 g additional food in their home cages on Saturday and Sunday, and this extra daily food ration is equivalent to the average amount of food earned in the operant sessions. Therefore, this ‘‘weekend’’ effect of increased responding is most directly attributable to the lack of training on the weekend rather than an increased level of food restriction.

On Tuesday through Friday of the Experimental Week (see Table 1 for details), rats were assigned to one of eight treatment groups ($n = 10$/group), categorized into one of the following experimental conditions: MORPHINE-NAIVE (1 group pretreated with VEH on each test day), Repeat Nal (4 groups pretreated with 0.56, 1.0, 3.3, or 5.6 mg/kg of morphine), and Single Nal (3 groups pretreated with 1.0, 3.3, or 5.6 mg/kg of morphine). Data from one subject in group Repeat Nal (5.6 mg/kg dose of morphine) were subsequently dropped due to a food hopper jam on

the final day (Day 4) of morphine/naloxone treatment, yielding a final sample size of 9 in that group and 10 in all other experimental groups.

For groups of rats in the Repeat Nal condition, rats were pretreated with their given dose of morphine on Tuesday through Friday (Days 1 to 4), and beginning 4 h after each morphine pretreatment were injected according to the following cumulative-dosing regimen: vehicle, followed by 0.03 mg/kg naloxone, 0.067 mg/kg naloxone (cumulative dose of 0.1 mg/kg), 0.23 mg/kg naloxone (cumulative dose of 0.33 mg/kg), and 0.67 mg/kg naloxone (cumulative dose of 1.0 mg/kg), successively at 15-min intervals. The 5-min operant window wherein rats could respond on the FR15 schedule began 10 min after each naloxone injection and ended immediately before the next injection. For groups of rats in the Single Nal condition, beginning 4 h after each morphine pretreatment rats received vehicle injections before each operant response window on Tuesday, Wednesday, and Thursday. However, on Friday of the Experimental Week these groups of rats also received the naloxone cumulative-dosing regimen 4 h after their given dose of morphine as described above for groups in the Repeat Nal condition. Therefore, rats received identical morphine experience whether tested under conditions Single Nal or repeat Nal, but received different degrees of naloxone experience. Finally, rats in the Morphine Naive group received vehicle pretreatment 4 h before the naloxone cumulative-dosing regimen (as described above) on each of the four experimental days (Tuesday through Friday). Inclusion of all these experimental groups allowed for the assessment of independent contributions of naloxone alone, morphine alone, and the combination of morphine and naloxone, in the progression of severity of operant response suppression across days (Tuesday through Friday).

Because naloxone (and other opioid antagonists) can produce sensitization to their own response-rate suppressant effects with repeated treatment (e.g., Schindler et al., 1992, 1993) even in the absence of morphine, we felt it important to truncate the naloxone dose–effect function at a dose that would produce minimal if any direct carry-over effects of repeated naloxone treatment on its own. In this way, the effects of repeated morphine treatment would not be confounded by pharmacological sensitization produced by naloxone itself. As a consequence, while some prior cumulative dose–effect studies (e.g., Adams and Holtzman, 1990; Young, 1986) of naloxone- or naltrexone-induced suppression of response rates during acute opioid dependence increased antagonist dose until a predetermined effect was obtained (e.g., >80% suppression), we chose instead to maintain a predetermined maximum dose of naloxone. Pilot studies (data not shown) revealed minimal shifts in naloxone potency upon repeated cumulative dosing to 1.0 mg/kg, whereas dosing to 3.0 mg/kg resulted in significant sensitization of response suppression by naloxone itself (i.e., in Morphine-Naive rats). Therefore, all cumulative dose–response functions in the current study were truncated at the same dose of 1.0 mg/kg of naloxone, regardless of the degree of response suppression achieved at that dose.

2.5. Statistical analysis

Data on all experimental treatment days were expressed as percent of response rate in the corresponding 5-min response window on the baseline days. Subsequently the converted percent baseline response rate data were entered into a number of analyses using ANOVA and/or quantitative probit dose–response analysis using the method of Litchfield and Wilcoxon (Tallarida and Murray, 1986). Details of each analysis performed are provided in the appropriate portion of the Results section.

3. Results

3.1. Effects of single and repeated morphine treatment on operant response rates at 4 h postmorphine (before naloxone)

As shown in Fig. 1, response rates 4 h after morphine pretreatment in the absence of naloxone show little change from baseline, even with repeated morphine treatment at 24-h intervals, except at the highest dose of morphine tested (5.6 mg/kg). The statistical reliability of this observation was assessed by mixed-design ANOVA with responding in the post-vehicle response window (first 5-min response window) as the dependent measure, morphine dose as a between-subjects factor, and treatment day as a within-subjects factor. This ANOVA revealed a significant main effect of morphine dose [$F(4,44) = 5.67$, $P < .001$] and a significant main effect of treatment day [$F(3,132) = 14.59$, $P < .0001$], but no significant interaction [$F(12,132) = 1.33$, $P > .20$]. Follow-up comparisons of the simple main effects of morphine dose on Days 1, 2, 3, and 4, corrected by the Bonferroni method for multiple comparisons, revealed a significant effect of morphine dose only on Day 4 [$F(4,44) = 6.67$, $P < .005$]. Further pairwise comparisons of all morphine dose groups to the vehicle (Morphine Naive) group on Day 4 revealed a significant decrease in response rates only in the group treated with the highest (5.6 mg/kg) dose of morphine. The suppression of response rates following naloxone treatment is considerably more pronounced than these subtle effects in the post-vehicle response window (see Fig. 1 and the following section).

3.2. Effect of single morphine treatment on potency of naloxone to suppress operant response rates at 4 h postmorphine

As shown in Fig. 1, the potency of naloxone to suppress operant response rates after a single morphine pretreatment (Day 1) appeared dependent on morphine

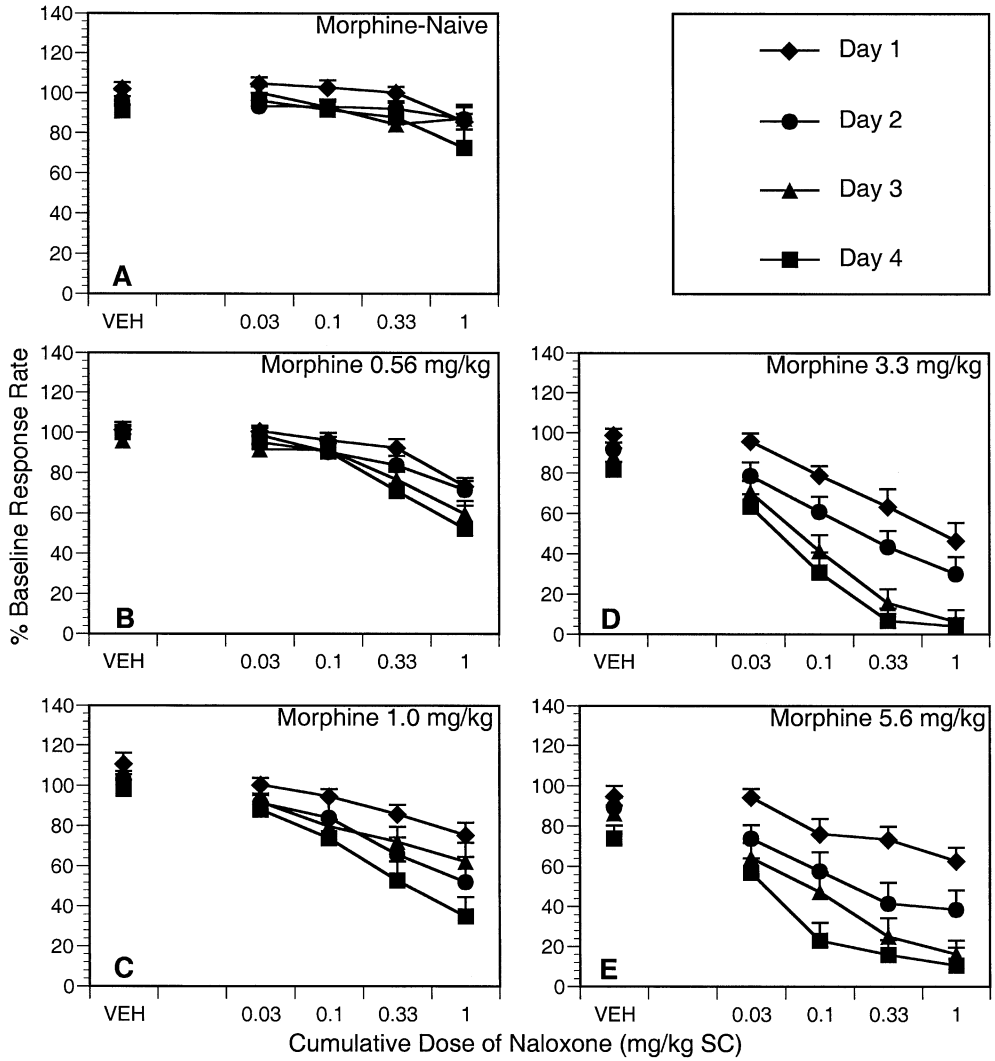

Fig. 1. Shift in naloxone potency as a function of morphine pretreatment dose across 4 days of repeated treatment at 24-h intervals. Data represent mean (± S.E.M.) percent of baseline response rate. As described in detail in the Materials and methods section, rats were treated with vehicle (Morphine-Naive, Panel A) or one of four different doses of morphine (0.56, 1.0, 3.3, 5.6 mg/kg, Panels B–E, respectively). Four hours after each morphine injection, an operant testing session was initiated in which rats received vehicle (VEH), followed by cumulative doses of naloxone from 0.03 to 1.0 mg/kg at 15-min intervals. Rats had 5-min response opportunities following each injection of VEH or naloxone. Note that increasing doses of morphine resulted in greater naloxone potency on Day 1 (diamonds). Note also that increasing doses of morphine resulted in increasing shifts in naloxone potency upon repeated exposure (Days 2–4, circles, triangles, squares, respectively). All groups had 10 rats, with the exception of the 5.6 mg/kg dose group (Panel E), where sample size was 9, one subject in this group was excluded because data on the final test day were compromised by a food hopper jam that prevented pellet delivery. Refer to Table 2 for statistical significance of shifts in naloxone potency as determined in a potency ratio analysis.

dose. This observation was tested using a two-factor mixed-design ANOVA, with responding in the postnaloxone response windows on Day 1 only (groups repeat Nal and MORPINE NAIVE) as the dependent measure, morphine dose as a between-subjects factor and cumulative naloxone dose as a within-subjects factor. A significant interaction [$F(3,132) = 3.19$, $P < .0005$] confirmed the positive relationship between morphine dose and potency of naloxone after a single morphine pretreatment. The main effect of morphine dose [$F(4,44) = 7.90$, $P < .0001$], and main effect of naloxone dose [$F(3,132) = 75.39$, $P < .0001$] were also significant.

3.3. Effect of repeated morphine treatment on potency of naloxone to suppress operant response rates at 4 h postmorphine

The degree of shift in naloxone potency with repeated morphine pretreatments at 24-h intervals also appeared dependent on morphine dose. This observation was tested using a three-factor mixed-design ANOVA, with responding in the postnaloxone response windows on Days 1 through 4 (groups Repeat Nal and MORPINE NAIVE) as the dependent measure, morphine dose as a between-subjects factor, and both treatment day and naloxone dose

serving as repeated measures. This ANOVA revealed a significant Morphine Dose × Treatment Day interaction [$F(12,132)=3.97$, $P<.0001$], a significant Morphine Dose × Naloxone Dose interaction [$F(12,132)=6.67$, $P<.0001$], and a significant Treatment Day × Naloxone Dose interaction [$F(9,396)=4.25$, $P<.0001$]. Significant main effects of all three factors (all Fs >26.48, Ps < .0001) were also observed; the only term that did not achieve significance was the three-way interaction between morphine dose, naloxone dose, and treatment day [$F(36,396)=1.28$, $P>.10$].

Rather than engaging in a large number of follow-up ANOVA comparisons, follow-up analysis consisted of quantitative probit dose–response analysis of naloxone dose–response functions on Days 1 and 4 using the method of Litchfield and Wilcoxon (Tallarida and Murray, 1986). Using this method, naloxone ED50 values with 95% confidence limits were determined, followed by calculation of potency ratios that could assess the statistical significance of shifts in naloxone dose–response function under different treatment conditions. A summary of the analysis can be found in Table 2, which clearly reveals that increases in naloxone potency from Days 1 to 4 are dependent on morphine pretreatment dose, with rats pretreated with 1.0, 3.3, and 5.6 mg/kg of morphine 4 h before each naloxone dose–response determination showing 8.85, 13.92, and 60.27-fold shifts in naloxone potency across treatment days, respectively (all $P<.05$).

The positive relationship between morphine pretreatment dose and degree of shift in naloxone potency (as measured by ED50) with repeated morphine pretreatment is also clearly demonstrated in Table 2. For example, after 4 days of morphine pretreatment, morphine doses of 1.0, 3.3, and 5.6 mg/kg produced roughly 21-, 600-, and 940-fold shifts, respectively, in naloxone potency relative to vehicle pretreatment (Morphine Naive), and all of these shifts were statistically reliable ($P<.05$). It is noteworthy that 4 days of pretreatment with 3.3 or 5.6 mg/kg of morphine could produce shifts in naloxone potency that are comparable to what has been reported in rats exposed chronically to morphine (e.g., Adams and Holtzman, 1990; Schulteis et al., 1994).

Table 2
ED50 values and potency ratios for naloxone-induced suppression of operant response rates following pretreatment with different doses of morphine or vehicle on Days 1 and 4 of treatment

Pretreatment condition	Treatment day	ED50 value (mg/kg) (95% CL)	Potency ratio, Day 4 naloxone dose–response following each dose of morphine vs.	
			Vehicle pretreatment on Day 4[a]	Same morphine pretreatment on Day 1[b]
Vehicle	Day 1	>8 mg/kg[c]		
	Day 4	8.32 (0.50–138)	–	N.D.
Morphine 0.56 mg/kg	Day 1	>8 mg/kg[c]		
	Day 4	1.12 (0.31–4.06)	7.46, N.S.	N.D.
Morphine 1.0 mg/kg	Day 1	3.51 (0.59–21.0)		
	Day 4	0.40 (0.13–1.20)	20.83 *	8.85 *
Morphine 3.3 mg/kg	Day 1	0.66 (0.02–5.66)		
	Day 4	0.05 (0.02–0.13)	599.57 *	13.92 *
Morphine 5.6 mg/kg	Day 1	1.92 (0.42–8.67)		
	Day 4	0.03 (0.01–0.11)	944 *	60.27 *

95% Confidence limits (CL) in parentheses.
N.S. = not significant; N.D. = could not be determined due to missing ED50 value (see footnote c for explanation).
[a] Potency ratio comparing the naloxone dose–response function under the specified pretreatment condition and specified treatment day to vehicle pretreatment on Day 4 (could not compare to vehicle Day 1, ED50 could not be calculated for this condition on this day; see footnote c below).
[b] Potency ratio comparing the naloxone dose–response function on Day 4 of the specified pretreatment condition to Day 1 of the same pretreatment condition.
[c] In some cases, ED50 values could not be calculated because the maximum naloxone dose of 1.0 mg/kg did not include enough of the linear region of the naloxone dose–response function to permit the calculations. Because the dose–response functions in these cases were more shallow than the one observed on Day 4 of vehicle pretreatment (ED50 value of 8.32 mg/kg on that day following vehicle), the ED50 values in those cases where one could not be calculated are listed as >8 mg/kg.
* $P<.05$ as determined by relative potency analysis (Tallarida and Murray 1986).

3.4. Effects of repeated naloxone experience on the degree of shift in naloxone dose–response functions following repeated morphine pretreatments

Data from groups Single Nal (three separate groups pretreated with 1.0, 3.3, or 5.6 mg/kg) and Repeat Nal (same three dose groups) collected during the final four response windows (following each naloxone cumulative-dose injection) were entered into this analysis. Data from Days 1 and 4 of the Repeat Nal groups and from Day 4 of the Single Nal groups (the only day on which a naloxone dose–response function was determined in these groups) were used. Due to the mixed nature of the data, with Days 1 and 4 of Repeat Nal being repeated measures in the same groups of animals, and Day 4 of Single Nal being from separate groups, an overall ANOVA could not be conducted. Rather than conduct multiple separate ANOVAs, these data were therefore subjected directly to quantitative probit dose–response analysis.

An inspection of Fig. 2 (Panel C) suggests that repeated treatment with naloxone on Days 1, 2, and 3 is not necessary to observe a progressive shift in naloxone potency following repeated pretreatment with 5.6 mg/kg of morphine. ED50 and potency ratio analysis summarized in Table 3 confirms this observation. In comparing the ED50 value from group Single Nal to the ED50 value from group repeat Nal on Day 1, a near-10-fold shift in potency of naloxone is seen as a consequence of repeated morphine pretreatment by itself,

and this shift is statistically significant ($P < .05$). However, it is also noteworthy that naloxone was about one-sixth as potent ($P < .05$) when administered for the first time on Day 4 (Single Nal) as when administered for the fourth time on Day 4 (Repeat Nal). Thus, whereas repeated morphine experience (5.6 mg/kg dose) by itself can produce a significant 10-fold increase in naloxone potency, a further 6-fold increase can be seen when naloxone is also administered on all treatment days.

Interestingly, when examining the effects seen following the lower doses of morphine (1.0 and 3.3 mg/kg, see Fig. 2, Panels A and B), naloxone experience on all 4 days of testing becomes necessary to observe shifts in naloxone potency across days of treatment. Thus, as shown in Table 3, when rats were pretreated with 1.0 or 3.3 mg/kg doses of morphine, naloxone potency on Day 4 in group Single Nal is not significantly different from naloxone potency on Day 1 in group Repeat Nal, whereas repeated treatment with both morphine and naloxone (Repeat Nal Day 4) results in dramatic shifts in naloxone potency, relative to both Day 1 in Repeat Nal and Day 4 in Single Nal groups.

Table 3
ED50 values and potency ratios for naloxone-induced suppression of operant response rates in rats administered naloxone on Day 4 only (Single Nal) vs. rats administered naloxone on all 4 days (Repeat Nal)

Pretreatment condition	Treatment group	ED50 value (mg/kg) (95% CL)[a]	Potency ratio vs.	
			Repeat Nal, Day 1	Repeat Nal, Day 4
Morphine 1.0 mg/kg	Repeat Nal, Day 1	3.51 (0.59–21.0)	–	0.11 *
	Repeat Nal, Day 4	0.40 (0.13–1.20)	8.85 *	–
	Single Nal, Day 4	5.27 (0.60–46.3)	1.50, N.S.	0.075 *
Morphine 3.3 mg/kg	Repeat Nal, Day 1	0.66 (0.02–5.66)	–	0.072 *
	Repeat Nal, Day 4	0.05 (0.02–0.13)	13.92 *	–
	Single Nal, Day 4	1.22 (0.19–7.74)	1.86, N.S.	0.26 *
Morphine 5.6 mg/kg	Repeat Nal, Day 1	1.92 (0.42–8.67)	–	0.017 *
	Repeat Nal, Day 4	0.03 (0.01–0.11)	60.27 *	–
	Single Nal, Day 4	0.19 (0.09–0.43)	9.95 *	0.16 *

N.S. = not significant.
[a] 95% confidence limits (CL) for calculated ED50 provided in parentheses.
* $P < .05$ as determined by relative potency analysis (Tallarida and Murray, 1986).

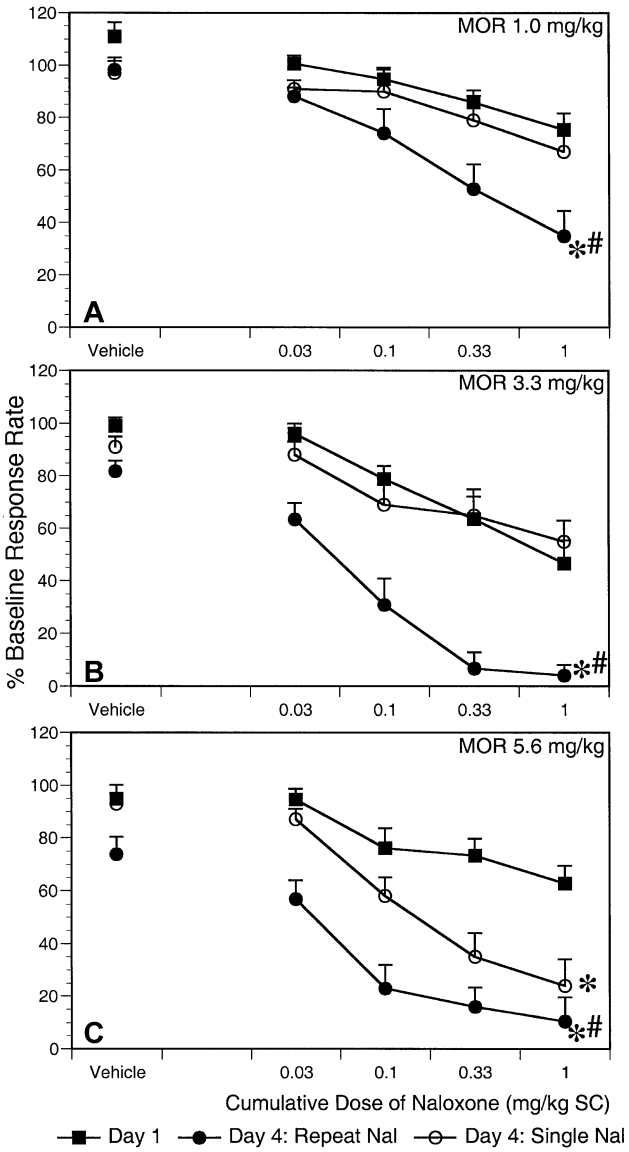

Fig. 2. Effect of repeated naloxone experience on shifts in naloxone potency. Data represent mean (± S.E.M.) percent of baseline response rate. As described in detail in the Materials and methods section and Table 1, rats were treated with one of three doses of morphine (1.0, 3.3, or 5.6 mg/kg) on each of four consecutive test days. Four hours after morphine pretreatment, rats in group Repeat Nal received the naloxone cumulative dose procedure described in the methods on all four test days. By contrast, rats in group Single Nal received vehicle substituted for each naloxone cumulative dose on Days 1–3, and received the naloxone cumulative dose regimen only on the fourth and final day of testing. Data for Day 1 (closed squares) are taken from the Repeat Nal condition; similar data were not available for group Single Nal because no naloxone was administered to this group on Day 1. For the highest dose of morphine tested (5.6 mg/kg, Panel C), note that repeated naloxone experience *was not* necessary to observe a shift in naloxone potency with repeated morphine exposure (compare group single Nal on Day 4 [open circles] to Day 1 in Panel C). However, a *greater* shift in potency was seen if naloxone dosing followed *all* morphine pretreatments (compare group Single Nal to Repeat Nal [closed circles] on Day 4 in Panel C). Note that for the lower doses of morphine tested (1.0 and 3.3 mg/kg), repeated naloxone experience *was* necessary to produce a shift in naloxone potency (Panels A and B); rats treated with naloxone only after the fourth and final morphine pretreatment (Single Nal Day 4) did not differ from rats treated with naloxone after the first morphine pretreatment (Repeat Nal Day 1). * $P < .05$ vs. Day 1; # $P < .05$ vs. Day 4: Single Nal. Note that data for all Repeat Nal groups were the same as that shown in Fig. 1. Refer to Table 3 for potency ratio analysis of these data.

4. Discussion

The current study supports and extends earlier work in reporting that single treatment with low to moderate doses of morphine (1.0–5.6 mg/kg sc) dose-dependently induces a state of acute dependence as demonstrated by antagonist-precipitated suppression of operant responding (Adams and Holtzman, 1990; Schulteis et al., 1997, 1999; White-Gbadebo and Holtzman, 1993, 1994; Young, 1986). A single pretreatment with a dose of morphine as low as 1.0 mg/kg produced a shift to the left in the cumulative naloxone dose–effect function relative to the naloxone dose–effect function determined in morphine-naive rats, and higher doses of morphine (3.3–5.6 mg/kg) produced proportionately greater shifts to the left (see Fig. 1 and Table 2). Only the lowest morphine dose tested (0.56 mg/kg sc) did not produce a significant shift in naloxone potency relative to morphine-naive conditions. These data suggest that acute exposure to very low doses of morphine can elicit rapid neuroadaptive responses, and that these initial neuroadaptations may reflect the early stages in the development of opioid dependence. Consistent with this notion, we have recently shown (Liu et al., 2002, Schulteis and Morse, 2001) that the neural substrates mediating antagonist-induced suppression of operant response rates in rats treated acutely with morphine (nucleus accumbens, bed nucleus of stria terminalis) are identical to the substrates mediating these effects in rats *chronically* exposed to morphine (e.g., Delfs et al., 2000; Gracy et al., 2001; Koob et al., 1989; Stinus et al., 1990; Walker et al., 2000).

In addition to reporting demonstrable signs of opioid dependence and withdrawal following a single treatment with low doses of morphine, the current study also demonstrates that naloxone-precipitated withdrawal increases progressively with repeated morphine pretreatments separated by 24-h intervals, also confirming earlier work with rodents (Schulteis et al., 1997, 1999) and humans (Azorlosa et al., 1994). Importantly, the degree of shift in naloxone potency produced by repeated morphine pretreatment was found to be dependent on morphine dose, with 1.0, 3.3, and 5.6 mg/kg doses of morphine, respectively, producing roughly 9-, 14-, and 60-fold shifts in naloxone potency from Day 1 to Day 4 of testing (see Fig. 1 and Table 2). Importantly, morphine-naive rats and rats treated repeatedly with the lowest dose of morphine tested (0.56 mg/kg) showed little if any change in naloxone potency even upon four determinations of the cumulative dose–effect function at 24-h intervals. Therefore, the shifts in naloxone potency observed following pretreatment with 1.0–5.6 mg/kg of morphine could not be attributed simply to sensitization to the rate-suppressing effects of naloxone produced by the antagonist itself (Schindler et al., 1992, 1993). As mentioned previously, we deliberately chose to truncate our naloxone dose–effect functions at 1.0 mg/kg even though this precluded ED50 determinations under certain conditions (e.g., Day 1 in Morphine Naive rats and rats treated with 0.56 mg/kg). This was an unfortunate but necessary and acceptable limitation because we felt it critical to eliminate sensitization produced by naloxone itself as an additional complicating factor in our analysis.

While discussing limitations of the cumulative-dosing procedure used herein, it must be noted that the total naloxone dose of 1.0 mg/kg is broken into four injections distributed over a 45-min period from the time of the first injection. Thus, it is possible that the naloxone ED50 values generated herein may be slightly different from those that would be obtained if bolus fixed doses of naloxone had been administered at exactly 4 h postmorphine to separate groups to generate the dose–effect function (e.g., Schulteis et al., 1997). However, it is noteworthy that the calculated ED50 of naloxone after Day 1 of pretreatment with 5.6 mg/kg of morphine in the present study (1.92 mg/kg; see Table 2) is nearly identical to that calculated from our earlier work wherein fixed doses of naloxone were use to generate the dose–effect function (1.88 mg/kg; Schulteis et al., 1997; see Azar et al., in press, for the ED50 calculations on these data). This suggests that our cumulative-dosing procedure provides a fairly accurate assessment of true naloxone potency, and provides the added benefit of reducing animal requirements fourfold (one group instead of five, vehicle + 4 doses).

The current paper extends beyond prior reports of shifts in naloxone cumulative dose–effect functions with acute or repeated morphine treatment (Adams and Holtzman, 1990; White-Gbadebo and Holtzman, 1993, 1994; Young, 1986) in seeking to determine directly the conditions under which repeated experience with naloxone in the presence of morphine (rather than simple repeated morphine experience itself) contributes to the observed shift in naloxone potency. Indeed, Adams and Holtzman (1990) had suggested, although not directly tested, the notion that repeated experience with the cumulative-dosing procedure could produce a type of conditioning to the interoceptive cues of low-dose naltrexone that might lead to a greater suppression with the higher doses that followed later in the session. They reasoned that such conditioning mechanisms contributed at least in part to the dramatic (more than 600-fold) shifts in naltrexone potency they observed when repeatedly testing animals with pretreatments of morphine separated by 1-week intervals. Consistent with this interpretation are reports (Easterling and Holtzman, 1999; France and Woods, 1985, 1987, 1988) that the discriminative stimulus cues of opioid antagonists are potentiated with acute or chronic morphine pretreatment.

In support of this conditioning hypothesis, Schulteis et al. (1999) reported that when each of three successive morphine (5.0 mg/kg) exposures was separated by intervals of 6 weeks, the response suppression produced by a fixed (0.3 mg/kg) dose of naloxone only increased if naloxone was administered after *each* of the three morphine exposures (Repeat Nal). Rats given naloxone only after the third and final morphine pretreatment at 6-week intervals (Single Nal)

showed a response to naloxone no different from that of rats treated with naloxone after a single morphine pretreatment. In that same study, however, it was found that at shorter intervals between successive morphine exposures (24 h, 1 week, or 3 weeks), repeated naloxone experience did not affect the magnitude of shift in naloxone-induced withdrawal; instead, the fixed 0.3-mg/kg dose of naloxone produced the same degree of response suppression after the third and final morphine pretreatment under Repeat Nal or Single Nal conditions. These data demonstrated that the influence of repeated naloxone experience varied as a function of the interval between successive morphine doses, and suggested that repeated naloxone experience was not necessary to observe an increase in naloxone effect when the interval between successive morphine (and successive naloxone) treatments was 24 h, as it was in the current study.

However, as noted by Schulteis et al. (1997), their "data cannot [entirely] rule out a role for naloxone-precipitated withdrawal experience.... It is quite possible that such experience-dependent mechanisms may play a facilitative, although not obligatory, role at shorter [e.g., 24-h] intervals between morphine treatments...." Consequently, a crucial component of the current study was a more systematic evaluation of morphine and naloxone dose conditions that might reveal such facilitative effects. The present findings extend the previous data of Schulteis et al. (1999) in reporting that morphine dose in addition to morphine intertreatment interval is a crucial factor that when varied systematically can identify conditions under which repeated naloxone experience is obligatory. As shown in Fig. 2 and Table 3, there was a definite relationship between the pretreatment dose of morphine and the degree to which repeated naloxone experience contributed to the shift in naloxone potency. At the 5.6-mg/kg dose of morphine, a single cumulative dose–response determination after the fourth and final morphine pretreatment (Single Nal) revealed a significant 10-fold shift in naloxone potency relative to its potency after a the first morphine pretreatment (Repeat Nal, Day 1). However, an additional 6-fold shift in naloxone potency was observed if naloxone cumulative dosing was repeated following all four morphine pretreatments (compare Repeat Nal Day 4 to Single Nal Day 4 in Fig. 2 and Table 3). Thus, at the highest dose of morphine tested, both naloxone experience-independent and -dependent effects were observed.

In contrast, at the 1.0 and 3.3 mg/kg pretreatment doses of morphine, naloxone experience following each morphine exposure appeared to be required in order to observe a shift in naloxone potency. Interestingly, from the results presented in Fig. 1 and Table 2, one might conclude that pretreatment with 3.3 and 5.6 mg/kg produces roughly equal effects on naloxone potency on Day 1 or Day 4 of treatment. However, from Fig. 2 and Table 3 it becomes evident that the shift in naloxone potency produced by four repeated pretreatments with the 3.3 mg/kg dose of morphine was entirely dependent on repeated naloxone experience, whereas there was a clear naloxone experience-independent component to the shift observed with the 5.6 mg/kg dose of morphine. It is noteworthy that there was no statistically reliable shift in naloxone potency, even with repeated naloxone treatment, at the lowest dose of morphine tested (0.56 mg/kg). It is likely that this low dose of morphine produces little or no potentiation of naloxone's discriminative stimulus effects (Easterling and Holtzman, 1999; France and Woods, 1985, 1987, 1988), and therefore limited ability for an association to be formed to these interoceptive cues.

While our data are consistent with an interpretation of conditioning processes potentiating naloxone potency, another possible explanation deserves some attention: that intermittent naloxone exposure following morphine pretreatment may directly (pharmacologically) potentiate the adaptive changes produced by morphine. For example, in rats made tolerant to the effects of morphine through continuous (7-day) intrathecal infusions, tolerance to the antinociceptive effects of morphine was potentiated if a single daily injection of naloxone (0.6 mg/kg sc) was given during the course of morphine exposure (Ibuki et al., 1997). Thus, transient naloxone antagonism of morphine at the spinal level actually potentiated rather than inhibited the development of morphine tolerance. This leads to the intriguing possibility that repeated bouts of naloxone-induced withdrawal during the development of opioid dependence could also increase subsequent severity of withdrawal. Research on alcohol withdrawal provides good evidence for such "kindling" of withdrawal. Clinical and preclinical research in ethanol-dependent subjects suggests that repeated episodes of abstinence can lead to a progressive increase in severity of withdrawal, in support of the "kindling" hypothesis of alcohol withdrawal (e.g., Ballenger and Post, 1978; Becker, 1996). For example, Becker and Hale (1993) and Becker et al. (1997) have shown that convulsions in mice are more severe if mice are exposed to repeated intermittent bouts (three or more) of ethanol exposure interspersed with brief periods of abstinence (e.g., 16 h exposure, 8 h abstinence) than mice exposed to the same amount and duration of ethanol in a single continuous bout.

A similar type of withdrawal "kindling" in the underlying neural substrates mediating the severity of opioid withdrawal during repeated naloxone experience under Repeat Nal conditions (vs. Single Nal) could be a contributing factor in the results described in the current study. However, arguing against an exclusively pharmacodynamic mechanism underlying the effects of repeated naloxone experience in our model of acute opioid dependence, and in favor of a significant role for conditioning processes, are recent findings from our laboratory (Schulteis and Nikpur, 2000; Schulteis et al., in preparation). These studies revealed that naloxone experience influences subsequent naloxone potency to suppress operant response rates *only* when the naloxone experience occurs in the operant envi-

ronment, demonstrating the formation of an association between context and naloxone-induced withdrawal, and not when an equal amount of naloxone exposure occurs in the home cage environment. If purely pharmacodynamic mechanisms of withdrawal "kindling" could account for the results with repeated naloxone, then the environment in which the naloxone injections occurred (home cage or operant context) should have been irrelevant with respect to the ultimate potency of naloxone to suppress response rates on the final day of treatment. This work is currently being extended to determine whether discrete cues (e.g., tone/light) in addition to context can be used as conditioned stimuli in our acute dependence model to produce a conditioned withdrawal response from acute morphine exposure, and to examine the neural substrates mediating both the unconditioned and conditioned responses.

In summary, the current study demonstrates that the shift in naloxone potency to suppress operant response rates following acute or repeated morphine exposure is dependent on morphine dose. Furthermore, the degree to which contextual conditioning processes contribute to the magnitude of naloxone-precipitated response suppression (conditioned withdrawal) upon repeated morphine pretreatment appears to be dependent on morphine pretreatment dose as well as the interval between successive morphine pretreatments (latter observation from Schulteis et al., 1999). Our observations of apparent conditioned withdrawal in our model of acute opioid dependence suggest that conditioning processes, long thought to play a role in addiction and relapse especially after periods of protracted abstinence (Childress et al., 1999; Koob and Le Moal, 1997; O'Brien et al., 1976; Schulteis and Koob, 1996; Wikler, 1973), may in fact play a significant role in the very early stages in the development of opioid dependence. Indeed, data with the conditioned place aversion model of acute opioid dependence (Azar et al., in press; Parker and Joshi, 1998) suggest that as few as one or two pairings of a specific environment with the aversive stimulus effects of naloxone-precipitated withdrawal from acute morphine can support a conditioned withdrawal aversion to that environment. Consequently, it is possible that conditioned withdrawal processes may contribute to the transition from initial experimentation and casual use of opioids (and perhaps other drugs of abuse) to ultimate loss of control and compulsive use.

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