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ORIGINAL INVESTIGATION

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Conditioning processes contribute to severity of naloxone-precipitated withdrawal from acute opioid dependence

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Abstract *Rationale:* 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 results in further increases in naloxone potency, and prior work has shown that this progressive shift in naloxone potency requires repeated naloxone experience under some but not all experimental conditions. *Objective:* The current study sought to further characterize the experimental conditions that support naloxone experience-dependent and experience-independent potentiation of precipitated suppression of operant responding in morphine pretreated rats, and to assess more directly whether conditioning mechanisms may contribute to the former process. *Methods:* Rats trained on an FR15 schedule for food received a total of five vehicle or morphine injections (5.6 mg/kg SC) at 4, 8, or 22 h prior to an operant session in which a cumulative dose-effect function for naloxone-induced suppression of responding was determined. Separate groups of animals at each interval between morphine and naloxone received cumulative naloxone dosing after all morphine pretreatments (NAL all days) or after just the first and last morphine pretreatment (NAL first/last). Additional groups of rats at the 4 h MOR–NAL interval received most of their naloxone cumulative dose-effect experience in either the home cage or in the operant context with levers retracted. *Results:* Vehicle-pretreated (Morphine-naive) rats showed little change in the naloxone dose-effect function even after five cumulative dose-effect determinations. With a single morphine pretreatment, naloxone potency was increased at 4 or 8 h post-morphine, but not at 22 h. With repeated morphine treatment, all MOR–NAL intervals resulted in significant shifts in naloxone potency across treatment days even when naloxone was administered only after the first and last morphine pretreatment. However, much greater shifts in naloxone potency were observed at 4-h and 8-h intervals when naloxone was administered on all treatment days. At the 22 h MOR–NAL interval, there was no further potentiation in naloxone potency with additional naloxone experience provided on the intermediate days. Finally, when the repeated naloxone experience occurred in the home cage at the 4-h interval, naloxone potency was identical to that seen after limited naloxone experience (NAL first/last), and significantly less than naloxone potency in groups receiving repeated naloxone experience in the operant context. *Conclusions:* The results suggest that conditioned withdrawal mechanisms may play a significant role in the initial development of opioid dependence.

Keywords Morphine · Dependence · Withdrawal · Addiction · Naloxone · Conditioning

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Introduction

Shifts to the left in opioid antagonist dose-effect functions as a consequence of opioid agonist exposure, have long been used as a valuable quantitative tool for the characterization of the neuroadaptative changes associated with opioid dependence (e.g. Way et al. 1969; Villereal and Castro 1979), with the magnitude of shift in the antagonist dose-effect function presumed to be a valid

index of the magnitude of the underlying state of opioid dependence. Using such an approach, a number of studies have demonstrated that even a single injection of an opioid agonist can elicit a state of "acute dependence" as measured by antagonist-precipitated withdrawal (Martin and Eades 1964; Cheney and Goldstein 1971; Wiley and Downs 1979; Jones 1980; Young 1986; Bickel et al. 1988; Heishman et al. 1989, 1990; Adams and Holtzman 1990; Azorlosa et al. 1994; White-Gbadebo and Holtzman 1994; Schulteis et al. 1997, 1999, 2003; Parker and Joshi 1998; Azar et al. 2003). Consistent with the quantitative utility of the antagonist-precipitation method, the degree of shift in the antagonist dose-effect function to elicit a variety of somatic, physiological, and subjective signs of opioid withdrawal varies in direct proportion to the dose of agonist used to induce dependence (Jones 1980; Bickel et al. 1988; Heishman et al. 1989; Adams and Holtzman 1990; Schulteis et al. 1997, 1999, 2003; Azar et al. 2003). In addition, studies in both human and animal models have suggested that peak signs of withdrawal in acute opioid dependence are generally observed when the antagonist is administered between 4 and 12 h post-morphine (Young 1986; Heishman et al. 1989, 1990; June et al. 1995).

As one would expect if acute dependence reflected the early stages in the development of a full opioid dependence state, repeated treatments with morphine at daily or weekly intervals can progressively increase the severity of withdrawal-like signs elicited upon antagonist administration (Adams and Holtzman 1990; White-Gbadebo and Holtzman 1994; Schulteis et al. 1997, 1999, 2003). 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, 2003) but not all conditions (Azorlosa et al. 1994; Schulteis et al. 1997, 2003). For example, repeated naloxone experience in acute morphine-dependent subjects is necessary for the potentiation of naloxone potency over days of treatment at low (1.0–3.3 mg/kg) but not higher (5.6 mg/kg) doses of morphine (Schulteis et al. 2003). In addition, repeated naloxone experience is necessary when successive morphine (and hence successive naloxone) treatments are separated by long intervals (6 weeks), but not when shorter (24 h to 1 week) intervals between successive treatments are employed (Azorlosa et al. 1994; Schulteis et al. 1997, 1999). These observations suggest the existence of both naloxone experience-dependent and naloxone experience-independent processes in the potentiation of withdrawal magnitude produced by repeated morphine exposure.

Naloxone experience-independent 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, 2003). Wikler (1973) and others (e.g. O'Brien et al. 1976) have clearly demonstrated that stimuli associated with opioid withdrawal in dependent humans can themselves come to elicit withdrawal signs through the formation of conditioned withdrawal responses. If naloxone experience-dependent processes in models of acute opioid dependence reflect the formation of conditioned withdrawal responses, then this suggests that conditioning may begin to play a role very early in the development of opioid dependence. In this regard, Villereal and Castro (1979) suggested that the ultra-rapid adaptation to opioid agonists they observed in their in vitro guinea pig ileum preparation, measured as a dramatic "superreactivity" to naloxone within minutes of opioid exposure represents changes in the function of existing neurochemical processes, rather than "something the chronic presence of the opioid generates de novo." While this statement may not be surprising, it does logically lead to the supposition "that yet other endogenous substances or physiological events could trigger the [superreactivity to naloxone]." Herein we speculate that conditioning processes may be one such "physiological event" that can trigger an exaggerated response to naloxone following acute morphine pretreatment.

The current study was designed to more completely identify the conditions under which naloxone experience-dependent (conditioning) processes contribute to the progressive shift in naloxone potency produced by repeated (5×) pretreatment with morphine, and to assess directly whether naloxone experience-dependent mechanisms do indeed reflect the formation of conditioned associations between naloxone and cues present in the operant testing environment by varying the context in which naloxone was administered.

Materials and methods

Subjects

Male Wistar rats (n=94, Harlan Labs, Indianapolis, Ind., USA) weighing 300–400 g at the time of testing were used. All rats were group housed (2–3/cage) in a temperature-controlled and humidity-controlled room with a 12 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 per day). All training and testing took place from 9:00 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).

Drugs

Morphine sulfate was purchased from King Pharmaceuticals, Inc. (Bristol, Tenn., USA), and naloxone HCl was purchased from Sigma (St Louis, Mo., USA). Both drugs were prepared for injection in sterile physiological saline, and all injections were made subcuta-

neously (SC) in a volume of 0.1 ml/100 g body weight. Doses of both drugs were expressed as the salt. Morphine was administered at a dose of 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 previously (Schulteis et al. 2003).

Operant training

Fourteen operant chambers (Coulbourn Instruments, Columbus, Ohio, USA) 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).

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.

Cumulative dosing procedure

Once baseline stability was achieved (defined as less than 10% variation from the mean of 5 consecutive test days), rats were acclimated to the injection procedure by receiving vehicle (saline) injections 10 min prior to each 5-min response window on 3 consecutive days. The experimental procedure was as follows: 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, and another vehicle injection was administered. The rats were returned to their operant chambers, and 10 min later the levers again were extended for 5 min to allow responding. This cycle of injection-timeout (10 min)-response window (5 min) was repeated a total of 5 times to complete the full session. Response rates in each 5-min response window on the last 2 days of vehicle baseline testing were averaged to provide the baseline response rates for each rat in the absence of any morphine or naloxone treatment. In the naloxone cumulative dosing regimen, the initial injection still consisted of vehicle, but the final four injections in the regimen were replaced by an escalating dose of naloxone, according to the following schedule: 0.03, 0.067 mg/kg (cumulative dose of 0.1 mg/kg), 0.23 mg/kg (cumulative dose of 0.33 mg/kg), and 0.67 mg/kg (cumulative dose of 1.0 mg/kg).

Acute dependence and withdrawal testing regimen

To begin the acute dependence regimen, rats were injected with 5.6 mg/kg morphine (all other groups; see Table 1 for details on experimental design) at 4, 8, or 22 h prior to receiving their first naloxone cumulative dosing regimen. A single Morphine-naive control group was injected with vehicle 4 h prior to the onset of the naloxone regimen. Thus, all groups of rats received naloxone cumulative dosing on this day, but differed in terms of whether morphine or vehicle (Morphine-naive) was administered, and in terms of the interval between morphine pretreatment and the onset of the cumulative dosing regimen. This initial cumulative naloxone dosing session always occurred on a Friday for all groups tested, followed by a weekend without any injections or testing.

On Monday of the following week, all rats were again tested with vehicle injections prior to all response windows in the operant schedule. The data from the Monday test session were 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. Beginning on the Tuesday after the first naloxone cumulative dose determination, four additional operant sessions were conducted for all groups (MOR–NAL 4, MOR–NAL 8, MOR–NAL 22, Morphine-naive) at daily intervals, with morphine or vehicle preceding the operant session by 4, 8 or 22 h depending on the specific group. For these additional sessions (Tuesday–Friday), all groups of rats were further divided into two subgroups as detailed in Table 1. One subgroup received the naloxone cumulative dosing regimen after each of the additional four morphine pretreatments (NAL all days). The second subgroup received vehicle prior to each operant response window on Tues–Wed–Thurs, and then received the naloxone cumulative dose regimen for the second time on Friday (NAL first/last). This latter subgroup therefore received naloxone only during the first and last test sessions, separated by exactly 1 week (Friday to Friday).

To more directly assess the potential role of contextual conditioning processes in the progressive shift in naloxone potency seen with repeated morphine and naloxone treatments, two additional groups of rats were tested at the 4-h interval between morphine and naloxone. These groups were as follows: (a) NAL home cage, wherein rats were tested with naloxone in the operant boxes only following the morphine pretreatments on days 1 and 5, and received the naloxone cumulative dosing regimen in their home cages in the

Table 1 Summary of experimental design. *MOR* morphine; *NAL* naloxone; *cumulative dose*, cumulative dosing at 15 min increments between successive naloxone doses

Morphine-naloxone interval (h)	Naloxone experience	Days 1 and 5[a]	Days 2, 3, and 4[b]
Morphine-naive	All days	NAL cumulative dose	NAL cumulative dose
	First/last	NAL cumulative dose	Vehicle
MOR–NAL 22	All days	NAL cumulative dose	NAL cumulative dose
	First/last	NAL cumulative dose	Vehicle
MOR–NAL 8	All days	NAL cumulative dose	NAL cumulative dose
	First/last	NAL cumulative dose	Vehicle
MOR–NAL 4	All days	NAL cumulative dose	NAL cumulative dose
	First/last	NAL cumulative dose	Vehicle
	Home cage	NAL cumulative dose	NAL cumulative dose in home cage
	Operant context	NAL cumulative dose	NAL cumulative dose in operant context, lever retracted

[a]Naloxone cumulative dosing always occurred with testing in operant sessions on days 1 and 5

[b]All treatments (NAL cumulative dose or Vehicle) occurred with testing in operant sessions on days 2, 3 and 4 unless otherwise indicated (NAL operant context, NAL home cage)

vivarium on days 2, 3, and 4; and (b) NAL operant context, wherein rats were tested with naloxone in the operant boxes only following morphine pretreatments on days 1 and 5, and received the naloxone cumulative dosing regimen in the operant testing chambers as other experimental groups, but with the levers retracted so no responding could occur, on days 2, 3, and 4 of testing. It should be noted that these treatment groups were tested concurrently in the same cohorts of rats with the MOR–NAL four groups (both NAL all days and NAL first/last), to enable direct comparisons among these groups.

It must be recognized that the current design results in some degree of repeated naloxone experience in all groups within the operant context on the first and last test sessions, and therefore we present and discuss the results in terms of *limited* (NAL first/last) versus *extra* naloxone experience in the operant environment (NAL all days, NAL operant context) or outside the operant context (NAL home cage). We chose this design, however, to ensure that naloxone potency in the limited and extra experience groups could be compared directly both prior to (day 1) and after (day 5) the varying degree of naloxone experience on days 2–4, thereby allowing us to attribute any observed differences to the varying naloxone experience, rather than potential pre-existing group differences. To minimize the possible carryover effects from the first naloxone cumulative dose response determination to the last in the NAL first/last group, we ensured that there would be a 1-week interval (Friday to Friday) between the first and the final morphine pretreatment (and naloxone cumulative dose sessions).

Data analysis

Data on all experimental treatment days were expressed as % of response rate in the corresponding 5-min response window on the baseline days prior to being entered into two- or three-factor mixed design ANOVAs as described in the appropriate portion of the "Results" section and the corresponding figure legends. All follow-up comparisons consisting of interaction contrasts, simple main effects, or direct comparisons of pairs of means were corrected by the Bonferroni method.

Results

Acute opioid withdrawal following a single morphine pretreatment

As shown in Fig. 1, the potency of naloxone to suppress operant response rates following the first morphine pretreatment varied as a direct function of the MOR–NAL interval, with shorter intervals producing greater shifts in naloxone potency relative to Morphine-naive conditions. This interval-dependent effect was confirmed by a significant interaction between MOR–NAL interval and cumulative naloxone dose [$F(9,219)=3.05$, $P<0.01$] in a two-factor mixed design ANOVA. Follow-up comparisons revealed that naloxone potency after a single morphine pretreatment 22 h earlier was no different from naloxone potency in Morphine-naive rats, but that naloxone potency at 8 h post-morphine was significantly increased, with a further significant increase at the 4-h interval post-morphine (see Fig. 1 for details of follow-up comparisons to support these findings). None of the treatment groups differed in their response rates in the first (post-vehicle) operant response window, prior to naloxone treatment [$F(3,73)=2.01$, $P>0.10$].

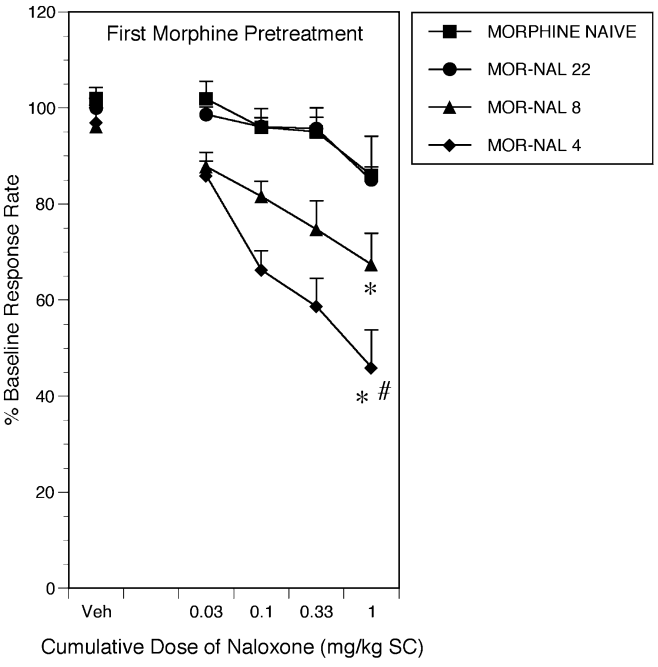

Fig. 1 Shift in naloxone potency after a single morphine pretreatment varies as a function of the interval between morphine and naloxone treatment, with shorter intervals producing greater shifts in potency relative to Morphine-naive conditions. Data represent mean (±SEM) percent of baseline response rate. Because rats had not been divided into subgroups (first/last, all days) yet, data from all subjects treated according to the regimen for Morphine-naive (*squares*), MOR–NAL 22 (*circles*), MOR–NAL 8 (*triangles*), and MOR–NAL 4 (*diamonds*) were included. This resulted in sample sizes of 18–22 per treatment condition. Significant symbols in the figure above refer to significant shifts in the cumulative naloxone dose-effect function, and resulted from interaction contrasts comparing the naloxone dose-effect function under pairs of treatment conditions as follows: *$P<0.01$ vs Morphine-naive and MOR–NAL 22, #$P<0.01$ vs MOR–NAL 8. The Morphine-naive and MOR–NAL 22 conditions did not differ from each other in terms of naloxone potency after a single morphine pretreatment. Finally, there was no significant difference in responding following vehicle administration between any of the treatment groups on this first day of testing [$F(3,73)=2.01$, $P>0.10$]

Potentiation of withdrawal magnitude upon repeated morphine and naloxone treatment

As shown in Fig. 2 in rats given naloxone cumulative dosing after every pretreatment with morphine (NAL all days), shorter intervals between morphine and naloxone produced greater shifts in the naloxone dose-effect function across treatment days than longer intervals. A three-factor mixed design ANOVA revealed a significant three-way interaction between treatment day, naloxone dose, and MOR–NAL interval [$F(36,420)=5.79$, $P<0.001$; all other two-way interactions $F>5.28$, $P<0.0001$]. Subsequent two-factor interaction contrasts for each treatment group revealed a significant treatment day×naloxone dose interaction at the 4 h [$F(12,108)=7.89$, $P<0.0001$], 8 h [$F(12,84)=6.26$, $P<0.0001$], and 22-h interval [$F(12,132)=5.59$, $P<0.0001$], but not in Morphine-naive rats [$F(12,96)=0.64$, $P>0.80$]. Thus, all three intervals between morphine and naloxone produced some degree of shift in

naloxone potency when morphine pretreatments were repeated. However, the 8-h and 4-h intervals supported significantly greater shifts in potency than the 22-h interval throughout all 5 days of testing (see Fig. 2 for full details on all follow-up comparisons in support of these findings).

Responding in the post-vehicle response window, prior to any naloxone administration on a given test day, also declined significantly across treatment days 1–5, but only at the 4-h and 8-h intervals between morphine and naloxone, as revealed by a significant interaction of MOR–NAL interval and treatment day [$F(12,140)=11.45$, $P<0.0001$] and follow-up interaction contrasts of naloxone potency on day 1 and day 5 in individual MOR–NAL groups (see Fig. 2 for full details on all follow-up comparisons). This decrease in responding following vehicle may be accounted for by contextual conditioning to the operant environment at the 4 and 8 h MOR–NAL intervals, as supported by more direct evidence of these conditioning processes in later experiments (see below).

Effects of varying naloxone experience on potentiation of withdrawal magnitude

Figure 3 reveals that MOR–NAL interval also influenced the magnitude of the shift in the naloxone dose-effect function across treatment days. An overall three-factor ANOVA resulted in significance of all main effects and interactions, including the three-way interaction of naloxone experience, naloxone dose, and MOR–NAL interval [$F(9,207)=10.21$, $P<0.0001$, all other $F>8.85$, $P<0.0001$]. A dramatically greater shift in naloxone potency with extra naloxone experience (NAL all days) versus limited experience (NAL first/last) was seen under MOR–NAL 8 and MOR–NAL 4 conditions, but not under Morphine-naive or MOR–NAL 22 conditions (see Fig. 3 for full details on all follow-up comparisons).

Extra naloxone experience (NAL all days) also reduced responding in the post-vehicle response window relative to responding in the NAL first/last condition, but only at MOR–NAL 8 and MOR–NAL 4 intervals, as revealed by a significant interaction of MOR–NAL interval and naloxone experience [$F(3,69)=23.45$, $P<0.0001$] and separate follow-up interaction contrasts for each MOR–NAL condition (see Fig. 3 for full details of follow-up comparisons). Importantly, no such differences in post-vehicle responding between NAL first/last and NAL all days conditions existed on day 1 of treatment [$F(3,69)=1.25$, $P>0.30$, data not shown], indicating specifically that differences between groups emerged as a result of varying degrees of naloxone experience on days 2–4. Once again, this finding is consistent with the interpretation that the decrease in responding following vehicle may

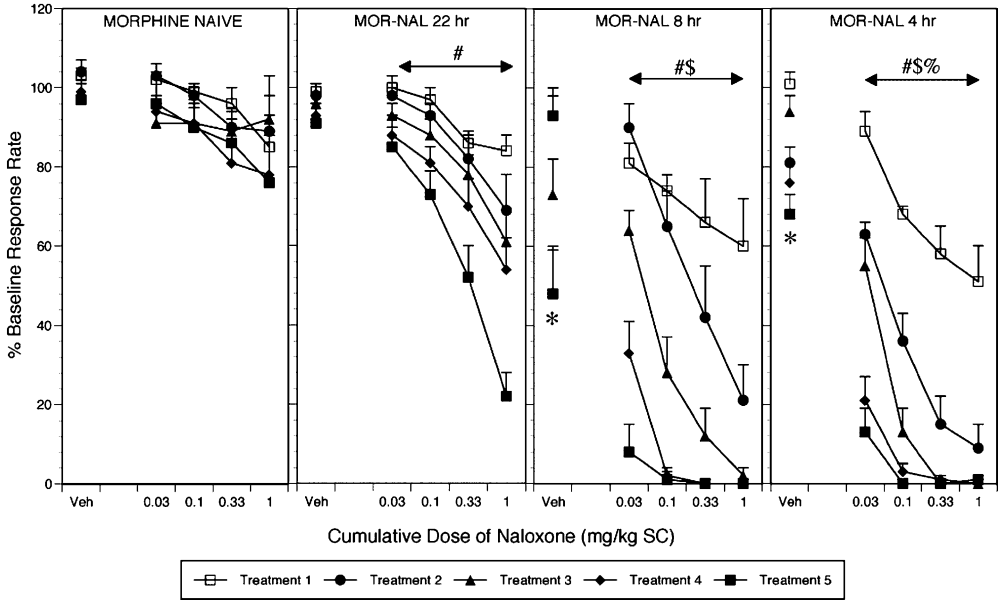

Fig. 2 Progressive shifts in naloxone potency with repeated morphine pretreatments vary as a function of the interval between morphine and naloxone treatment, with shorter intervals producing greater shifts in naloxone potency across treatment days. Data represent mean (±SEM) percent of baseline response rate. Only data from subjects treated with naloxone on all test days could be included in this analysis, resulting in sample sizes of 8–12 per treatment condition. Significant symbols below the day 5 data point in the vehicle data column of each figure panel represent significant shifts in post-vehicle responding across treatment days, as determined by tests of simple main effects of treatment day at each MOR–NAL interval (*$P<0.05$). Significant symbols above the naloxone dose-effect functions of each figure panel represent significant interactions of MOR–NAL treatment interval, naloxone dose, and treatment day in three-factor interaction contrasts comparing pairs of MOR–NAL treatment conditions. Individual symbols represent the following significant three-way interactions (all $F>9.22$, $P<0.0001$): #$P<0.05$ vs Morphine-naive, \$$P<0.05$ vs MOR–NAL 22. %$P<0.05$ MOR–NAL 8 h vs MOR–NAL 4 h refers to the following: while three-way interaction was not significant for this comparison [$F(12,192)=1.24$, $P=0.26$], the two-way treatment day×MOR–NAL interval interaction was significant [$F(4,64)=3.49$, $P<0.015$]; this interaction was accounted for by a simple main effect of MOR–NAL interval on days 2–3 [$F(1,16)=4.99$, $P<0.05$], but not on days 4–5 [$F(1,16)=0.68$, $P>0.42$]

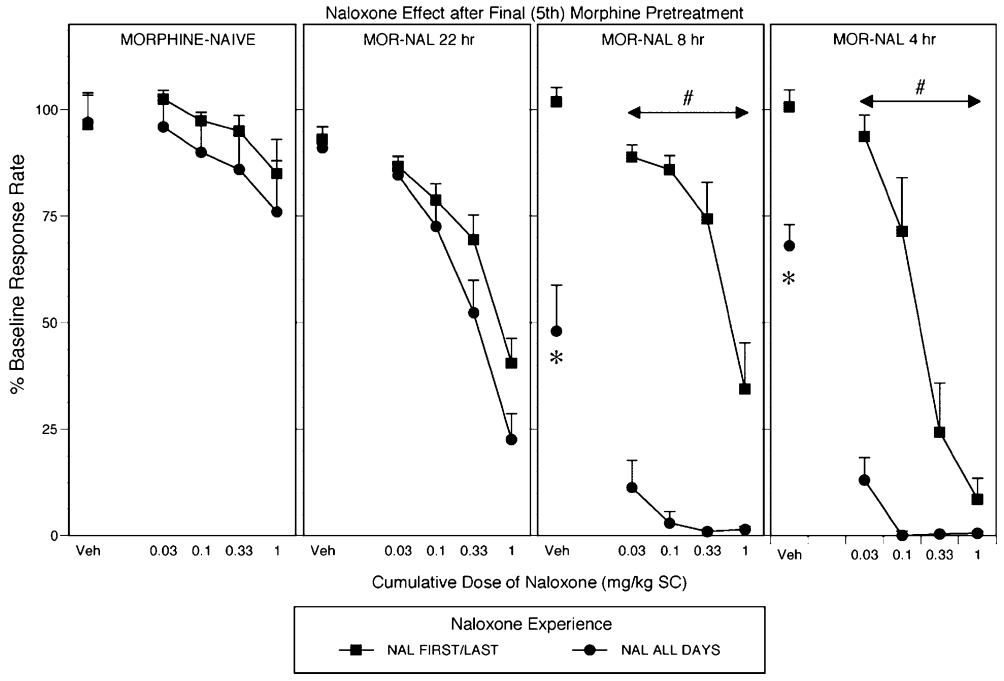

Fig. 3 Repeated morphine treatment with limited naloxone experience (*NAL first/last*) produces a significant shift in naloxone potency at 4-h, 8-h, or 22-h intervals between morphine and naloxone, compared to naloxone potency in Morphine-naive rats. Repeated experience with naloxone (*NAL all days*, *circles*) after each morphine pretreatment further increases naloxone potency, but the effects of repeated naloxone experience are seen only at short (4-h and 8-h) intervals between morphine and naloxone. Data represent mean (±SEM) percent of baseline response rate of 8–12 subjects per treatment condition. Significant symbols below NAL all days data point in the vehicle data column of each figure panel represent significant decreases in post-vehicle responding produced by repeated naloxone experience (*P<0.05 NAL all days vs NAL first/last). Significant symbols above the naloxone dose-effect functions of each figure panel represent significant interactions of naloxone experience and naloxone dose in two-factor mixed design ANOVAs (#P<0.05 NAL all days vs NAL first/last). Note: data for the NAL all days condition are the same as those summarized in Fig. 2, but data from NAL all days and NAL first/last groups represented in this figure were collected concurrently to permit direct comparisons

be accounted for by contextual conditioning to the operant environment at the 4 and 8 h MOR–NAL intervals when repeated naloxone experience in the operant context is available (NAL all days).

Effects of varying context in which naloxone is experienced on potentiation of withdrawal magnitude

As shown in Fig. 4, extra experience with naloxone on days 2–4 of morphine pretreatment at a fixed 4 h MOR–NAL interval increased naloxone potency to suppress operant response rates *only* when the extra experience occurred in the operant context (NAL operant context, NAL all days), not when the extra naloxone experience was provided in the home cage environment (NAL home cage). This observation was confirmed by a significant interaction of naloxone experience and naloxone dose [F(9,93)=7.28, P<0.0001] on day 5, and follow-up comparisons consisting of interaction contrasts among pairs of treatment conditions confirmed this hypothesis. However, opportunity to respond on the levers on days 2–4 (NAL all days) resulted in a further increase in naloxone potency on day 5 compared with the group where naloxone experience was given in the operant context but levers were retracted on days 2–4 (NAL operant context) (see Fig. 4 for full details on follow-up comparisons). Importantly, none of the four treatment groups differed from each other on day 1 of testing [F(9,93)=0.76, P>0.60, data not shown], indicating that observed group differences on day 5 could not be attributed to pre-existing differences in sensitivity to naloxone prior to manipulation of naloxone experience on days 2–4.

Discussion

Acute opioid withdrawal following a single morphine pretreatment

The current study supports and extends earlier work in reporting that single treatment with 5.6 mg/kg morphine induces a state of acute dependence as demonstrated by antagonist-precipitated suppression of operant responding (Young 1986; Adams and Holtzman 1990; White-Gbadebo and Holtzman 1994; Schulteis et al. 1997, 1999, 2003). Earlier work by Young (1986) had reported increased potency of naloxone or naltrexone to suppress operant response rates if a single morphine pretreatment at 10 mg/kg was administered 4 h prior to determination of the cumulative dose-effect function for the opioid antagonists, but not if the pretreatment was delayed to

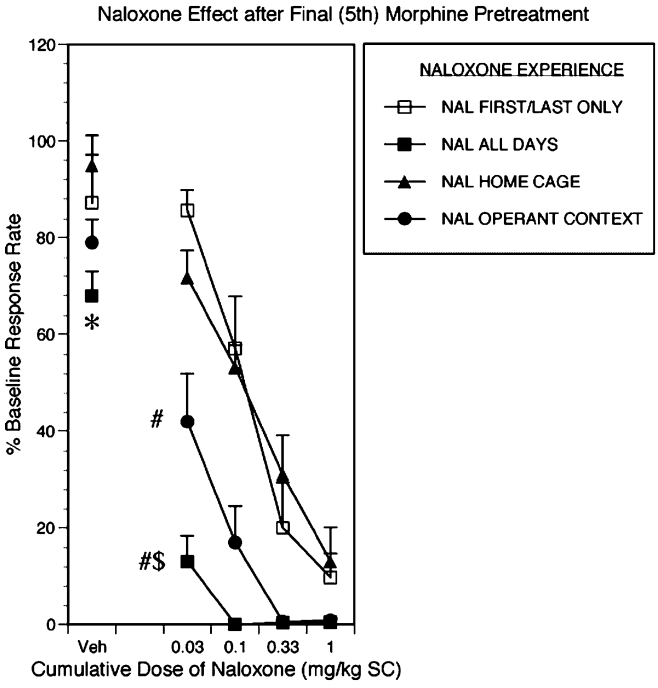

Fig. 4 Extra experience with naloxone on days 2–4 of morphine treatment at a fixed 4 h MOR–NAL interval increases naloxone potency to suppress operant response rates *only* when the extra experience occurs in the operant context (NAL operant context, NAL all days), not when the extra naloxone experience is provided in the home cage environment. Data represent mean (±SEM) percent of baseline response rate of 8–12 subjects per treatment condition. Significant symbols below NAL all days data point in the vehicle data column represent significant decreases in post-vehicle responding observed in group NAL all days (*P<0.05 NAL all days vs NAL first/last and NAL home cage, all F>4.15, P<0.05). Post-vehicle responding in group NAL operant context was intermediate between the NAL first/last and NAL all days conditions and did not differ significantly from either group (all F<1.30, P>0.26). Significant symbols to the left of a naloxone dose-effect function represent significant interactions of naloxone experience and naloxone dose in two-factor mixed design ANOVAs comparing pairs of treatment groups as follows: #P<0.05 vs NAL first/last and NAL home cage (F>9.04, P<0.0001); \$$P$<0.05 NAL all days vs NAL operant context [F(3,93)=5.19, P<0.025]. Note: data for the NAL all days and NAL first/last conditions are the same as those summarized in Fig. 3, but data from all four groups represented in this figure were collected concurrently to permit direct comparisons

12 h. Subsequently the 4 h MOR–NAL interval became the standard in most animal studies of acute dependence (Adams and Holtzman 1990; White-Gbadebo and Holtzman 1994; Schulteis et al. 1997, 1999, 2003). Using a lower dose of morphine (5.6 mg/kg) than that employed by Young (1986) and slightly different MOR–NAL intervals (4, 8, 22 h), the current study confirmed and extended findings of a time-limited "window" in which naloxone could elicit opioid withdrawal-like signs following a single morphine injection. Specifically, significant shifts in the naloxone cumulative dose-effect function were produced by morphine pretreatment at 4 or 8 h, but not 22 h, prior to naloxone administration and operant testing in opioid-naive subjects.

In seeming contrast to our negative findings at the 22 h MOR–NAL interval, work with human subjects had reported naloxone to be effective in eliciting subjective and somatic ratings of opioid withdrawal when administered as long as 24 h post-morphine (Heishman et al. 1990; June et al. 1995), although withdrawal signs had typically declined significantly by 24 h post-morphine, and had disappeared completely by 36 h (Kirby et al. 1990). It is difficult to equate morphine dose and naloxone dose range precisely between human and rodent studies, and this may account for the apparent discrepancy. However, it is important to note that while all subjects in the studies with human subjects (Heishman et al. 1990; Kirby et al. 1990; June et al. 1995) were confirmed to be non-dependent at the time of acute dependence testing, they all had a history of prior opioid use and dependence ranging from 7 to 30+ years. In the current study, when morphine was administered 4 additional times to rats at daily intervals, there was a significant shift in naloxone potency even at the 22 h MOR–NAL interval, regardless of whether or not naloxone was administered after each morphine pretreatment (see Fig. 3). Moreover, naloxone given 24 or even 48 h after a single high (20 mg/kg) dose of morphine can elicit a significant conditioned place aversion (Parker and Joshi 1998; Parker et al. 2002), a highly sensitive index of the aversive stimulus effects of withdrawal from acute or chronic opioid dependence (e.g. Schulteis et al. 1994; Azar et al. 2003). Therefore, under the correct experimental conditions, the time course over which naloxone is effective in eliciting signs of opioid withdrawal in rats following acute bolus doses of morphine appears comparable to the time course observed in human post-addicts.

Potentiation of withdrawal magnitude upon repeated morphine and naloxone treatment

With regard to naloxone potency upon repeated exposure to morphine at 24-h intervals, it was found that 22-h, 8-h, and 4-h intervals between morphine and naloxone supported progressive shifts in naloxone potency across treatment days (see Fig. 2), with significantly greater shifts observed at 4 and 8 h than at 22 h. These effects were directly attributable to the interaction of naloxone with morphine pretreatment, because our naloxone cumulative dosing regimen by itself (Morphine-naive subjects) did not produce any sensitization of naloxone-induced suppression of response rates (see Schulteis et al. 2003 for further discussion).

Effects of varying naloxone experience on potentiation of withdrawal magnitude

As shown in Fig. 3, at the 4- and 8-h MOR–NAL intervals, providing extra experience with naloxone after all five morphine pretreatments (NAL all days) produced a greater shift in naloxone potency than when naloxone dose-effect determinations were limited to just the first and last treatment days (NAL first/last). If one compares the potency of naloxone under NAL first/last conditions from

groups treated at the 22, 8, and 4 h MOR–NAL intervals, there is relatively little difference in naloxone potency on day 5 (see Fig. 3), indicating that morphine was producing an equivalent degree of naloxone experience-independent neuroadaptation in these groups. However, when one compares the NAL all days conditions from groups treated at the three MOR–NAL intervals, it becomes clear that at shorter intervals (4 and 8 h) but not the longest interval (22 h), there was an additional contribution of naloxone experience-dependent processes to the full magnitude of shift in naloxone potency. In summary, our recent findings indicate that naloxone experience-dependent mechanisms and experience-independent mechanisms contribute to the magnitude of withdrawal from acute morphine dependence to a varying degree depending on: (1) the duration of the interval between successive morphine pretreatments (Schulteis et al. 1999); (2) the dose of morphine (Schulteis et al. 2003); and (3) the interval between morphine and naloxone administration (see Fig. 3, current study).

Effects of varying context in which naloxone is experienced on potentiation of withdrawal magnitude

What remained unclear from this collective body of work was whether the naloxone experience-dependent mechanisms truly reflected the functioning of conditioning processes, as has been argued (Adams and Holtzman 1990; Schulteis et al. 1999, 2003). Adams and Holtzman (1990) had suggested conditioning to the interoceptive stimulus properties of opioid antagonists, wherein the interoceptive cues associated with low doses of the antagonist in the cumulative dose-effect function acquire predictive value of the withdrawal state elicited by the higher doses that follow later in the session. Siegel and colleagues (Sokolowska et al. 2002) have similarly argued that interoceptive cues can contribute to other conditioned drug responses, such as conditioned hyperalgesia to morphine. The current data in which extra naloxone experience resulted in potentiation of naloxone potency only when extra experience was associated with the operant context would suggest that any predictive value of interoceptive antagonist cues is acquired in a context-specific fashion. Thus, when naloxone was administered in the home cage on days 2–4 of morphine treatment, naloxone-induced suppression of responding was no greater after five morphine treatments than it was after a single morphine treatment. In contrast, naloxone administered in the operant context, even with levers retracted, on days 2–4 of morphine treatment, resulted in a significant shift in naloxone potency across treatment days. Moreover, operant response rates in the post-vehicle response window declined only under those experimental conditions where extra naloxone experience also shifted naloxone potency (see Figs 2, 3, 4), providing further evidence that the operant context could be associated with naloxone-precipitated withdrawal through conditioning mechanisms.

Notably, this conditioning occurred in association with an operant context that was familiar to the subjects long before the first withdrawal episode was experienced. This might at first seem surprising, since one would expect that extensive pre-exposure to the operant chamber would lead to latent inhibition, a process whereby pre-exposure to a conditioned stimulus or an environment inhibits their ability to become associated with an unconditioned stimulus through Pavlovian conditioning (see Lubow 1989). However, in the current study, the majority of pre-exposure to the operant chambers during training and establishment of baseline stability occurred without removal of the animals from the chambers or injections; rather, habituation to the injection schedule of the cumulative dosing regimen occurred in only three operant sessions prior to the onset of morphine treatment and withdrawal testing.

Recent theories of contextual conditioning emphasize that a "context" consists not merely of geometric features of the environment (e.g. distances, direction, relative locations) but also includes multi-modal sensory (visual, tactile, olfactory etc.) cues and temporal or episodic context (e.g. Sharp 1999; Anagnostaras et al. 2001; Moser and Paulsen 2001; Anderson and Jeffery 2003). So-called hippocampal "place" cells are critical in the formation of "maps" or representations of all of these distinct contextual elements (Wood et al. 2000; Moser and Paulsen 2001). Firing patterns of these "place" cells appear to be defined not merely by location within an environment, but rather firing patterns can change dramatically with differing experiences within the same spatial context (Wood et al. 2000; Anderson and Jeffery 2003), suggesting multiple possible representations of a given spatial context depending on the other contextual elements that may be present or absent at a given point in time ("episode").

Within the framework of the current study, one can postulate that the initiation of the injection regimen created a distinct new context which had been experienced by the animals only twice prior to the initial acute withdrawal episode. Many models of contextual fear conditioning incorporate limited (1–3 day) pre-exposure (habituation) to the context prior to conditioning, and still demonstrate potent conditioned fear (e.g. conditioned freezing; see Sparks and LeDoux 1995; Thomas et al. 2002); thus, our experimental procedure is quite similar to such contextual fear conditioning paradigms. In addition, the onset of actual naloxone dosing, and the interoceptive stimulus cues provided by lower doses early in the cumulative regimen, which were predictive of the withdrawal state elicited at the higher doses that followed (Adams and Holtzman 1990), could be argued to have provided yet another episodic context that was entirely unique to experience with conditioned withdrawal.

In further support of this notion of multi-faceted contextual representations, recent studies suggest that hippocampal "place" cells show distinct firing patterns based upon specific behaviors performed by the subjects within a given portion of their spatial environment (Wood

et al. 2000; Hollup et al. 2001). In that regard, the current study found that providing animals with operant response opportunity during all five cumulative dose determinations (NAL all days) resulted in slightly but significantly greater potency of naloxone on the fifth and final determination than did confinement to the operant context without opportunity to respond (NAL operant context). This suggests that permitting the animals to engage in operant responding may provide additional salient cues that can be associated with the withdrawal response, and subsequently engender a stronger context-conditioned response.

Taken together, these observations lead us to suggest that experience of all unique elements or cues provided by naloxone cumulative dosing regimen resulted in the formation of a new episodic context within an otherwise familiar operant environment, and this novel contextual representation reliably predicted the onset of an aversive motivational state of opioid withdrawal, with a corresponding shift to withdrawal-related behaviors (e.g. suppression of responding) upon subsequent exposure to this withdrawal-predictive context. Delineation of the neural circuits and mechanisms that support these rapid shifts in contextual significance in acute opioid dependence may have important implications for the role of conditioned drug-like and drug-opposite (e.g. withdrawal) responses in the development and maintenance of patterns of compulsive drug use (Wikler 1973; O'Brien et al. 1976; Childress et al. 1999; Di Chiara et al. 1999; Everitt et al. 2001).

Recent findings (see current study and Schulteis et al. 1997, 1999, 2003; Parker and Joshi 1998; Parker et al. 2002; Azar et al. 2003) using the place aversion and suppression of operant responding models of acute opioid dependence have significant implications for the role of conditioned withdrawal in drug abuse, dependence, and addiction. Conditioning mechanisms have received considerable attention in terms of contributing to the maintenance of compulsive drug use once dependence is established, as well as to relapse after periods of acute or protracted abstinence (Wikler 1973; O'Brien et al. 1976; Childress et al. 1999; Di Chiara et al. 1999; Everitt et al. 2001), but this recent work with acute dependence models suggests a critical role of conditioning very early in the development of opioid dependence. Context-specific associations with precipitated acute opioid withdrawal as measured with suppression of responding or place aversion paradigms appear to be rapidly formed and quite robust. Indeed, naloxone potency to suppress operant response rates (Schulteis et al. 2003, present study) after as few as four acute bolus doses of morphine (5.6 mg/kg) is comparable to its potency after chronic exposure to high levels of morphine (Schulteis et al. 1994), but only if conditions are present that permit context-specific conditioning. In addition, as few as one pairing of a unique environment with naloxone-precipitated acute withdrawal (from 4 to 48 h post-morphine) results in robust conditioned place aversions (Parker and Joshi 1998; Parker et al. 2002; Azar et al. 2003). Finally, the robustness of context-specific conditioning of acute opioid withdrawal is strikingly evident in our findings (Schulteis et al. 1999) that context-specific conditioned withdrawal-like responses were formed even when successive morphine treatments (and conditioning opportunities) were separated by intervals of 6 weeks.

Taken together, these data suggest that the neural substrates mediating the response-disruptive and aversive stimulus effects of naloxone in opioid-dependent rats show particularly rapid and long-lasting neuroadaptive response to limited acute treatment with morphine, and that conditioning processes make a significant contribution to the development of this response. Recent findings in our laboratory (Liu et al. 2002) suggest that the neural substrates mediating these responses after acute morphine exposure include elements of the "extended amygdala," most notably the nucleus accumbens and the bed nucleus of the stria terminalis. Further delineation of the neural substrates that mediate the unconditioned and conditioned neuroadaptive response to acute morphine, and the degree to which these critical substrates of acute dependence parallel those involved in expression of withdrawal from an established, chronic state of dependence, may be critical to our understanding of the neuroadaptive mechanisms that contribute to the transition from the use of opioids and other drugs to loss of control, compulsive used, and a spiraling state of addiction and relapse (e.g. Koob and Le Moal 2001). It becomes increasingly clear that a complete understanding of this process will require an understanding of the neuroanatomical, neurochemical, and cellular/molecular substrates of both direct (unconditioned) as well as conditioned neuroadaptive responses.

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