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Abuse-related effects of mu opioid analgesics in an assay of intracranial self-stimulation in rats: modulation by chronic morphine exposure

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Abstract

Intracranial self-stimulation (ICSS) is an operant procedure in which responding is maintained by electrical brain stimulation. Stimulation frequency can be rapidly varied to maintain a wide range of baseline response rates, and drugs effects can be simultaneously evaluated on both low ICSS rates maintained by low stimulation frequencies and high ICSS rates maintained by high stimulation frequencies. ICSS "facilitation" denotes drug-induced increases in low ICSS rates and is often interpreted as an abuse-related effect, whereas ICSS "depression" denotes decreases in high ICSS rates and may indicate abuse-limiting effects. This study examined the roles of drug efficacy and of prior mu agonist exposure as determinants of mu agonist effects on ICSS in rats with electrodes implanted in the medial forebrain bundle. The high-, intermediate- and low-efficacy mu agonists methadone, fentanyl and nalbuphine were tested during escalating regimens of morphine exposure (Vehicle, 3.2, 18 mg/kg/day). During vehicle treatment, methadone and fentanyl primarily depressed ICSS, whereas nalbuphine produced weak facilitation that was not dose-dependent. Chronic morphine produced tolerance to ICSS depression and increased expression of ICSS facilitation. These results suggest that mu agonist exposure increases expression of abuse-related ICSS facilitation by mu agonists with a broad range of efficacies at mu receptors.

Keywords

Drug abuse; Methadone; Fentanyl; Nalbuphine; Chronic morphine; Tolerance; Intracranial self-stimulation; Rat

Introduction

Mu opioid receptor agonists constitute one class of medications that are extensively used in the clinic for their analgesic properties. One limitation for their use is their abuse potential (Cicero *et al.*, 2007; Denisco *et al.*, 2008). Multiple preclinical procedures have been developed to examine abuse-related effects of opioids, one of which is intracranial self-

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stimulation (ICSS) (Kornetsky *et al.*, 1979; Wise, 1998). ICSS is an operant procedure in which experimental animals learn to self-administer brief electrical pulses into brain regions that are considered to be part of the brain's reward pathways. The frequency of electrical stimulation can be rapidly varied to manipulate the magnitude of reinforcement and to maintain a wide range of baseline response rates. Drugs effects can then be efficiently evaluated on both low ICSS rates maintained by low stimulation frequencies and high ICSS rates maintained by high stimulation frequencies. ICSS "facilitation" denotes drug-induced increases in low ICSS rates and is often interpreted as an abuse-related effect, whereas ICSS "depression" denotes decreases in high ICSS rates and may be indicative of abuse-limiting effects (Miliaressis *et al.*, 1986; Carlezon and Chartoff, 2007; Vlachou and Markou, 2010; Altarifi and Negus, 2011; Bauer *et al.*, 2013). Evaluation of the variables that are involved in opioid-induced changes in ICSS may contribute to improved understanding of opioid mechanisms in reward and motivation.

Accordingly, the purpose of the present study was to extend our previous research on determinants of mu opioid agonists on ICSS in rats (Altarifi and Negus, 2011; Altarifi *et al.*, 2012). Specifically, our previous studies identified two general phenomena. First, effects of mu agonists on ICSS in subjects with no prior history of opioid exposure were dependent on the efficacy of the opioid at mu receptors [with in vitro efficacy to stimulate GTPγS binding as the metric of efficacy; (Selley *et al.*, 1998)]. High- to intermediate-efficacy mu agonists such as methadone and morphine produced biphasic effects that included weak ICSS facilitation manifested as increases in low ICSS rates maintained by some low brain stimulation frequencies and robust ICSS depression manifested as decreases in high ICSS rates maintained by high brain stimulation frequencies. Conversely, lower efficacy mu agonists such as nalbuphine were less effective and reliable to produce both rate-increasing and rate-decreasing effects, and the opioid antagonist naltrexone did not alter ICSS at doses that blocked effects of mu agonists. Second, with morphine, chronic treatment produced tolerance to rate-decreasing effects and enhanced expression of rate-increasing effects (Altarifi *et al.*, 2012). Taken together, these findings suggest that abuse liability of high-efficacy mu agonists may be constrained by abuse-limiting effects in opioid-naive subjects, but regimens of opioid exposure may produce tolerance to abuse-limiting effects and enhanced expression of abuse-related effects.

The present study examined the interaction between these two phenomena by evaluating effects of chronic morphine exposure on changes in ICSS produced by four mu opioid receptor ligands that vary in efficacy at mu receptors: the high-efficacy mu agonist methadone, the intermediate efficacy mu agonist fentanyl, the low-efficacy mu agonist nalbuphine, and the mu antagonist naltrexone (Selley *et al.*, 1998). We hypothesized that chronic morphine would produce (1) tolerance to ICSS depression and enhanced expression of ICSS facilitation by the high-efficacy mu agonists methadone and fentanyl, and (2) dependence as indicated by the emergence of withdrawal-associated ICSS depression by naltrexone. The impact of opioid exposure on ICSS effects of nalbuphine was more difficult to predict, but studies on other behavioral endpoints suggest that chronic treatment with higher efficacy mu agonists can produce tolerance to agonist effects and sufficient levels of opioid dependence to expose antagonist effects of nalbuphine (Woods and Gmerek, 1985; Preston *et al.*, 1989; Oliveto *et al.*, 1991; Young *et al.*, 1991).

Methods

Subjects and ICSS electrode implantation

Male Sprague-Dawley rats (Harlan, Fredrick, MD, USA) weighing 310-350 g at the time of surgery were used for these studies. Rats were individually housed and maintained on a 12-h light/dark cycle with lights on from 6:00 a.m. to 6:00 p.m. Rats had free access to food and

water except during testing. Animal maintenance and research were in compliance with National Institutes of Health guidelines on care and use of animal subjects in research, and all animal use protocols were approved by the Virginia Commonwealth University Institutional Care and Use Committee.

Rats were anesthetized with isoflurane gas (2.5-3% in oxygen; Webster Veterinary, Phoenix, AZ) for the implantation of stainless steel electrodes. The cathode of each bipolar electrode (Plastics One, Roanoke, VA) was 0.25 mm in diameter and covered with polyamide insulation except at the flattened tip. The anode was 0.124 mm in diameter and uninsulated. The cathode was implanted in the left medial forebrain bundle at the level of the lateral hypothalamus (2.8 mm posterior and 1.7 mm lateral from bregma, and 8.8 mm below the skull). The anode was wrapped around one of three skull screws to serve as the ground, and the skull screws and electrode assembly were secured with orthodontic resin. Animals were allowed to recover for at least 7 days prior to commencing ICSS training.

Experimental procedure

Apparatus and training—Experiments were conducted in sound attenuating chambers that contained modular acrylic test chambers (29.2 × 30.5 × 24.1) equipped with a response lever (4.5 cm wide, extended 2.0 cm through the center of one wall, 3 cm off the floor), stimulus lights (three lights colored red, yellow and green positioned 7.6 cm directly above the lever), a 2-W white house light, and an ICSS stimulator (Med Associates, St. Albans, VT). Electrodes were connected to the stimulator via bipolar cables and a commutator (Model SL2C, Plastics One, Roanoke, VA). A computer and software (Med Associates, St. Albans, VT) controlled the stimulator, programming parameters and data collection.

Rats were trained under a continuous reinforcement schedule of brain stimulation using procedures identical to those described previously (Altarifi and Negus, 2011; Altarifi *et al.*, 2012). Each lever press resulted in the delivery of a 0.5-s train of square wave cathodal pulses (0.1-ms pulse duration), and stimulation was accompanied by illumination of the stimulus lights above the lever. Responses during the 0.5-s stimulation period did not result in additional stimulation. During the initial phase of training, sessions lasted 30 to 60 min, the frequency of stimulation was held constant at 158 Hz, and the stimulation intensity was adjusted to the lowest value that would sustain at least 30 stimulations per minute. Once this criterion was met, frequency manipulations were introduced during sessions that consisted of sequential 10 min components. During each component, a descending series of 10 current frequencies (158-56 Hz in 0.05 log increments) was presented, with a 60-s trial at each frequency. A frequency trial began with a 5-s time out followed by a 5-s “priming” phase during which animals received 5 non-contingent stimulations with a 0.5-s interval between each stimulation. This non-contingent stimulation was followed by a 50-s “response” phase, during which responding produced electrical stimulation under a continuous reinforcement schedule. Training continued with three to twelve sequential components per day, and the current intensity was adjusted until rats reliably responded during the first three to four frequency trials of all components for at least three consecutive days. This intensity (range: 110-220 μA) was held constant for the remainder of the study. In addition, rats were habituated to saline injections until these injections had no effect on ICSS frequency-rate curves as determined by two-way analysis of variance (see Data Analysis).

Testing—Once training and habituation to saline injections were completed, “pre-drug baseline” sessions were conducted over a period of 3 consecutive days to establish baseline ICSS performance before administration of any mu agonists. Each pre-drug baseline session consisted of 3 components as described above. Rats were then distributed into 3 different groups. Each group received a different test drug (methadone 0.032-5.6 mg/kg; fentanyl

0.003-0.1 mg/kg; or nalbuphine 0.1-10 mg/kg). Testing in each group proceeded in three phases to evaluate test drug effects before chronic morphine (phase 1), during daily treatment with 3.2 mg/kg/day morphine (phase 2) and during treatment with 18 mg/kg/day morphine (phase 3). In addition to being tested with their designated test mu agonist during each phase, all rats were also tested with 0.1 mg/kg naltrexone after mu agonist testing during phase 3. Rats in the methadone and nalbuphine groups were also tested again 3 weeks after termination of repeated morphine (phase 4). Table 1 summarizes the sequence of treatments in all groups.

The first phase started immediately after the third pre-drug baseline session and lasted for 15-20 days. Daily ICSS sessions in this and all subsequent phases consisted of (a) three consecutive daily-baseline components, (b) a 30 min time out, with administration of saline or drug at the beginning of the time out, and (c) two more test components. Thus, ICSS was assessed twice each day: once during the daily-baseline components before that day's injection of saline or drug (and approximately 23 hr after the previous day's injection), and once during the test components that began 30 min after that day's injection. After the last component, subjects were removed from the test chamber and returned to their home cages. Test sessions involving administration of active doses of the test drug were separated by at least three days in the fentanyl group, which was the first group studied. A modification in experimental design was introduced for the later methadone and nalbuphine groups (see below for rationale), and in these groups, test sessions with active doses were separated by at least two days. For all groups, saline was administered instead of test drug on intervening days. ICSS sessions were sometimes omitted on weekends.

The second phase started with a 7-day maintenance period, during which 3.2 mg/kg/day morphine was administered during the time out of each daily ICSS session. On day 8, test sessions were resumed, and test drug effects were redetermined using the same dose order and intervals as the first phase. In addition, the fentanyl group was also tested with an additional higher dose in phase two after testing with the original doses was completed. On days that subjects did not receive test drug, they received 3.2 mg/kg morphine. This morphine injection was usually administered during the time out of an ICSS session as described above; however, ICSS sessions were occasionally omitted during the weekends, and on these days, the morphine injection was administered without ICSS. As in phase 1, test sessions for fentanyl were separated by at least three days, so that each test session was preceded by at least two days of treatment with the chronic morphine dose. In the methadone and nalbuphine groups, an alternative design was implemented to minimize protracted opioid withdrawal on days when saline or low test-drug doses were examined. Thus, when subjects were tested with saline vehicle or with low doses of methadone (0.32 mg/kg) or nalbuphine (0.1-0.32 mg/kg), they also received a supplemental dose of 3.2 mg/kg morphine after the final component on that day, before returning to their home cages. Supplemental doses were not administered after higher doses of methadone or nalbuphine to minimize the potential for opioid overdose. Phase two lasted 20-25 days.

The third phase began with a gradual increase in the morphine dose administered during the time out of consecutive daily ICSS sessions. Subjects received 5.6 mg/kg/day morphine for 2 days, followed by 10 mg/kg/day morphine for 2-4 days, followed by the terminal dose of 18 mg/kg/day for the remainder of the third phase. The rate of dose escalation was individually determined in each rat to assure expression of ICSS at a given dose before proceeding to a higher dose, and the only variability in rate of dose escalation was whether 2, 3 of 4 days were required to complete 10 mg/kg/day treatment and advance to the terminal dose of 18 mg/kg/day. Once the terminal dose of 18 mg/kg/day morphine was achieved, it was maintained for seven days. Subsequently, test sessions were resumed, and test drug effects were redetermined using the same dose order and intervals as in the second

phase. In addition, the methadone group was also tested with an additional higher dose in phase 3 after testing with the original doses was completed. Also, all subjects were tested with 0.1 mg/kg naltrexone after testing with the designated mu agonist was completed. This naltrexone dose was selected as a dose that did not alter ICSS in non-dependent rats but that blocked methadone-induced facilitation of ICSS in a previous study (Altarifi *et al.*, 2012). Subjects in this phase received 18 mg/kg/day morphine on the days that they did not receive test drug, and 18 mg/kg morphine was also administered at the end of test sessions during which saline vehicle or low methadone (0.32-1.0 mg/kg), or nalbuphine (0.1-3.2 mg/kg) doses were tested. Phase three lasted between 20-25 days.

At the end of the third phase, daily morphine injections were terminated, but daily ICSS sessions continued for at least three days. No further experiments were conducted in the fentanyl group. For the methadone and nalbuphine groups, ICSS sessions and drug treatments were suspended for two weeks. Training was then resumed for three days, after which an extra phase (phase four) was conducted in these subjects identical to phase 1.

Data Analysis

The primary dependent variable was the reinforcement rate in stimulations/trial during each frequency trial. To normalize these raw data, reinforcement rates from each trial in each rat were converted to Percent Maximum Control Rate (%MCR) for that rat. The maximum control rate was determined for each rat during the pre-drug baseline sessions at the beginning of the experiment. The first component from these sessions (and from all other sessions) was considered to be an acclimation component, and data were discarded. The maximum control rate was defined as the mean of the maximal rates observed during any frequency trial of the second and third components of the three pre-drug baseline sessions (six total pre-drug baseline components). Subsequently, %MCR for each trial was calculated as (Reinforcement Rate During a Frequency Trial ÷ Maximum Control Rate) × 100. Graphs show mean frequency-rate curves, with brain stimulation frequency on the abscissa, and ICSS rate expressed as %MCR on the ordinate.

Frequency-rate curves from test sessions during each phase of the study were submitted for analysis. As noted above, these frequency-rate curves were assessed twice on each test day: once during daily baseline components before that day's injection (and approximately 23 hr after the previous day's injection), and again during test components that began 30 min after that day's injection. Daily baseline data and test data from test sessions were analyzed separately. The daily baseline data provided information on changes in baseline ICSS produced by the chronic treatment (saline, 3.2 mg/kg/day morphine, or 18 mg/kg/day morphine). More specifically, because daily baseline components were conducted approximately 23 hr after the most recent injection of the chronic treatment, they provided data on changes in ICSS produced by 23 hr withdrawal from that treatment. Because rats in all three test drug groups received the same progression of chronic morphine treatments during sequential phases of the study, daily baseline data from test sessions within each phase were averaged across all rats to yield mean baseline ICSS data during chronic treatment with saline, 3.2 mg/kg/day morphine and 18/mg/kg/day morphine. Mean daily-baseline data during each phase were compared to the pre-drug baseline data using two-way ANOVA, with phase of chronic treatment as one factor and ICSS frequency as the other factor. A significant ANOVA was followed by a Holm-Sidak post hoc test, and the criterion for significance was set at $p < 0.05$. To facilitate within-subject data analysis, data were included only for those rats that completed all three phases of chronic morphine treatment (N=15, 5 from each group).

Test data from each test session were analyzed to assess dose effects of each test drug (methadone, fentanyl, nalbuphine) on ICSS frequency-rate curves during each phase of

chronic morphine treatment. Within each phase, ICSS test data for a given dose were averaged and compared by two-way ANOVA, with drug dose as one factor and ICSS frequency as the other factor. A similar approach was used to compare effects of saline and 0.1 mg/kg naltrexone treatment during phase 3. A significant ANOVA was followed by a Holm-Sidak post hoc test, and the criterion for significance was set at $p < 0.05$. Data were included for all rats that completed a given phase.

To provide an additional summary measure of baseline and test ICSS performance, the total number of stimulations per component was calculated as the average of the total stimulations delivered across all 10 frequency trials of each component. Baseline and test data were expressed as a percentage of the total stimulations per component earned during the "pre-drug baseline" components (% Control). Thus, % Control was calculated as (Mean Total Stimulations During Daily Baseline or Test Components ÷ Mean Total Stimulations During Pre-Drug Baseline Components) × 100.

Drugs

Morphine sulfate, methadone HCl, naltrexone HCl and fentanyl HCl were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). Nalbuphine HCl was provided by Dr. Kenner Rice (Chemical Biology Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD). All drugs were dissolved in saline and delivered subcutaneously in a volume of 1 ml/kg body weight.

Results

Baseline ICSS before and during chronic morphine

During pre-drug baseline sessions, the mean ± SEM maximum control rate (MCR) for all rats in the study was 57.2±9.8 stimulations per trial, and the mean ± SEM control number of total stimulations delivered across all frequencies was 286.7±70.7. The mean ± SEM MCR values for the methadone, fentanyl and nalbuphine groups were 56.5 ± 6.8, 55.8 ± 11.1, and 59.7 ± 12.7, respectively. The mean ± SEM control numbers of total stimulations delivered across all frequencies for the methadone, fentanyl and nalbuphine groups were 282.5 ± 61.9, 263.5 ± 52.6, and 315.5 ± 96, respectively. Figure 1 shows mean frequency-rate ICSS curves before treatment and during all phases of chronic morphine treatment. During baseline (i.e. before any drug administration), little responding was maintained by the lower frequencies of stimulation (56-79 Hz), and ICSS increased at the intermediate and higher frequencies (89-158 Hz). Chronic treatment with vehicle and 3.2 mg/kg morphine did not significantly alter ICSS. Treatment with 18 mg/kg/day morphine produced a decrease in ICSS relative to the pre-drug baseline (significant at 100-126 Hz). ICSS partially recovered within three days after termination of chronic morphine, although ICSS rates were still significantly decreased at one frequency (112 Hz).

Effects of methadone on ICSS before and during chronic morphine

Figure 2 shows the effect of methadone on ICSS. During vehicle treatment, methadone failed to increase ICSS at any brain stimulation frequency. Rather, methadone dose-dependently decreased ICSS maintained by high frequencies of brain stimulation, with significant decreases at 126-158 Hz after 1.0 mg/kg methadone and at 100-158 Hz after 3.2 mg/kg methadone (figure 2 a,b). Lower doses of methadone (0.032-0.1 mg/kg) were also tested, but as with 0.32 mg/kg methadone, they failed to alter ICSS (data not shown). Repeated treatment with 3.2 mg/kg/day morphine reduced expression of methadone's rate-decreasing effects and increased expression of its rate-increasing effects. Thus, 1.0 mg/kg methadone no longer decreased ICSS at any frequency of brain stimulation, but rather increased ICSS at intermediate frequencies (71-100 Hz). Similarly, 3.2 mg/kg methadone

decreased ICSS across a narrower range of high frequencies (141-158 Hz), and it increased ICSS at intermediate frequencies (71-79 Hz). One of eight subjects died before completion of this phase, so only seven rats completed testing with all methadone doses during chronic treatment with 3.2 mg/kg/day morphine and advanced to higher morphine dose. During chronic treatment with 18 mg/kg/day morphine, 1.0 and 3.2 mg/kg methadone produced exclusive rate-increasing effects at the intermediate frequencies 89-100 Hz and 63-89 Hz, respectively. A higher dose of 5.6 mg/kg methadone was introduced during this phase, and it produced biphasic effects similar to the effects of 3.2 mg/kg during treatment with 3.2 mg/kg/day morphine (figure 2 e,f). Two subjects died during high-dose morphine treatment, so only 5 rats completed testing with all methadone doses in this phase.

Effects of fentanyl on ICSS before and during chronic morphine

Figure 3 shows the effect of fentanyl on ICSS. During vehicle treatment, a low dose of 0.003 mg/kg fentanyl increased ICSS at one frequency (89 Hz), but only rate-decreasing effects were produced by higher fentanyl doses of 0.01 mg/kg (158 Hz) and 0.03 mg/kg (100-158 Hz) (figure 3 a,b). A lower fentanyl dose of 0.001 mg/kg was also tested, and it did not alter ICSS (data not shown). During chronic 3.2 mg/kg/day morphine, 0.003 mg/kg fentanyl did not produce any significant change in ICSS compared to vehicle. However, effects of 0.01 and 0.03 mg/kg fentanyl changed from producing only decreases in ICSS at high frequencies during vehicle treatment to exclusive increases in ICSS at intermediate frequencies during 3.2 mg/kg/day morphine treatment. Thus, fentanyl at 0.01 and 0.03 mg/kg exclusively increased ICSS at 89-100 Hz and 71-100 Hz, respectively (figure 3 c,d). A higher fentanyl dose of 0.1 mg/kg was also introduced during this phase, and this dose nearly eliminated responding and significantly reduced ICSS at the highest five frequencies (100-158 Hz). One of six rats died during this phase, so only five rats completed testing with all fentanyl doses and advanced to next phase. During treatment with 18 mg/kg/day morphine, neither 0.003 nor 0.01 mg/kg fentanyl altered ICSS at any frequency. However, 0.03 mg/kg fentanyl still increased ICSS at intermediate frequencies (71-100 Hz), and 0.1 mg/kg fentanyl still decreased ICSS at high frequencies (112-158 Hz) (figure 3 e,f).

Effects of nalbuphine on ICSS before and during chronic morphine

Figure 4 shows the effects of nalbuphine on ICSS. During vehicle treatment, nalbuphine primarily increased ICSS, although these effects were not monotonically related to dose. Thus, exclusive increases in ICSS were produced by nalbuphine doses of 0.1 (79 Hz), 0.32 (71-89 Hz) and 3.2 mg/kg (71-89 Hz), and the highest dose of 10 mg/kg produced biphasic effects (increased ICSS at 63-79 Hz and decreased ICSS at 158 Hz). Conversely, 1.0 mg/kg nalbuphine (the first dose tested) did not increase ICSS at any frequency and significantly decreased ICSS at the highest frequency (158 Hz). Repeated treatment with 3.2 mg/kg/day morphine eliminated expression of nalbuphine's rate-decreasing effects and enhanced the dose-dependence and magnitude of nalbuphine's rate-increasing effects. For example, 10 mg/kg nalbuphine increased ICSS at frequencies of 63-100 Hz and did not decrease ICSS at any frequency (figure 4 c,d). During treatment with 18 mg/kg/day morphine, nalbuphine produced similar effects consisting of exclusive increases in ICSS across all nalbuphine doses. One of six rats died during treatment with 18 mg/kg/day nalbuphine, so only five rats completed testing with all nalbuphine doses in this phase.

Effect of naltrexone on ICSS during chronic 18 mg/kg/day morphine

Before termination of treatment with 18 mg/kg/day morphine, 0.1 mg/kg naltrexone was tested, and results are shown in figure 5. Naltrexone decreased ICSS at frequencies of 112 and 126 Hz.

Effect of methadone and nalbuphine after 3 weeks of morphine abstinence

The effects of methadone (N=5) and nalbuphine (N=5) were redetermined beginning after 3 weeks of morphine abstinence. As noted above, daily baseline ICSS recovered toward pre-drug baseline levels within three days after termination of morphine treatment (Figure 1), and daily baseline ICSS persisted at pre-drug baseline levels throughout testing during abstinence. For example, for the 10 rats that completed abstinence testing, the pre-drug control ± SEM number of stimulations per component was 297.8 ± 83.6, and the mean number of stimulations per component during abstinence testing was 293.0 ± 163.0. Figure 6 shows that methadone and nalbuphine effects during abstinence testing were generally similar to their effects during treatment with 3.2 mg/kg/day morphine. Thus, 0.32 mg/kg methadone did not significantly alter ICSS, 1.0 mg/kg methadone increased ICSS at intermediate frequencies (63-100 Hz), and 3.2 mg/kg methadone tended to produce biphasic effects, with increased ICSS at 71-79 Hz and a non-significant decrease in mean ICSS rates at high frequencies (126-158 Hz) (figure 6 a,b). Similarly, nalbuphine produced dose-dependent increases in ICSS, although effects of the lowest dose of 0.1 mg/kg nalbuphine did not achieve statistical significance (figure 6 c,d). In addition, diarrhea was not detected after any nalbuphine dose during this phase (data not shown).

Discussion

This study examined the impact of graded morphine exposure on changes in ICSS produced by agonists with high efficacy (methadone), intermediate efficacy (fentanyl) or low efficacy (nalbuphine) at mu opioid receptors. There were three main findings. First, in agreement with previous results (Altarifi *et al.*, 2012) the higher efficacy mu agonists methadone and fentanyl produced primarily rate-decreasing effects in opioid-naïve subjects, whereas the low-efficacy mu agonist nalbuphine produced primarily rate-increasing effects that did not vary systematically as a function of dose. Second, repeated morphine produced cross tolerance to the rate-decreasing effects and enhanced expression of the rate-increasing effects of all three mu agonists. Lastly, the daily morphine dosing regimen used here produced withdrawal-associated decreases in baseline ICSS determined approximately 23 hr after morphine. Repeated morphine also enhanced rate-decreasing effects of the antagonist naltrexone. However, this evidence of opioid dependence and withdrawal was not sufficient to account for enhanced expression of mu agonist-induced rate-increasing effects. Taken together, these results provide further evidence to suggest that repeated opioid exposure increases the degree to which mu agonists produce abuse-related facilitation of ICSS.

Opioid effects in opioid-naïve rats

The constellation of rate-increasing and rate-decreasing effects produced by methadone, fentanyl and nalbuphine in opioid-naïve subjects in this study agrees with effects reported previously for these and other mu agonists that vary in efficacy at mu receptors (Altarifi and Negus, 2011; Altarifi *et al.*, 2012). Specifically, rate-decreasing effects predominated for high-efficacy agonists; lower efficacy agonists produced weaker and more variable rate-decreasing effects; and antagonists such as naltrexone failed to alter ICSS at doses that antagonize effects of mu agonists. The efficacy-dependent rate-decreasing effects of mu agonists in this ICSS procedure agree with the efficacy-dependent magnitude and/or variability in rate-decreasing effects of mu agonists in other assays of responding maintained by other reinforcers (e.g. food) under other schedules (Oliveto *et al.*, 1991; Pitts *et al.*, 1996). Moreover, in the ICSS literature, drug-induced facilitation of ICSS is often interpreted as evidence of an abuse-related effect, whereas drug-induced depression of ICSS is often interpreted as evidence of abuse-limiting dysphoric effects or motor impairment (Carlezon and Chartoff, 2007; Bauer *et al.*, 2013). From this perspective, the present results could be interpreted to suggest that abuse-limiting dysphoric and/or motor effects often

predominate over abuse-related rewarding effects of mu agonists in opioid-naïve rats. This finding in ICSS may be related to the observation that mu agonists are often more efficacious to produce dysphoric subjective effects and behavioral impairment than abuse-related euphoric effects in opioid-naïve/inexperienced human subjects (Lasagna *et al.*, 1955; Walker *et al.*, 2001)

Opioid effects in morphine-treated rats

Repeated morphine has been shown previously to produce tolerance to the ICSS-decreasing effects of morphine (Lorens and Mitchell, 1973; Altarifi and Negus, 2011), and the present study found that repeated morphine also produced cross-tolerance to the ICSS-decreasing effects of the other mu agonists methadone and fentanyl. This agrees with previous reports of morphine-induced cross tolerance to other effects of methadone and/or fentanyl, including their rate-decreasing effects in assays of schedule-controlled responding for food reinforcement (Picker *et al.*, 1991; Hughes *et al.*, 1995; Smith *et al.*, 1997) or their morphine-like discriminative stimulus effects (Young *et al.*, 1991; Walker *et al.*, 1997). Nalbuphine produced only small and inconsistent rate-decreasing effects before morphine treatment, and as a result, cross tolerance was difficult to assess. Nonetheless, the complete absence of nalbuphine-induced rate-decreasing effects during morphine treatment suggests that morphine also produced cross tolerance to any rate-decreasing effects of nalbuphine. Previous studies have failed to reveal cross tolerance between the rate-decreasing effects of morphine and nalbuphine in assays of schedule-controlled responding for food (Oliveto *et al.*, 1991; Picker and Yarbrough, 1991; Smith *et al.*, 1997), but nalbuphine rate-decreasing effects in these studies occurred only at high doses, were variable across subjects, and may have been associated with precipitated withdrawal during morphine treatment (see below). The present findings agree with previous reports of cross tolerance between other effects of morphine and nalbuphine, such as discriminative stimulus effects (Walker *et al.*, 1997) and thermal antinociceptive effects (Gringauz *et al.*, 2001).

In addition to producing tolerance to mu agonist-induced rate-decreasing effects, repeated morphine treatment also enhanced expression of mu agonist-induced facilitation of ICSS. This agrees with previous studies reporting that repeated morphine enhances expression of its own ICSS-facilitating effects (Carlezon and Wise, 1993; Altarifi *et al.*, 2012), and extends this phenomenon to other mu agonists with a broad range of efficacies at mu receptors. There are at least two possible explanations for the enhanced expression of abuse-related rate-increasing effects of mu agonists during morphine treatment. First, mu agonist effects on ICSS may reflect an integration of rate-increasing and rate-decreasing effects, and repeated morphine may produce selectively greater tolerance to rate-decreasing effects and unmask enhanced expression of rate-increasing effects. In support of this possibility, a previous study using targeted intracranial drug injections found that different brain areas mediate morphine-induced rate-increasing effects (forebrain areas proximal to striatum) and rate-decreasing effects (brain stem areas proximal to periaqueductal gray) (Broekkamp *et al.*, 1976), and other studies reported that chronic morphine produces greater mu receptor desensitization in areas that mediate rate-decreasing effects (Sim *et al.*, 1996). Alternatively, or in addition, repeated morphine may produce sensitization to neural substrates that mediate rate-increasing effects. For example, repeated morphine produced sensitization to morphine-induced increases in mesolimbic dopamine release in nucleus accumbens (Spanagel and Shippenberg, 1993). Regardless of the underlying mechanism, these data suggest a shift in morphine effects that favors expression of abuse-related rate-increasing effects relative to abuse-limiting rate-decreasing effects. This shift apparent in ICSS may be related to the finding that regimens of mu agonist exposure can also increase expression of abuse-related rewarding effects in preclinical assays of place conditioning (Lett, 1989; Shippenberg *et al.*, 1996), increase reinforcing effects in preclinical assays of self-administration (Thompson

and Schuster, 1964; Yanagita, 1978; Carrera *et al.*, 1999; Negus and Rice, 2009), and increase expression of abuse-related subjective effects and reinforcing effects in humans (Comer *et al.*, 2010; Cooper *et al.*, 2012).

The regimen of repeated morphine treatment used in this study produced little evidence of tolerance to mu agonist-induced ICSS facilitation, even when the daily morphine dose was increased to 18 mg/kg/day. For example, the low-efficacy mu agonist nalbuphine produced the greatest and most reliable ICSS facilitation during vehicle treatment, and it continued to produce equivalent or greater ICSS facilitation during morphine treatment. It is possible that more intensive treatment regimens (e.g. more frequent morphine dosing, or longer treatment times) may have produced tolerance to rate-increasing effects, but the present results suggest that mu agonist-induced facilitation of ICSS is relatively resistant to tolerance.

ICSS facilitation has been interpreted as an abuse-related drug effect (Wise, 1998; Carlezon and Chartoff, 2007; Vlachou and Markou, 2010). In agreement with this proposition, we recently reported that the overall magnitude of ICSS facilitation produced in rats by a series of monoamine releasers correlated with another, more extensively validated measure of abuse liability: progressive-ratio measures of reinforcement in drug self-administration procedures in nonhuman primates (Bauer *et al.*, 2013). However, the present results with different efficacy mu agonists do not align so clearly with findings from nonhuman primate drug self-administration results. Specifically, ICSS facilitation produced by nalbuphine was similar to or greater than facilitation produced by methadone or fentanyl across morphine treatment regimens in the present study, but in drug self-administration studies in nonhuman primates, the reinforcing effects of nalbuphine were weaker than those of the higher efficacy mu agonist alfentanil (Hursh and Winger, 1995; Rowlett *et al.*, 2002). Future studies will be required to clarify the relationship between ICSS and drug self-administration measures of opioid abuse liability.

Role of morphine dependence and withdrawal

Treatment with the highest daily morphine dose (18 mg/kg/day) produced evidence of morphine dependence in this study insofar as ICSS was depressed at a time of spontaneous withdrawal (23 hr after each daily dose). Moreover, this withdrawal-associated depression of ICSS could be reversed by all three mu agonists and exacerbated by treatment with the mu opioid antagonist naltrexone. These findings agree with previous studies showing that similar morphine treatment regimens can produce signs of opioid dependence (Kishioka *et al.*, 1996) and that depression of ICSS is one sign of opioid withdrawal (Easterling and Holtzman, 1997; Liu *et al.*, 2008; Altarifi and Negus, 2011). However, three findings suggest that enhanced expression of mu agonist-induced ICSS facilitation could not be attributed completely to reversal of morphine withdrawal. First, this enhanced expression of ICSS facilitation was observed during treatment with a lower dose of 3.2 mg/kg/day morphine, which did not produce evidence of dependence or withdrawal. Second, during treatment with 18 mg/kg/day morphine, nalbuphine reliably facilitated ICSS despite the informal observation in all rats that it precipitated diarrhea, a common sign of opioid withdrawal. Lastly, the enhanced expression of methadone- and nalbuphine-induced facilitation of ICSS was also observed more than 2 weeks after termination of repeated morphine, a time when diarrhea was no longer evident.

Acknowledgments

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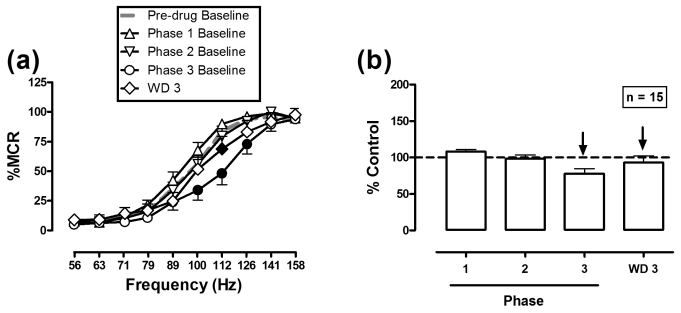

Fig. 1.

ICSS performance before, during and after chronic morphine treatment. ICSS curves were analyzed during pre-drug baseline sessions (grey dashed line), daily baseline components from test sessions during phases 1-3, and day 3 after termination of chronic morphine treatment (WD3: Withdrawal Day 3) for subjects that finished all three phases. The left panel shows ICSS frequency-rate curves. Abscissae: frequency of electrical brain stimulation in hertz (log scale). Ordinates: ICSS rate expressed as percent maximum control rate (%MCR). Filled symbols indicate frequencies at which ICSS rates were lower than those observed during the pre-drug baseline components, as determined by the Holm-Sidak post-hoc test following a significant two-way ANOVA. Summary data in the right panel show the total number of stimulations per test component expressed as a percentage of total pre-drug baseline control stimulations. Abscissae: phase of the treatment. Ordinates: percent control stimulations per test component. Downward arrows indicate the presence and direction of significant differences from pre-drug baseline as determined by analyses of frequency-rate data in the left panel, such that downward arrows indicate significant depression of ICSS at ≥1 frequency of the frequency-rate curve. ANOVA results were as follows: Significant main effect of frequency [$F(9,126)=158.1$; $P<0.001$], significant main effect of phase [$F(3,42)=11.0$; $P<0.001$], and significant phase X frequency interaction [$F(27,378)=4.9$; $P<0.001$]. All points show mean ± SEM for 15 rats.

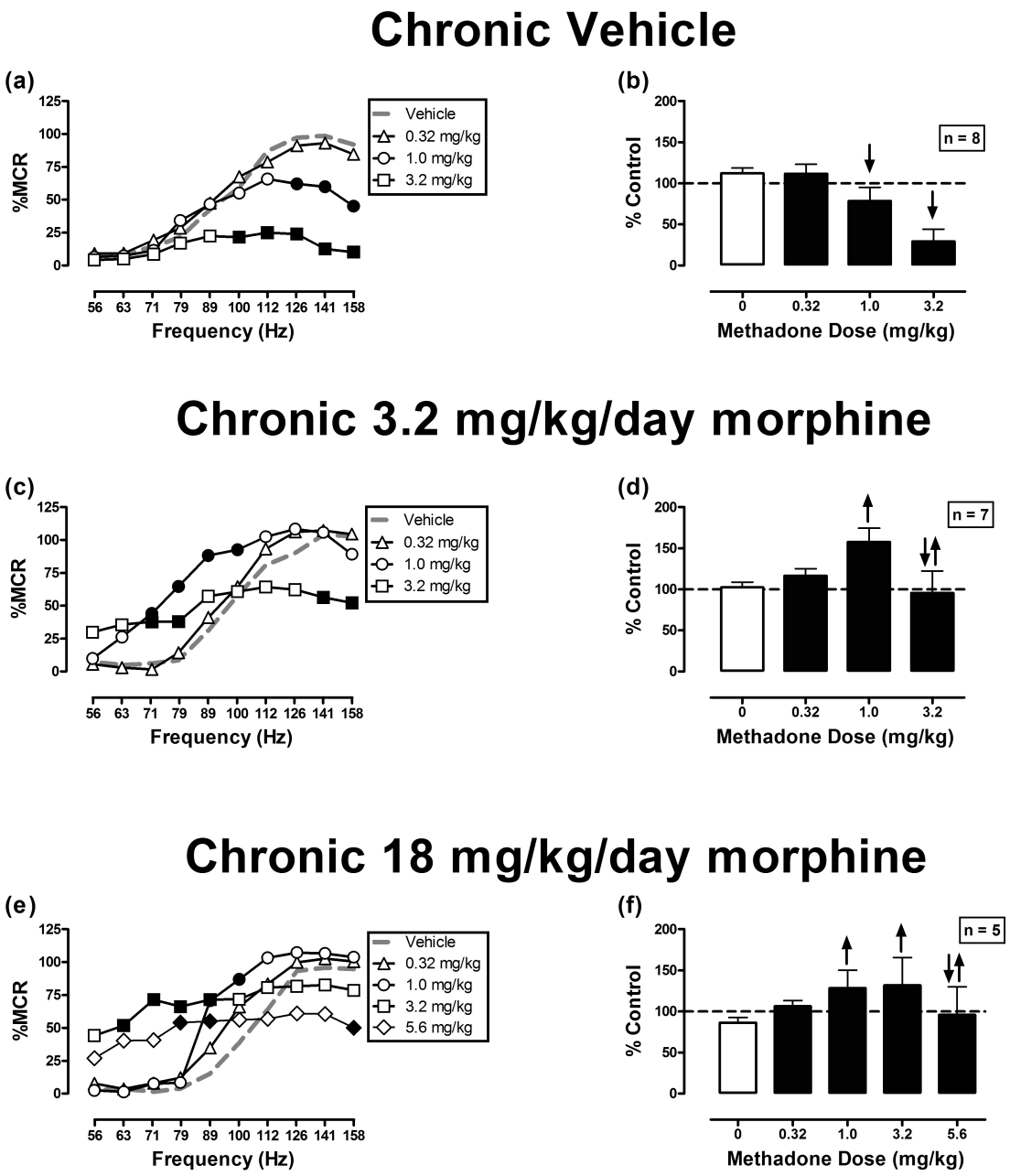

Fig. 2.

Effects of methadone on ICSS before and during chronic morphine treatment. Methadone doses (or vehicle) were administered during treatment with repeated vehicle (phase 1; a,b), repeated 3.2 mg/kg/day morphine (phase 2; c,d), and repeated 18 mg/kg/day morphine (phase 3; e,f). Left panels show full frequency-rate curves. Left abscissae: frequency of electrical brain stimulation in hertz (log scale). Left ordinates: ICSS rate expressed as percent maximum control rate (%MCR). Filled symbols indicate frequencies at which ICSS rates after methadone were different than those observed after vehicle, as determined by the Holm-Sidak post-hoc test following a significant two-way ANOVA. Summary data in the right panels show the total number of stimulations per test component expressed as a

percentage of total pre-drug baseline control stimulations. Abscissae: dose of methadone in mg/kg. Ordinates: percent control stimulations per test component. Upward and/or downward arrows indicate the presence and direction of significant differences from vehicle treatment as determined by analyses of frequency-rate data in the left panels. All points show mean ± SEM for 5-8 rats. For description of axes and symbols, please refer to figure 1. ANOVA results were as follows: Chronic vehicle: Significant main effect of frequency [$F(9,63)=31.4$; $P<0.001$], significant main effect of dose [$F(3,21)=9.9$; $P<0.001$], and significant dose X frequency interaction [$F(27,189)=9.1$; $P<0.001$]. Repeated 3.2 mg/kg/day morphine: Significant main effect of frequency [$F(9,54)=67.2$; $P<0.001$], no significant main effect of dose [$F(3,18)=2.6$; $P=0.086$], and significant dose X frequency interaction [$F(27,162)=11.2$; $P<0.001$]. Repeated 18 mg/kg/day morphine: Significant main effect of frequency [$F(9,36)=91.5$; $P<0.001$], no significant main effect of dose [$F(4,16)=1.2$; $P=0.365$], and significant dose X frequency interaction [$F(36,144)=10.4$; $P<0.001$].

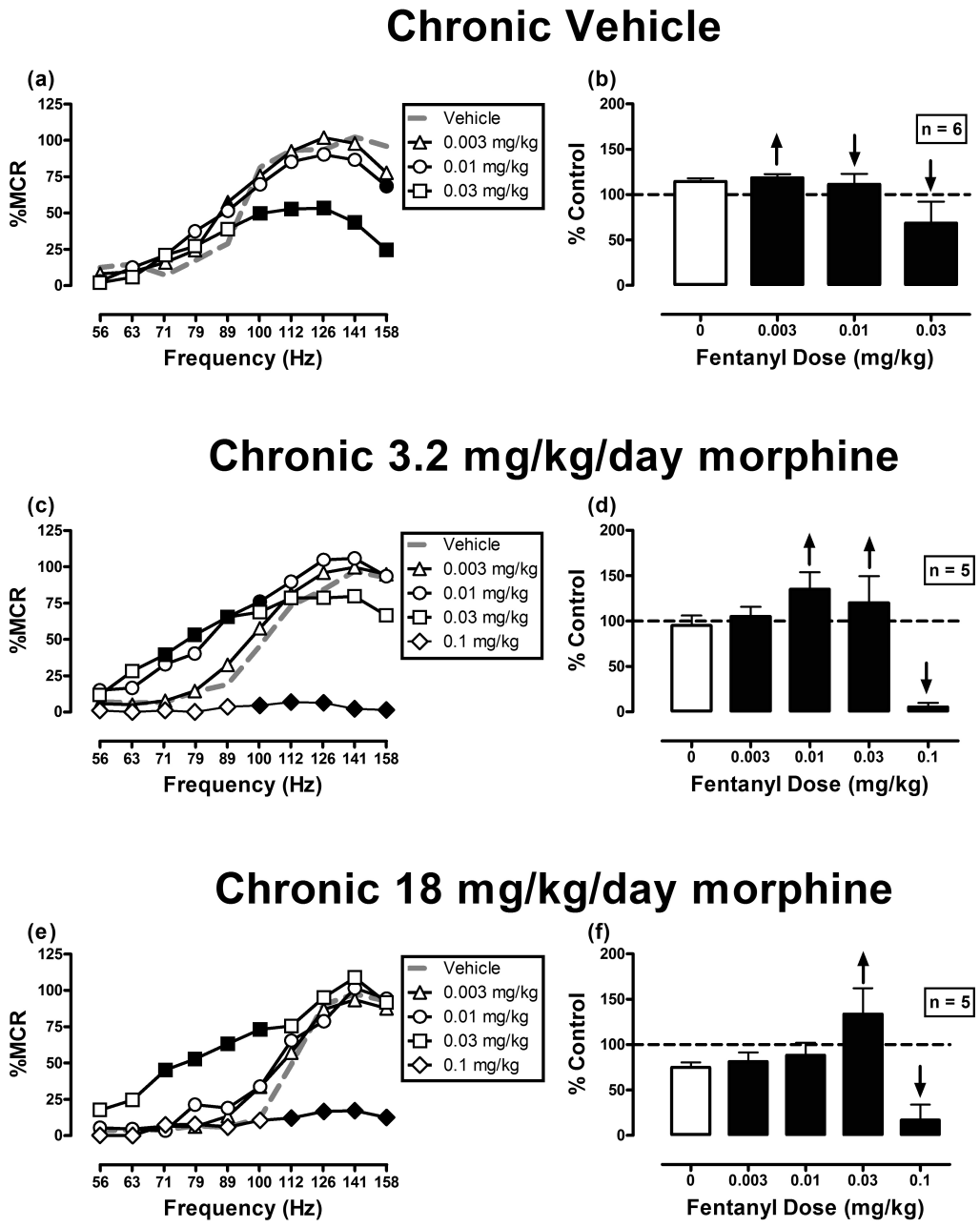

Fig. 3.

Effects of fentanyl on ICSS before and during chronic morphine treatment. Fentanyl doses (or vehicle) were administered during treatment with repeated vehicle (phase 1; a,b), repeated 3.2 mg/kg/day morphine (phase 2; c,d), and repeated 18 mg/kg/day morphine (phase 3; e,f). All points show mean ± SEM for 5-6 rats. For description of axes and symbols, please refer to figures 1 and 2. ANOVA results were as follows: Chronic vehicle: Significant main effect of frequency [$F(9,45)=93.2$; $P<0.001$], significant main effect of dose [$F(3,15)=4.3$; $P=0.022$], and significant dose X frequency interaction [$F(27,135)=5.6$; $P<0.001$]. Repeated 3.2 mg/kg/day morphine: Significant main effect of frequency [$F(9,45)=30.6$; $P<0.001$], significant main effect of dose [$F(4,20)=15.7$; $P<0.001$], and

significant dose X frequency interaction [$F(36,180)=10.0$; $P<0.001$]. Repeated 18 mg/kg/day morphine: Significant main effect of frequency [$F(9,36)=37.7$; $P<0.001$], significant main effect of dose [$F(4,16)=12.1$; $P<0.001$], and significant dose X frequency interaction [$F(36,144)=5.3$; $P<0.001$].

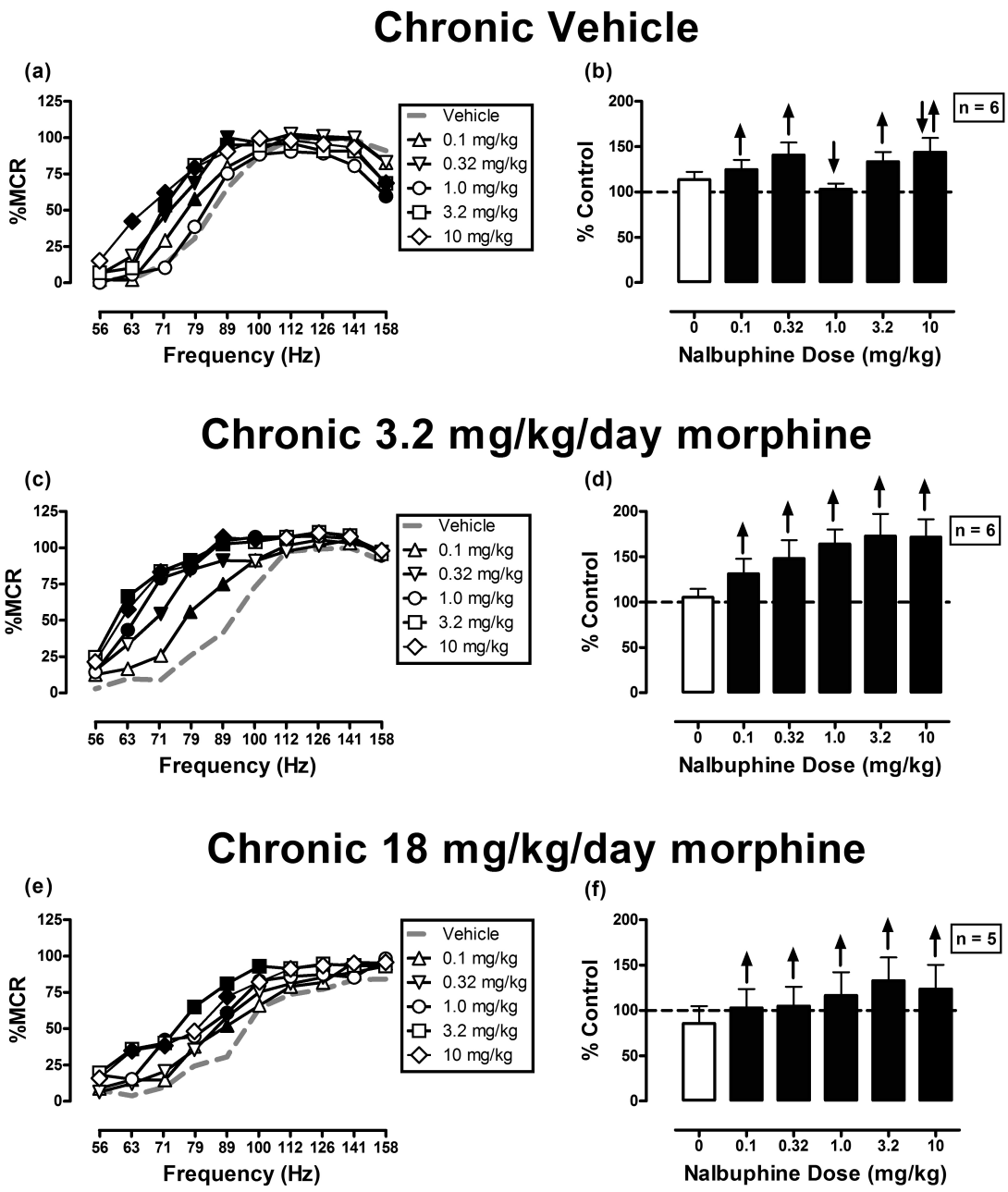

Fig. 4.

Effects of nalbuphine on ICSS before and during chronic morphine treatment. Nalbuphine doses (or vehicle) were administered during treatment with repeated vehicle (phase 1; a,b), repeated 3.2 mg/kg/day morphine (phase 2; c,d), and repeated 18 mg/kg/day morphine (phase 3; e,f). All points show mean ± SEM for 5-6 rats. For description of axes and symbols, please refer to figures 1 and 2. ANOVA results were as follows: Chronic vehicle: Significant main effect of frequency [$F(9,45)=63.0$; $P<0.001$], significant main effect of dose [$F(5,25)=6.6$; $P<0.001$], and significant dose X frequency interaction [$F(45,225)=3.0$; $P<0.001$]. Repeated 3.2 mg/kg/day morphine: Significant main effect of frequency [$F(9,45)=18.9$; $P<0.001$], significant main effect of dose [$F(5,25)=14.9$; $P<0.001$], and

significant dose X frequency interaction [$F(45,225)=2.9$; $P<0.001$]. Repeated 18 mg/kg/day morphine: Significant main effect of frequency [$F(9,36)=13.2$; $P<0.001$], significant main effect of dose [$F(5,20)=9.0$; $P<0.001$], but no significant dose X frequency interaction [$F(45,180)=1.0$; $P=0.532$].

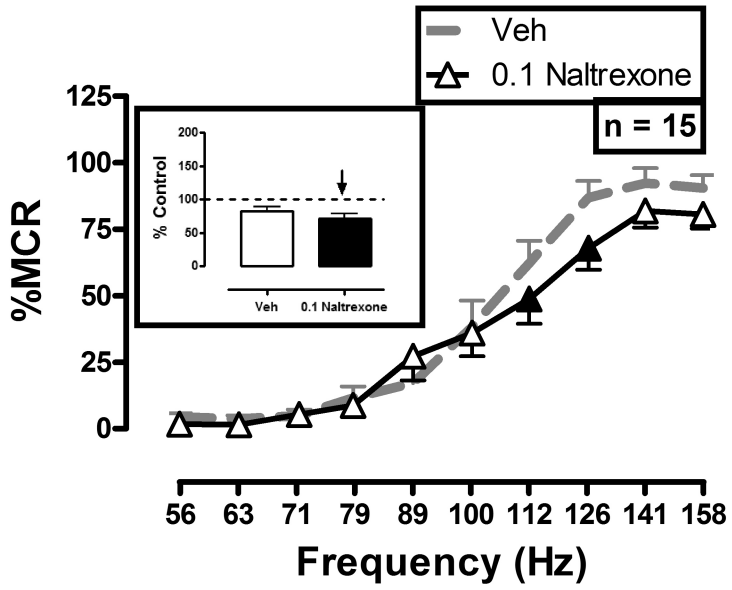

Fig. 5.

Effects of 0.1 naltrexone on ICSS during chronic 18 mg/kg/day morphine treatment (phase 3). All points show mean ± SEM for 15 rats from all groups. For description of axes and symbols, please refer to figures 1 and 2. There was significant main effect of frequency [$F(9,126)=74.0$; $P<0.001$], significant main effect of treatment [$F(1,14)=6.7$; $P=0.021$], and significant dose X frequency interaction [$F(9,126)=3.7$; $P<0.001$].

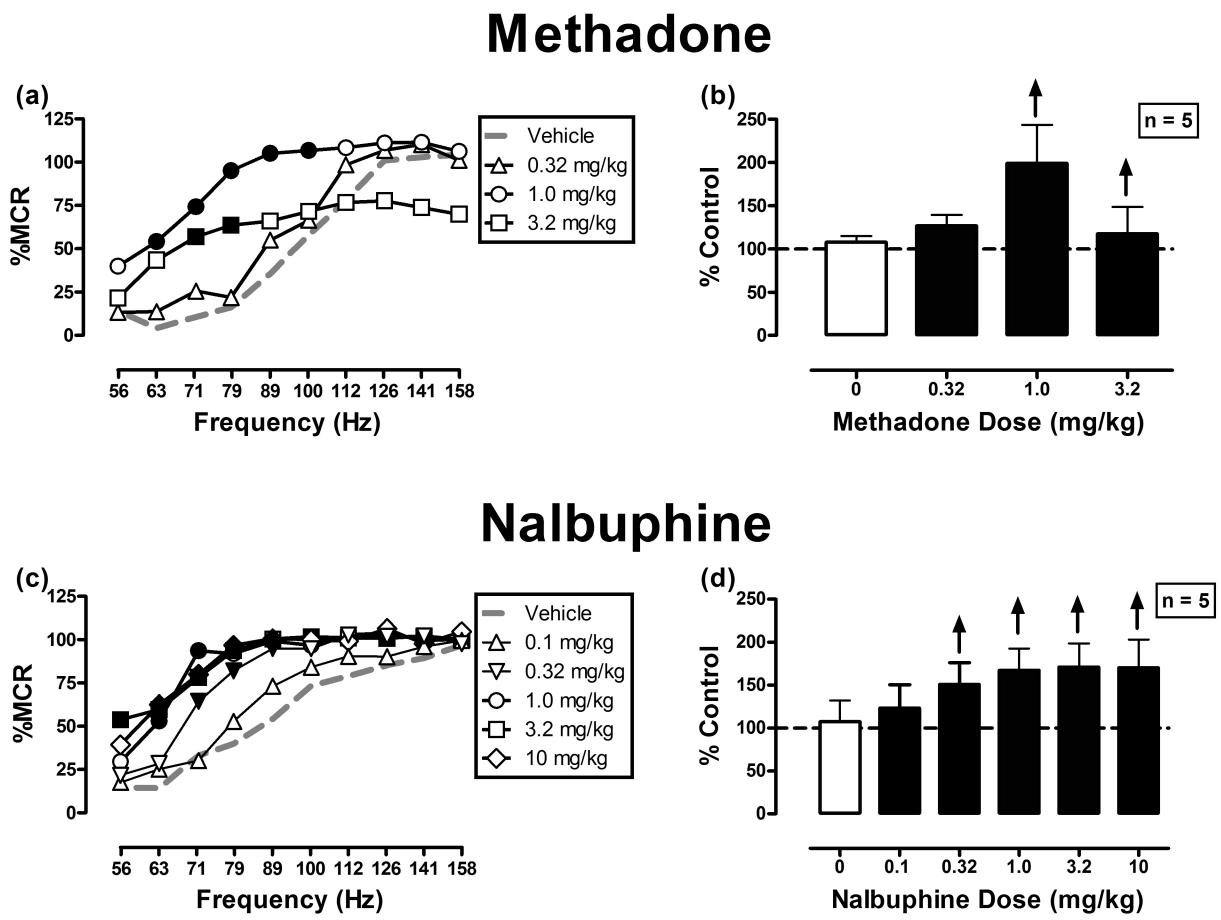

Fig. 6.

Effects of methadone and nalbuphine on ICSS after termination of repeated morphine treatment. Methadone (a,b) and nalbuphine (c,d) were tested after 3 weeks of morphine abstinence. All points show mean ± SEM for 5 rats. For description of axes and symbols, please refer to figures 1 and 2. ANOVA results were as follows: Methadone: Significant main effect of frequency [$F(9,36)=22.6$; $P<0.001$], no significant main effect of dose [$F(3,12)=2.6$; $P=0.097$], and significant dose X frequency interaction [$F(27,108)=5.7$; $P<0.001$]. Nalbuphine: Significant main effect of frequency [$F(9,36)=7.8$; $P<0.001$], significant main effect of dose [$F(5,20)=11.3$; $P<0.001$], and significant dose X frequency interaction [$F(45,180)=2.0$; $P<0.001$].

Table 1

Summary table showing the experimental design, drug doses, and number of subjects used in each group.

Group	Test Drug	Variable	Phase I	Phase II	Phase III	Phase IV
1	Methadone (n = 5-8)	Chronic treatment	Vehicle	3.2 mg/kg/day morphine	18 mg/kg/day morphine	Vehicle
		Doses (mg/kg)	0.032-3.2	0.032-3.2	0.032-5.6	0.032-3.2
2	Fentanyl (n = 5-6)	Chronic treatment	Vehicle	3.2 mg/kg/day morphine	18 mg/kg/day morphine	N/A [a]
		Doses (mg/kg)	0.001-0.03	0.001-0.1	0.001-0.1	N/A [a]
3	Nalbuphine (n = 5-6)	Chronic treatment	Vehicle	3.2 mg/kg/day morphine	18 mg/kg/day morphine	Vehicle
		Doses	0.1-10	0.1-10	0.1-10	0.1-10

[a] N/A=not applicable. Subjects were euthanized at the end of phase III