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ORIGINAL INVESTIGATION

Anxiolytic-like effects of morphine and buprenorphine in the rat model of fear-potentiated startle: tolerance, cross-tolerance, and blockade by naloxone

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Abstract
Rationale Morphine and buprenorphine have analgesic and anxiolytic-like properties. While their analgesic effects have been well characterized, their anxiolytic-like properties have not.
Objectives Effects of acute morphine and buprenorphine on the expression of acoustic fear-potentiated startle (FPS) and naloxone pretreatment were assessed. Effects of chronic morphine and buprenorphine on tolerance, cross-tolerance, and withdrawal were also examined.
Materials and methods Fear-conditioned rats were given subcutaneous drug treatment immediately before testing for FPS. Experiment 1, rats were administered morphine (0.03, 0.25, 0.63, 2.5, or 10 mg/kg) or buprenorphine (0.004, 0.0075, 0.015, 0.03, or 0.25 mg/kg). Experiment 2, rats were given saline or naloxone (0.5 mg/kg) and 5min later given saline, morphine (2.5 mg/kg), or buprenorphine (0.03 mg/kg). Experiment 3, rats received once-daily injections of saline, morphine (10 mg/kg), or buprenorphine (0.25 mg/kg) for 7 days. Immediately before testing, saline-treated rats were given saline, morphine (2.5 mg/kg), or buprenorphine (0.03 mg/kg), morphine-treated rats were given morphine (2.5 mg/kg) or buprenorphine (0.03 mg/kg), and buprenorphine-treated rats were given buprenorphine (0.03 mg/kg) or morphine (2.5 mg/kg). Tolerance and cross-tolerance in analgesia were assessed via the tail-flick test, as were naloxone-precipitated withdrawal.
Results Morphine and buprenorphine had parallel dose–response curves in blocking FPS, with buprenorphine 40 times more potent than morphine. Naloxone reversed these effects. Morphine and buprenorphine showed tolerance and cross-tolerance in their anxiolytic-like and analgesic effects. Chronic buprenorphine produced less withdrawal than chronic morphine.
Conclusions Cross-tolerance between morphine and buprenorphine suggests a common receptor mediating their anxiolytic-like and analgesic effects.

Keywords Fear conditioning · Startle · Anxiety · Opioid · Tolerance · Withdrawal

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Morphine, the prototypical μ-opioid receptor agonist, and buprenorphine, a partial μ- and δ-opioid receptor agonist, full opioid receptor-like receptor (ORL_1) agonist, and κ-opioid receptor antagonist, are potent analgesics that are widely used clinically for the management of pain. The pharmacology of morphine and buprenorphine in analgesia, reward, and physical dependence is well characterized, and the μ-opioid receptor has been identified as playing a critical role in these actions (Matthes et al. 1996; Sora et al. 1997).

Morphine and related agonists are also known to possess anxiolytic-like properties. However, the pharmacology of opioids in their anxiolytic-like actions is far less characterized. Using animal models of unconditioned and conditioned fear, several studies have described anxiolytic-like effects of opioids. Morphine (Motta and Brandao 1993; Koks et al. 1999; Anseloni et al. 1999; Sasaki et al. 2002; Uriguen et al. 2002; Shin et al. 2003), as well as selective agonists for the μ-opioid receptor (Asakawa et al. 1998; Uriguen et al. 2002), δ-opioid receptor (Saitoh et al. 2004),

κ-opioid receptor (Privette and Terrian 1995; Wright and Ingenito 2001; Wall and Messier 2000), and ORL_1 receptor (Jenck et al. 1997; Jenck et al. 2000; Wichmann et al. 2000, but see Fernandez et al. 2004) have been shown to increase the percentage of entries and time spent in the open arms of a plus maze, indicating an anxiolytic-like action.

Morphine also attenuates conditioned fear responding to a context previously paired with a footshock (Kameyama and Nagasaka 1982; Bartoletti et al. 1990). Our laboratory uses the fear-potentiated startle paradigm to assess conditioned fear. In this task, animals show an increased acoustic startle response elicited in the presence of a conditioned stimulus (CS; i.e., light) that had been previously paired with a footshock unconditioned stimulus. Davis (1979a) showed that systemic morphine depressed startle amplitude on CS trials (i.e., when startle was elicited in the presence of a light that had been previously paired with a footshock) but not during noise-alone trials (i.e., when startle was presented in the absence of the light), suggesting that morphine suppresses fear-potentiated startle but not baseline startle. Fentanyl (Fendt and Mucha 2001), a μ-opioid receptor agonist estimated to be 80 times more potent than morphine as an analgesic, and the selective ORL_1 agonist, Ro 64-6198 (Jenck et al. 2000), also attenuate fear-potentiated startle without affecting baseline startle.

The fear-potentiated startle paradigm has been established for its sensitivity to several conventional anxiolytic compounds, which differ considerably in structure and mechanisms of action (Davis et al. 1993). Specifically, our lab has demonstrated that diazepam, clonidine (Davis et al. 1979), and buspirone (Kehne et al. 1988), all block fear-potentiated startle. That opioid agonists show anxiolytic-like properties in this paradigm further establishes their anxiolytic-like action. Collectively, these studies strongly implicate the opioid system in the regulation of fear- and anxiety-related behaviors.

The anxiolytic-like properties of opioids may hold clinical significance. Persistent pain is significantly associated with depression and anxiety disorders (Huyser and Parker 1999; McWilliams et al. 2003). Withdrawal symptoms engendered by opioid dependence are also associated with depression and anxiety (Hall 1984). In rats, both spontaneous and naloxone-precipitated withdrawal from chronic morphine administration result in anxiogenic-like effects in the plus maze (Schulteis et al. 1998) and the fear-potentiated startle (Harris and Gewirtz 2004) models of anxiety. Interestingly, buprenorphine has been shown to block the acquisition of opioid-withdrawal-induced conditioned place aversion in rats (Stinus et al. 2005). Furthermore, buprenorphine is showing promise for the treatment of anxiety-related and other adverse behaviors associated with the withdrawal syndrome (Gowing et al. 2002; Bailey 2004) and may also hold promise for the treatment of a population of patients refractory to conventional anxiolytics (Bodkin et al. 1995). Improving our understanding of the behavioral pharmacology of buprenorphine's anxiolytic-like actions could advance the clinical success of this drug.

The present study aims to pharmacologically characterize the anxiolytic-like effects of morphine and buprenorphine by generating full dose–response curves for their effect on fear-potentiated startle and examining the effects of naloxone pretreatment. In addition, the current study addresses whether or not the anxiolytic-like effects of morphine and buprenorphine show tolerance and cross-tolerance after chronic administration.

The degree to which tolerance develops in the anxiolytic-like actions of buprenorphine and morphine is not yet known. A high degree of cross-tolerance develops between different opioid compounds that are acting through the same receptor (Craft and Dykstra 1990; Moulin et al. 1988). Morphine and buprenorphine show considerable cross-tolerance to analgesia (Barrett et al. 2001; Gringauz et al. 2001; Walker and Young 2001), consistent with findings that the μ-opioid receptor is critical for the analgesic effects of both agonists (Ide et al. 2004; Sora et al. 1997). However, the extent to which buprenorphine and morphine show cross-tolerance in their anxiolytic-like effects is not known.

Compared to morphine, buprenorphine has been described as having a more favorable clinical profile with minimal abuse potential (Cowan 2003; Tzschentke 2002). The current study assesses the dependence liability of morphine and buprenorphine by quantifying various signs of naloxone-precipitated withdrawal after chronic administration. The overall findings of this study could not only add to our understanding of the neural basis of anxiety regulation but might also have therapeutic implications for the treatment of opioid abuse.

Materials and methods

Animals

Male Sprague-Dawley rats ($N = 233$; Charles River, Raleigh, NC, USA), weighing 350 to 450 g at testing, were housed four to a cage and maintained on a 12:12-h light–dark cycle with food and water available ad libitum. All behavioral procedures took place during animals' light cycle.

Drugs

Morphine hydrochloride, buprenorphine hydrochloride, and naloxone hydrochloride were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Morphine and naloxone were dissolved in physiological saline, and buprenorphine was dissolved in a 10% dimethyl sulfoxide, 90% saline solution. All drugs were injected subcutaneously (s.c.) and delivered in a volume of 0.1 ml/100-g body

weight. Doses were based on the salt weight of the compounds.

Apparatus

Fear conditioning Rats were trained and tested in two identical 8 × 15 × 15-cm Plexiglas and wire mesh cages as described by Cassella and Davis (1986). Background noise (60-dB wideband) and startle stimuli (95dB, 50 ms white-noise bursts; rise decay, 5 ms) were delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX, USA). Startle response amplitudes were quantified using an Endevco (San Juan Capistrano, CA, USA) 2217E accelerometer. Cage movement produced by the rat's startle response resulted in displacement of the accelerometer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 300 ms after onset of the startle-eliciting noise burst. The visual conditioned stimulus was a 3.7-s light (200 lx) produced by a 25-W incandescent bulb located 25 cm above each cage. The unconditioned stimulus was a 0.5-s 0.4-mA shock delivered through the four floor bars. The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads, Newton, CT, USA).

Tail flick Analgesia measures were taken with a 17 × 17 × 5.25-in. Model 33 Tail Flick Analgesia Meter (IITC, Inc., Life Science Instruments, Woodland Hills, CA, USA). Radiant heat was delivered from an overhead 24-V, 150-W halogen light bulb prefocused to a 4 × 6-mm stimulation area. A built-in sensor detects autonomic tail flick, and a built-in timer displays reaction times. The apparatus can be controlled digitally from the front panel, which contains keypad buttons for start, stop, and reset of tests.

Procedure

Fear conditioning

Matching For two consecutive days, rats were placed in the startle chamber and after a 5-min acclimation period, received 30 presentations of startle stimuli (95-dB noise burst) separated by a 30-s intertrial interval. Their mean startle amplitudes were calculated and marked as their pretraining startle baseline. Rats were then divided into treatment groups according to their startle baselines, such that the mean startle amplitudes were balanced across groups.

Training The next day, rats were returned to the same startle chambers. After 5 min of acclimation, rats received a series of ten light–shock pairings (3.7-s light CS coterminates with a 0.5-s footshock), with 4-min intertrial interval.

Testing Twenty-four to 48h later (Experiments 1 and 2) or 7 days later (Experiment 3), rats were returned to the startle cages, and after 5min, were presented with 30 startle stimuli (leaders), used to habituate their startle response to a stable baseline. Thirty seconds after the final leader stimulus, rats received 15 startle stimuli presented alone (noise-alone trial) and 15 startle stimuli 3.2 s after onset of the 3.7-s light (light–noise trials). The two trial types were presented in a balanced mixed order, with 30-s intertrial interval.

Drug treatment

Experiment 1 Immediately before testing, rats were given s.c. administration of saline (n = 45) or one of five different doses of either morphine (n = 43; 0.03, 0.25, 0.63, 2.5, or 10 mg/kg) or buprenorphine (n = 45; 0.004, 0.0075, 0.03, or 0.25 mg/kg).

Experiment 2a Rats (n = 32) were first given s.c. administration of either saline or naloxone (0.5 mg/kg) and 5 min later given either saline, morphine (2.5 mg/kg), or buprenorphine (0.03 mg/kg) and immediately thereafter tested for fear-potentiated startle.

Experiment 2b A separate group of rats (n = 31) were given s.c. administration of saline or naloxone (2 mg/kg) and 5 min later tested for fear-potentiated startle.

Experiment 3 Twenty-four hours after training, and for the next 7days, rats (n = 100) received once-daily s.c. injections of saline, morphine (10 mg/kg), or buprenorphine (0.25 mg/kg). Twenty-four hours after the final injection, rats were tested for fear-potentiated startle. Immediately before testing, saline-treated rats were challenged with saline, morphine (2.5 mg/kg), or buprenorphine (0.03 mg/kg); the morphine-treated rats were challenged with either morphine (2.5 mg/kg) or buprenorphine (0.03 mg/kg), and the buprenorphine-treated rats were challenged with either buprenorphine (0.03 mg/kg) or morphine (2.5 mg/kg).

Antinociception tests

A cohort of rats (n = 35) was given an antinociception test 24 h after being tested for fear-potentiated startle. Follow-

ing a methodology previously described by Gellert and Holtzman (1978), rats were tested for their responsivity to radiant heat pain using the tail-flick test. At the beginning of the test, radiant heat from a 24-V, 150-W bulb was focused on the lower third of the rats tail. An automatic timer was also activated. Withdrawal of the tail activated a photocell, which subsequently turned off the light source and the timer. Tail-flick latency was recorded to the nearest tenth of a second. The light intensity was set so that the baseline latencies were between 2.0 and 3.0 s. If a response was not made within 8.0s, the test was terminated in order to minimize tissue damage (i.e., 8.0-s cutoff). Baseline reaction times were determined twice for each rat, 5 min apart, and averaged to give a single predrug baseline value. The rats were then given morphine (10 mg/kg) or buprenorphine (0.25 mg/kg) and returned to their home cages. Thirty minutes later the reaction times were redetermined with a single test. This 30-min interval corresponds with the approximate time that rats had drug in their system before receiving their first test trial during fear-potentiated startle testing.

Withdrawal assessment

Twenty-four hours after antinociception testing, the same cohort of rats (n = 35) was tested for naloxone-precipitated withdrawal. Following a modified version of the method reported in Le Guen et al. (2001), rats were administered s.c. injections of naloxone (2 mg/kg) and 5 min later observed for the following withdrawal signs: chewing, grooming, teeth chattering, wet dog shakes, penis licking, eye twitching, ptosis, and diarrhea. For each rat, indices of withdrawal signs were counted for 1 h, over 5-min blocks. The total number of withdrawal signs was quantified by assigning a score of "1" for each aforementioned sign if it occurred once during a 5-min block, giving a maximum score of "12" for each sign over an hour period. As an additional measure of withdrawal severity, the total number of fecal boluses was counted for each rat at the end of the observational period.

Statistical analyses

Depending on the experiment, all data were analyzed by analysis of variance (ANOVA) followed by individual mean comparison using Tukey's post hoc tests or independent sample t-tests. A significance level of $p < 0.05$ is taken for all results.

Fear-potentiated startle

The mean startle amplitude on noise-alone and light–noise trials was calculated for each rat. Difference scores were calculated as: (light–noise minus noise-alone trials). Percent fear-potentiated startle was calculated as: [(light–noise minus noise-alone trials) / (noise-alone trials)] × 100.

The mean percent block of fear-potentiated startle by morphine or buprenorphine was calculated for each rat as: [(mean percent potentiation under saline minus mean percent potentiation under the drug at each dose) / mean percent potentiation under saline] × 100. These data were collected from several different cohorts of animals in different experiments. The mean percent blockade for each agonist dose was calculated against the saline groups associated with those particular cohorts of animals used in respective studies.

In experiment 1, the fear-potentiated startle data were plotted on a log scale as a function of agonist dose. The dataset for these dose–response curves were also fitted to a theoretical sigmoidal distribution, using a sigmoidal dose–response curve-fit algorithm in GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA). Each curve was defined by four parameters: the minimum response (bottom), the maximum response (top), the slope, and the drug dose that produced a response halfway between minimum and maximum. The dose that produced a 50% maximum effect was taken as the $\log ED_{50}$ for each dose–response curve. Prism software fitted the data to the following equation:

$$Y = \text{bottom} + (\text{Top} - \text{Bottom})/(1 + 10\,\hat{}\,((\text{LogED}_{50}) \times \text{HillSlope}))$$

The slopes and $\log ED_{50}$s of morphine vs. buprenorphine were statistically compared to determine if the curves were parallel and statistically different, respectively. The four aforementioned curve parameters were fitted and compared across agonists groups using curve fit for nonlinear regression to determine if the curves were statistically different.

Antinociception testing

Analgesia data were expressed in terms of the percentage of the maximum possible effect (%MPE) using the formula:

$$\%\text{MPE} = \frac{\text{test latency} - \text{baseline latency}}{\text{cut off time} - \text{baseline latency}} \times 100$$

Withdrawal assessment

The overall severity of withdrawal syndrome was quantified in each rat by summating the number of withdrawal signs and fecal boluses observed over an hour period and averaging the score to obtain a global rating for each treatment group (Table 1).

Table 1 Withdrawal assessment

Behavioral signs	Mean number of events (±SEM)					
	SAL–MOR (n=6)	SAL–BUP (n=6)	MOR–MOR (n=6)	MOR–BUP (n=5)	BUP–BUP (n=7)	BUP–MOR (n=5)
Chewing	2.7±0.5	3.2±0.5	5.0±0.4	4.2±0.6	3.6±0.4	3.4±0.5
Grooming	1.7±0.6	2.5±0.3	4.3±0.5	5.2±0.4	3.4±0.4	3.8±0.7
Teeth chattering	1.5±0.3	1.0±0.4	2.5±0.4	3.0±0.8	1.7±0.4	2.2±0.7
Wet dog shake	2.0±0.4	1.3±0.4	2.7±0.8	4.0±0.4	2.0±0.4	2.2±0.6
Penis licking	1.2±0.2	1.2±0.3	3.2±0.7	3.0±0.6	2.3±0.6	2.2±0.7
Abdominal spasms	0.2±0.2	0	1.0±0.4	1.2±0.6	0.7±0.4	0.2±0.2
Eye twitching	0.3±0.2	0	0.8±0.5	1.2±0.6	0.6±0.4	0.6±0.4
Ptosis	0	0.2±0.2	0.8±0.2	1.0±0	0.3±0.2	0.2±0.2
Diarrhea	0	0	0.7±0.2	0.4±0.2	0.1±0.1	0.2±0.2
Global rating	9.5±0.8	9.3±0.9	21.0±2.4	23.2±1.3	14.7±2.0	15.0±2.2

Results

Experiment 1: dose–response function of morphine and buprenorphine in fear-potentiated startle

Figure 1 shows the mean startle amplitude for noise-alone (black) and light–noise (white) trials and the difference between the two (stripe) for separate treatment groups representing five doses of morphine (Fig. 1a) or buprenorphine (Fig. 1b) and saline groups corresponding to each agonist group. An ANOVA was carried out with trial type (light–noise vs. noise alone) as a within subject factor, and dose as a between subject factor. For morphine, there was a significant trial effect $F(1, 88) = 39.79$, dose effect $F(5, 88) = 4.66$, and a trial × dose interaction $F(5, 88) = 8.88$. Tukey's post hoc tests indicated that the saline group is significantly different from the morphine groups, except at doses of 0.03mg/kg and 0.25mg/kg (Fig. 1a). For buprenorphine, there was also a significant trial effect $F(1, 135) = 67.19$, dose effect $F(5, 135) = 7.16$, and a trial × dose interaction $F(5, 135) = 16.07$. Tukey's post hoc tests indicated that the saline group was significantly different from all buprenorphine groups with the exception of dose of 0.004 mg/kg (Fig. 1b).

To more easily compare the dose–response effects for the two agonists, the mean percent blockade of fear-potentiated startle at each of the five doses of morphine and buprenorphine were plotted as a function of agonist dose (Fig. 2). A one-way ANOVA comparing doses of the agonists revealed a significant fit to a linear trend $F(4, 254) = 9.27$. The dose–response curves of morphine and buprenorphine were statistically parallel, evidenced by the finding that there was no significant difference between their slopes, $F(1, 6) = 1.089$, $p > 0.05$. However, the $\log ED_{50}$s for morphine and buprenorphine were significantly different, $F(1, 6) = 52.77$. The best-fit ED_{50}s were morphine = 0.40 mg/kg and buprenorphine = 0.01 mg/kg, indicating a 40-fold difference. Together, these findings show that morphine and buprenorphine blocked the expression of fear-potentiated startle in a dose-dependent manner. Further, morphine and buprenorphine have parallel dose–response curves, and buprenorphine is 40 times more potent than morphine in their anxiolytic-like actions.

Experiment 2. Effect of naloxone pretreatment on the anxiolytic-like effects of morphine and buprenorphine

Figure 3a shows the mean startle amplitude for groups that received saline (SAL–SAL, SAL–MOR, SAL–BUP) or naloxone (0.5 mg/kg; NAL–MOR, NAL–BUP) pretreatment, with morphine (2.5 mg/kg) or buprenorphine (0.03 mg/kg) posttreatment. A one-way ANOVA of the difference scores showed a significant difference among groups, $F(4, 27) = 4.38$. Tukey's post hoc tests revealed that saline pretreatment groups (SAL–MOR and SAL–BUP), but not naloxone pretreatment groups (NAL–MOR and NAL–BUP), differed significantly from saline-only groups (SAL–SAL).

Figure 3b shows the mean percent blockade of fear-potentiated startle by morphine or buprenorphine following saline or naloxone pretreatment. There was no significant difference between saline pretreatment groups (SAL–MOR and SAL–BUP) or between naloxone pretreatment groups (NAL–MOR and NAL–BUP). These groups were collapsed, and an independent-samples t-test revealed a significant difference between saline pretreatment groups compared with naloxone pretreatment groups, $t(24) = 2.87$.

Figure 4 shows the mean startle amplitude for groups that received saline or naloxone (2 mg/kg) immediately before testing. An independent-samples t-test showed that there is no significant difference between groups. Thus, while naloxone by itself has no effect on the expression of fear-potentiated startle, naloxone pretreatment attenuates the anxiolytic-like effects of morphine and buprenorphine in the fear-potentiated startle task.

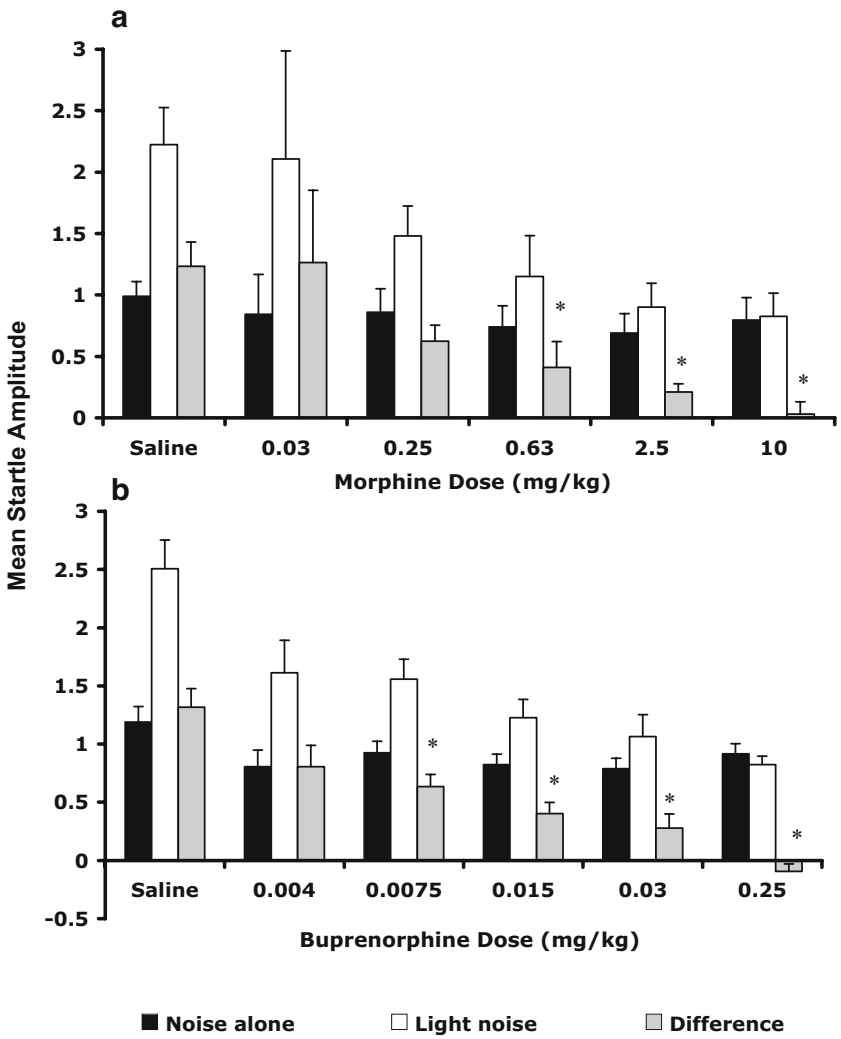

Fig. 1 Morphine and buprenorphine dose-dependently block fear-potentiated startle without affecting baseline startle. Mean startle amplitude for noise-alone (*black*) and light–noise (*white*) trials and the difference (SEM) between the two (*gray*) are shown for **a** morphine (n=43) and **b** buprenorphine (n=45). Fear-potentiated startle in the saline group (n=45) was significantly different from morphine groups (0.63. 2.5, and 10 mg/kg) and buprenorphine groups (0.0075, 0.015, 0.03, and 0.25 mg/kg), * = $p<0.05$ compared to saline

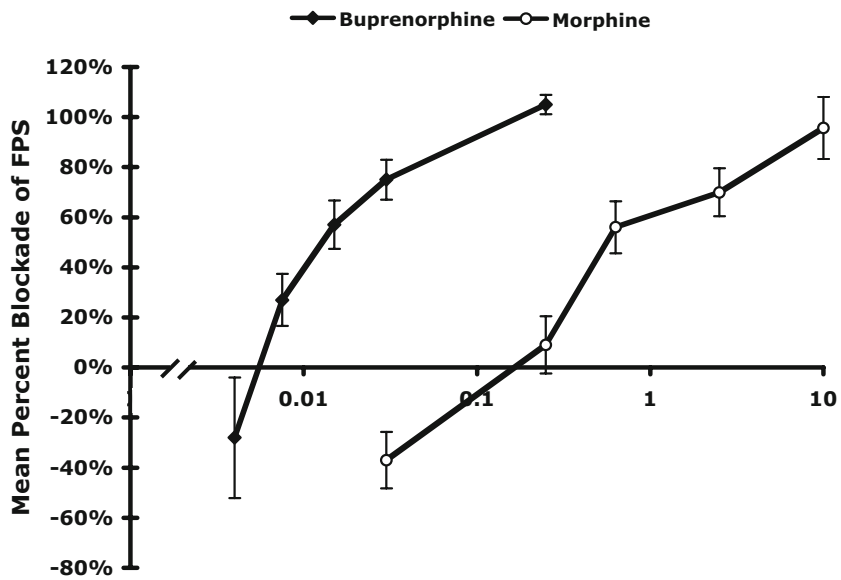

Fig. 2 Buprenorphine is 40 times more potent than morphine as an anxiolytic in parallel dose–response curves. The mean percent blockade of fear-potentiated startle (±SEM) by buprenorphine, n=45, (*black diamond*) and morphine, n=43, (*white circle*) was plotted as a function of dose (milligram per kilogram). The dose–response curves of morphine and buprenorphine were statistically parallel. The best-fit ED_{50}s were morphine=0.40 mg/kg and buprenorphine=0.01 mg/kg, indicating a 40-fold difference

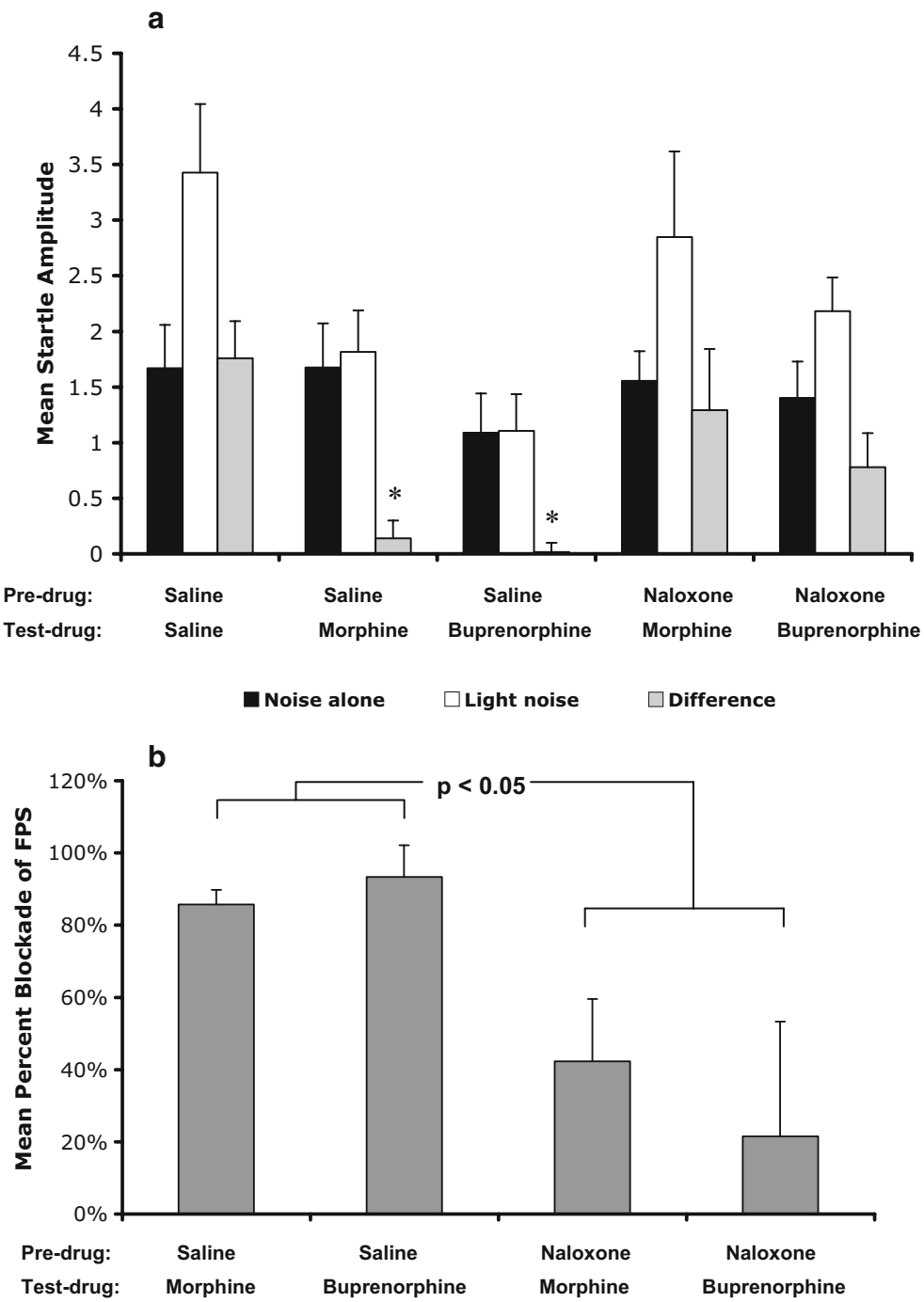

Fig. 3 Naloxone pretreatment reverses the anxiolytic-like effects of morphine and buprenorphine. **a** Mean startle amplitude for noise-alone (*black*) and light–noise (*white*) trials and the difference (±SEM) between the two (*gray*) are shown for groups that received saline (SAL–SAL, n=6; SAL–MOR, n=6; SAL–BUP, n=6) or naloxone (0.5 mg/kg; NAL–MOR, n=7; NAL–BUP, n=7) pretreatment, with morphine (2.5 mg/kg) or buprenorphine (0.03 mg/kg) posttreatment, * = p<0.05. **b** The mean percent blockade of fear-potentiated startle (±SEM) by morphine or buprenorphine following saline or naloxone pretreatment is shown

Experiment 3. The effect of chronic morphine and buprenorphine on tolerance and cross-tolerance of anxiolytic-like effects and analgesia and on naloxone-precipitated withdrawal

Figure 5a shows the mean startle amplitude for groups given saline, morphine (2.5 mg/kg), or buprenorphine (0.03 mg/kg) following chronic administration of saline (SAL–SAL, SAL–MOR, or SAL–BUP), morphine (10 mg/kg; MOR–MOR, MOR–BUP), or buprenorphine (0.25 mg/kg; BUP–BUP, BUP–MOR). A one-way ANOVA showed a significant difference among groups $F(6, 93) = 3.69$. Tukey's post hoc tests indicated that groups given acute opioid treatment (SAL–MOR, SAL–BUP) showed significantly less fear-potentiated startle than groups given saline only (SAL–SAL), while groups given chronic opioid treatment (MOR–MOR, MOR–BUP, BUP–BUP, BUP–MOR) did not.

Figure 5b shows the mean percent blockade of fear-potentiated startle by morphine, or buprenorphine following chronic administration of saline, morphine, or buprenor-

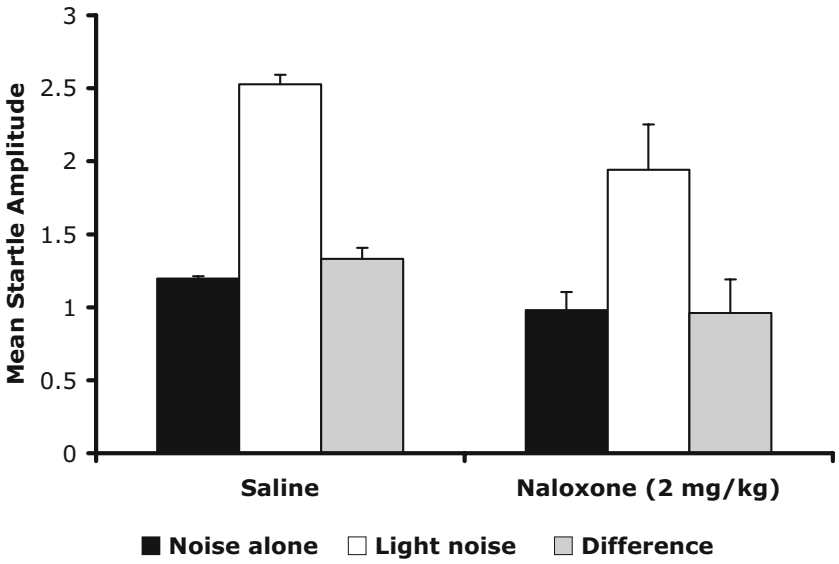

Fig. 4 Naloxone alone has no effect on the expression of fear-potentiated startle. Mean startle amplitude for noise-alone (*black*) and light–noise (*white*) trials and the difference (±SEM) between the two (*gray*) are shown for groups that received saline (n=16) or naloxone (2 mg/kg; n=15). There is no significant difference between groups

phine. A 3 × 2 ANOVA, with pretreatment (saline, morphine, or buprenorphine) and posttreatment (morphine or buprenorphine) as factors showed a significant main effect of pretreatment $F(2, 88) = 5.09$, but not posttreatment, and no interaction among the two. Tukey's post hoc tests indicated that saline pretreatment groups (SAL–MOR, SAL–BUP) differed significantly from morphine (MOR–MOR, MOR–BUP) and buprenorphine (BUP–BUP, BUP–MOR) pretreatment groups. However, there was no significant difference between morphine pretreatment and buprenorphine pretreatment groups. Thus, the capacity of morphine and buprenorphine to block the expression of fear-potentiated startle is reduced in rats given prior chronic administration of either morphine or buprenorphine.

With regard to the analgesia results, Fig. 6 shows the percentage of the maximum possible effect for groups given saline, morphine (2.5 mg/kg), or buprenorphine (0.03 mg/kg) following chronic administration of saline (SAL–SAL, SAL–MOR, or SAL–BUP), morphine (10 mg/kg; MOR–MOR, MOR–BUP), or buprenorphine (0.25 mg/kg; BUP–BUP, BUP–MOR). A 3 × 2 ANOVA with pretreatment (saline, morphine, or buprenorphine) and posttreatment (morphine or buprenorphine) as factors showed a significant main effect of pretreatment $F(2, 35) = 58.60$, but not posttreatment, and no interaction between the two. In Tukey's post hoc tests, saline pretreatment groups (SAL–MOR, SAL–BUP) differed significantly from morphine (MOR–MOR, MOR–BUP) or buprenorphine (BUP–BUP, BUP–MOR) pretreatment groups. However, there was no significant difference between morphine pretreatment groups and buprenorphine pretreatment groups. These findings show that the analgesic effects of both morphine and buprenorphine are attenuated in rats given prior chronic administration of either morphine or buprenorphine.

Figure 7 shows the mean number of withdrawal signs (Fig. 6a) and number of fecal boluses (Fig. 6b) observed after acute or chronic administration of morphine and buprenorphine. A 3×2 ANOVA of mean withdrawal signs showed a significant main effect of pretreatment $F(2, 54)=4.70$, but not posttreatment, and no interaction between the two. In Tukey's post hoc tests, morphine pretreatment groups showed significantly more withdrawal signs than saline pretreatment groups, while buprenorphine pretreatment groups did not differ significantly from saline pretreatment groups. The same pattern of results was found with the mean number of boluses. A 3×2 ANOVA showed a significant main effect of pretreatment $F(2, 35)=8.00$, but not posttreatment, and no interaction between the two. Tukey's post hoc tests showed that morphine pretreatment groups had significantly more boluses than saline pretreatment groups. Again, buprenorphine pretreatment groups did not differ significantly from saline pretreatment groups. Thus, rats given chronic administration of buprenorphine showed less signs of dependence than those given chronic morphine administration.

Discussion

The fear-potentiated startle paradigm has been validated as a behavioral model of conditioned fear (Davis et al. 1993). It has also been pharmacologically validated with prototypical anxiolytic compounds (i.e., diazepam). Using this well-characterized, highly controlled experimental procedure, we generated full dose–response curves for the anxiolytic-like effects of morphine and buprenorphine. While previous studies demonstrated dose-dependent effects of opioid agonists on anxiety-related behaviors, none have produced *full* dose–response curves for opioid

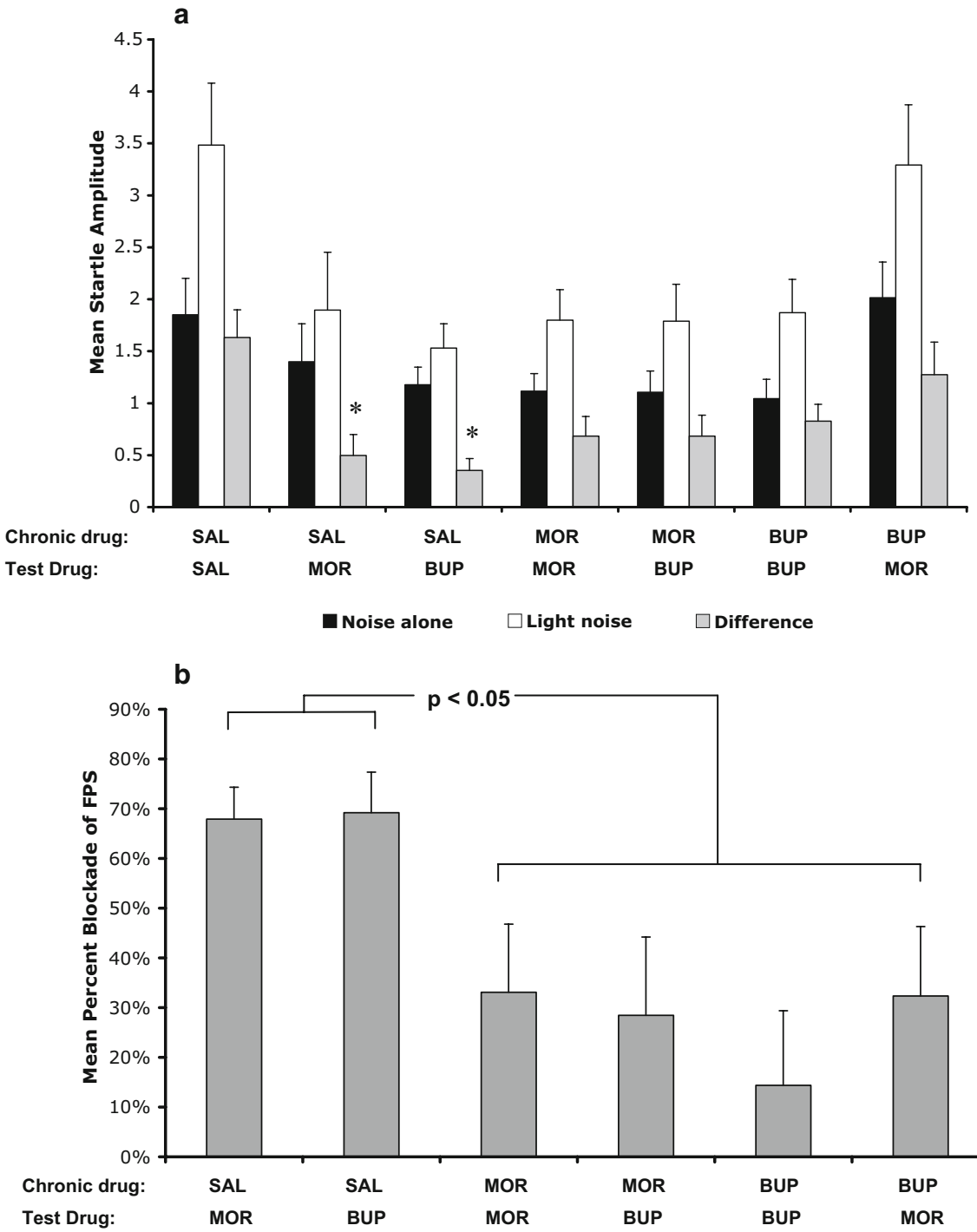

Fig. 5 Chronic administration of morphine and buprenorphine produces tolerance and cross-tolerance in their anxiolytic-like effects. **a** Mean startle amplitude for noise-alone (*black*) and light–noise (*white*) trials and the difference (±SEM) between the two (*stripe*) are shown for groups given saline, morphine (2.5 mg/kg), or buprenorphine (0.03 mg/kg) following 7-day pretreatment with saline (SAL–SAL, $n=12$; SAL–MOR, $n=9$; or SAL–BUP, $n=13$), morphine (10 mg/kg; MOR–MOR, $n=16$; MOR–BUP, $n=14$), or buprenorphine (0.25 mg/kg; BUP–BUP, $n=19$; BUP–MOR, $n=17$), * = $p<0.05$. **b** The mean percent blockade of fear-potentiated startle (±SEM) by morphine or buprenorphine following chronic administration of saline, morphine, or buprenorphine

effects. Thus, until now, it has not been established how the dose–response profiles of opioids in anxiety-related behaviors compare to that of other well-described physiological effects of opioids, such as analgesia.

Here, we show that both morphine and buprenorphine robustly blocked the expression of fear potentiated in a dose-dependent manner, without affecting baseline startle (Fig. 1). The dose–response curves of morphine and

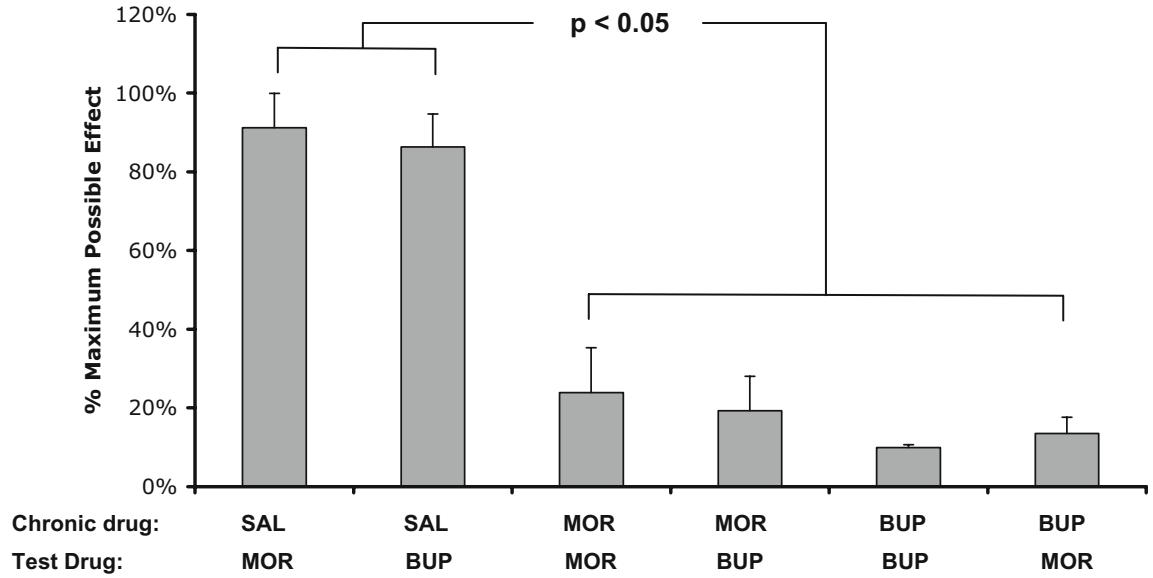

Fig. 6 Chronic administration of morphine and buprenorphine produces tolerance and cross-tolerance in their analgesic effects. Percentage of maximum possible effect (±SEM) is shown for groups given saline, morphine (2.5 mg/kg), or buprenorphine (0.03 mg/kg) following 7-day pretreatment with saline (SAL–MOR, n=6; or SAL–BUP, n=6), morphine (10 mg/kg; MOR–MOR, n=6; MOR–BUP, n=5), or buprenorphine (0.25 mg/kg; BUP–BUP, n=7; BUP–MOR, n=5)

buprenorphine were parallel, evidenced by their statistically similar hill slopes (Fig. 2). Buprenorphine was 40 times more potent than morphine in blocking the expression of fear-potentiated startle, evidenced by the rightward position of morphine's ED_{50} relative to buprenorphine on the dose–response curve (Fig. 2). It has been demonstrated that buprenorphine is 25–40 times more potent than morphine as an analgesic (Cowan et al. 1977; Lewis 1985). The similarity in order and magnitude of potency of morphine and buprenorphine in their effects on conditioned fear and analgesia suggests that there may be some overlap in the brain regions that mediate the analgesic and anxiolytic-like pharmacology of opioids. Two notable sites, the midbrain periaqueductal gray and the amygdaloid complex, are both known to have dual functions in processing analgesia as well as fear (Behbehani 1995; Davis and Whalen 2001). Lesions of the central nucleus of the amygdala (Ce) strongly attenuate antinociception responses (Helmstetter and Bellgowan 1993; Fox and Sorenson 1994) and dampen the analgesic effect of systemically administered morphine (Manning and Mayer 1995) in rats. Lesions of the Ce also block the expression of conditioned fear (Hitchcock and Davis 1986). Microinjection of morphine into the rat amygdala evokes an analgesic response (Helmstetter et al. 1993; Good and Westbrook 1995) and attenuates unconditioned (Rodgers 1978; File and Rodgers 1979) and conditioned (Good and Westbrook 1995) fear expression.

An important question to consider is whether or not morphine and buprenorphine are acting on the same receptor to exert their anxiolytic-like effects. Supporting previous findings (i.e., Davis 1979a, b; Kameyama and Nagasaka 1982; Koks et al. 1999), we show that morphine's anxiolytic-like effect is naloxone reversible, thus, further implicating a role of classic opioid receptors in mediating morphine's anxiolytic-like effects (Fig. 3). The role of opioid receptors in mediating buprenorphine's anxiolytic-like effect is unclear because buprenorphine's pharmacological profile is not restricted to the μ-opioid receptor. Buprenorphine binds to all classic opioid receptors with high affinity and moderate to low efficacy and can act as an antagonist at the κ-opioid receptor (Lutfy and Cowan 2004), a full agonist at the ORL_1 receptor (Wnendt et al. 1999; Bloms-Funke et al. 2000; Hawkinson et al. 2000; Huang et al. 2001). Interestingly, there is increasing evidence that drugs acting at the ORL_1 receptor may have clinical relevance for the treatment of anxiety-related behaviors (Jenck et al., 1997, 2000; Meis and Pape 1998). Nevertheless, we found that naloxone—which does not bind to the ORL_1 receptor—reverses the anxiolytic-like action of buprenorphine, suggesting a role of classic opioid receptors in mediating buprenorphine's anxiolytic-like effect (Fig. 3). Nevertheless, because the nonselective antagonist, naloxone, binds to all classic opioid receptors, it is not clear which receptor is mediating the anxiolytic-like effects of morphine and buprenorphine. Further research is needed to address this question. Future studies should use selective opioid receptor antagonists to delineate the role of each receptor type in modulating the expression of fear.

Although both morphine and buprenorphine reduced fear-potentiated startle at most doses, very low doses of each compound (0.03 mg/kg of morphine and 0.004 mg/kg

of buprenorphine) appear to facilitate the expression of fear-potentiated startle (Fig. 2). That is, at these low doses, the magnitude of fear-potentiated startle was actually higher than in the saline condition. The fact that it occurred with both drugs at these low doses is especially noteworthy. Why this occurred is not clear. One possibility is that these low doses were inhibiting tonic release of enkephalins by acting via autoreceptors on enkephalin neurons. However, the fact that naloxone did not facilitate fear-potentiated startle is not consistent with this hypothesis. Another possibility is that these low doses were activating autoreceptors on neurons that release some other neurotransmitters that tonically inhibit the expression of fear-potentiated startle. More experiments are needed to address these issues.

We further explored the pharmacology of morphine and buprenorphine by assessing tolerance and cross-tolerance in fear-potentiated startle. Cross-tolerance develops between

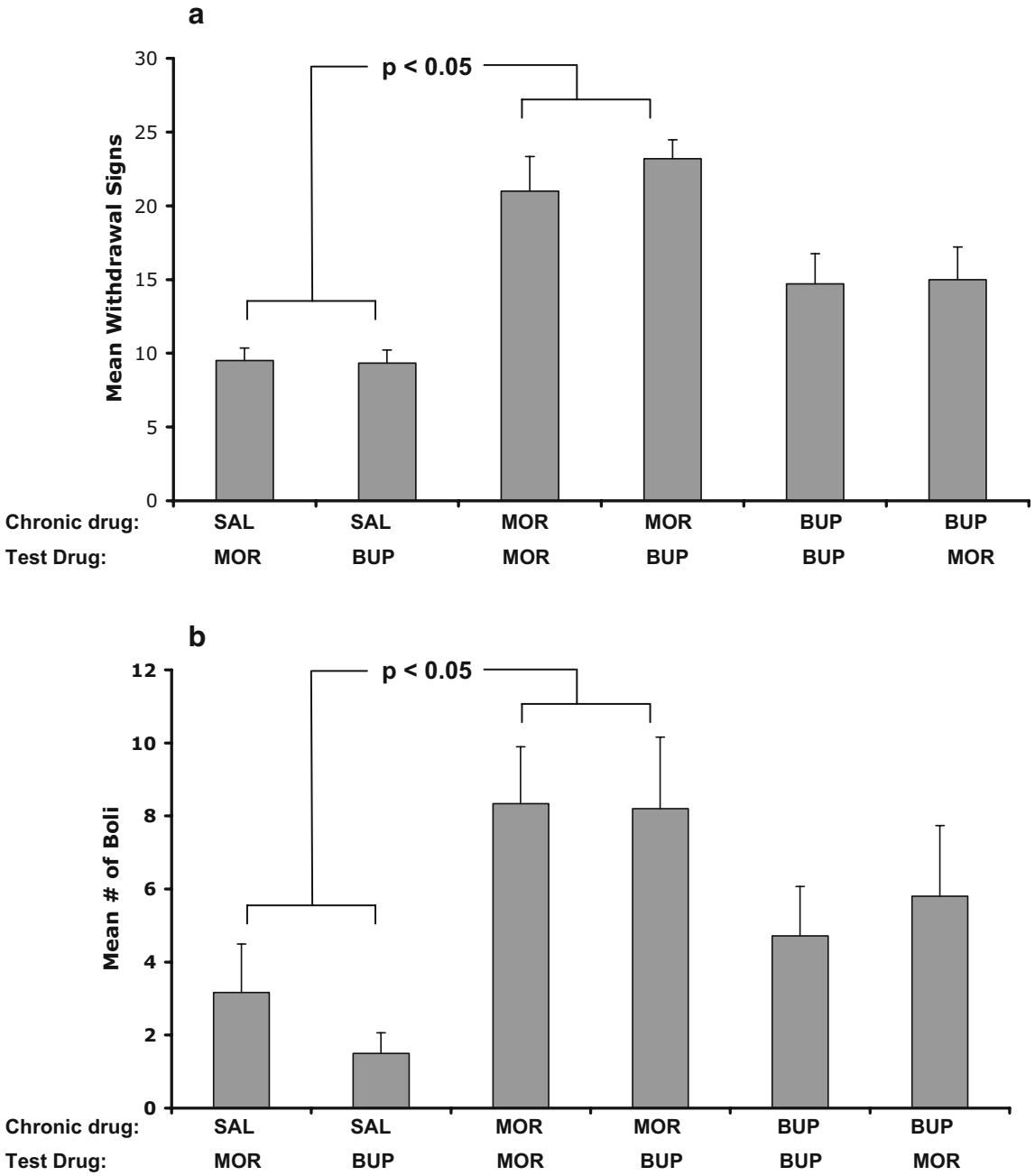

Fig. 7 Morphine produces more signs of dependence than buprenorphine after chronic administration. **a** The mean number of withdrawal signs (±SEM) and **b** mean number of fecal boluses (±SEM) observed after acute or chronic pretreatment with morphine (acute, $n=6$; chronic, $n=11$) or buprenorphine (acute, $n=6$; chronic, $n=12$), * = $p<0.05$

opioid agonists that act through the same receptor (Craft and Dykstra 1990; Moulin et al. 1988). We demonstrated that repeated morphine and buprenorphine treatment produced significant tolerance in their anxiolytic-like effects. Importantly, we found that morphine and buprenorphine show cross-tolerance in these effects (Fig. 5). That is, the ability of morphine or buprenorphine to block fear-potentiated startle was significantly attenuated in animals given prior repeated treatment with buprenorphine or morphine, respectively. Thus, it is argued that morphine and buprenorphine are acting on the same receptor to modulate the expression of conditioned fear. Using the tail-flick assay as a positive control, we confirmed that our chronic treatment regimen was sufficient to induce robust tolerance and cross-tolerance in analgesia (Fig. 6).

There is considerable interest in buprenorphine's development as a therapeutic agent. This is largely because buprenorphine is associated with limited physical dependence liability and thus has a more favorable clinical profile than morphine and related agonists (Cowan 2003; Fudala et al. 1990; Jasinski et al. 1978; Tzschentke 2002). The current study extends this idea by demonstrating that chronic buprenorphine treatment produces less naloxone-precipitated withdrawal signs than comparable morphine treatment (Fig. 7).

In summary, we have shown that both morphine and buprenorphine dose-dependently blocked the expression of fear-potentiated startle, with buprenorphine being 40 times more potent than morphine. Very low doses of each compound slightly increased fear-potentiated startle for reasons that are not clear. The anxiolytic-like effects of both compounds were disrupted by naloxone. Tolerance and cross-tolerance between morphine and buprenorphine were also seen, suggesting that a common receptor mediates their anxiolytic-like and analgesic effects. Finally, buprenorphine was shown to produce fewer withdrawal symptoms than morphine. Together, these findings add new information regarding the role of opioid receptors in the expression of fear- and anxiety-related behaviors and provide further support for the clinical promise of buprenorphine.

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