Conditioned Withdrawal Drives Heroin Consumption and Decreases Reward Sensitivity

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Aspects of drug withdrawal may become conditioned to previously neutral environmental stimuli via classical conditioning processes. Nevertheless, the significance of conditioned withdrawal effects in motivating drug intake remains largely unexplored. Here, we investigated the effects of conditioned withdrawal in modulating heroin consumption and brain reward sensitivity in rats. Rats intravenously self-administered heroin (20 $\mu\text{g}/\text{infusion}$) during 0 h (control), 1 h (nondependent), or 23 h (dependent) sessions and had daily intracranial self-stimulation (ICSS) thresholds assessed. ICSS thresholds remained stable and unaltered in control rats. In nondependent rats, heroin self-administration induced a transient activation of reward systems, reflected in lowering of ICSS thresholds. In dependent rats, heroin intake escalated across sessions and was associated with a gradual decrease in reward sensitivity, reflected in progressively elevated ICSS thresholds. Thus, as dependence develops, heroin may be consumed not only for its acute reward-facilitating effects, but also to counter persistent deficits in reward sensitivity. In nondependent rats, the opioid receptor antagonist naloxone (30 $\mu\text{g}/\text{kg}$) increased heroin consumption and reversed heroin-induced lowering of ICSS thresholds, effects resistant to classical conditioning. In contrast, in dependent rats naloxone (30 $\mu\text{g}/\text{kg}$) increased heroin consumption and also elevated ICSS thresholds above their already elevated baseline levels (i.e., precipitated withdrawal). Most importantly, stimuli repeatedly paired with naloxone-precipitated withdrawal provoked heroin consumption and elevated ICSS thresholds in dependent rats. Thus, conditioned stimuli predicting the onset of heroin withdrawal, and hence the reward deficits coupled with this state, may play a critical role in provoking craving and relapse in human opiate addicts.

Key words: heroin; opiate; conditioned withdrawal; classical conditioning; pavlovian conditioning; addiction; craving; relapse

Introduction

Drug-seeking behavior has long been linked to craving from the perspective of conditioned positive reinforcement, where previously neutral stimuli linked to the positive reinforcing or rewarding effects of addictive drugs acquire motivational significance (Stewart et al., 1984; Shaham et al., 1994). However, as addiction develops, negative reinforcement mechanisms have been hypothesized to emerge that drive the compulsivity of drug addiction (Koob and Le Moal, 2005). Conditioned withdrawal, in which previously neutral stimuli repeatedly paired with drug withdrawal alone precipitate a withdrawal-like state, has been observed in both animal (Baldwin and Koob, 1993; Schulteis et al., 2000; Kenny and Markou, 2005) and human laboratory situations (O'Brien et al., 1977). Essentially unexplored, however, is the hypothesis that stimuli paired with negative reinforcement can acquire motivating properties for drug seeking.

Withdrawal from opiates and other addictive drugs decreases the sensitivity of brain reward systems, measured as elevated intracranial self-stimulation (ICSS) thresholds in rats, and can occur even when physical signs of withdrawal are not manifested (Epping-Jordan et al., 1998; Kenny and Markou, 2005). Decreases in reward sensitivity revealed during withdrawal are hypothesized to reflect compensatory (homeostatic) adaptations in brain reward circuitries that arise to counteract prolonged overstimulation of these systems by drugs of abuse (Solomon and Corbit, 1974; Koob and Le Moal, 2005). Recently, we found that reward deficits observed during withdrawal may be conditioned to environmental stimuli through classical (pavlovian) conditioning processes (Kenny and Markou, 2005). It was hypothesized that such cue-induced reward deficits may serve as a powerful source of craving, motivating drug intake in human opiate addicts (Kenny and Markou, 2005). However, although anecdotal evidence is compelling (Wikler, 1973; Khantzian, 1985; Bradley et al., 1989; Unnithan et al., 1992), little empirical evidence exists supporting a role for withdrawal or conditioned withdrawal in motivating drug intake in humans or animals (Shaham et al., 2003). Indeed, the role of withdrawal in perpetuating the drug habit has remained a fundamental, yet unresolved, question in drug addiction research (Wikler, 1973; Shaham and

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Stewart, 1995; Hutcheson et al., 2001; Ahmed et al., 2002; Robinson and Berridge, 2003).

Here, we explored the role of conditioned withdrawal in motivating heroin intake in rats. First, we investigated whether the quantities of heroin consumed by rats during unlimited (23 h) daily self-administration sessions were sufficient to induce withdrawal-like deficits in brain reward systems. Recently, we have shown that rats with this schedule of unlimited daily access to heroin demonstrated behavioral alterations, including physical signs of opiate withdrawal and motivational signs of withdrawal such as reduced food intake, reminiscent of human heroin addicts (Chen et al., 2006). Next, we tested the hypothesis that cues paired with precipitated opiate withdrawal would alter heroin intake. Finally, we examined the state of brain reward systems, reflected in alterations of ICSS thresholds, during cue-induced motivational states.

Materials and Methods

Subjects

Male Wistar rats ($n = 36$; Charles River, Kingston, NY) weighing 200–250 g at the start of the experiments were housed in groups of three in a humidity- and temperature-controlled (22°C) vivarium on a 12 h light/dark cycle (lights off at 6:00 P.M.) with access to food and water *ad libitum*. The animals were allowed to acclimate to these conditions for at least 7 d. All procedures were conducted in strict adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Drugs

Heroin hydrochloride was obtained from the National Institute on Drug Abuse, and naloxone was obtained from Sigma (St. Louis, MO). Heroin was dissolved in 0.9% sterile saline for injection in a volume of 20 $\mu\text{g/kg}$ /0.1 ml infusion. Naloxone was dissolved in 0.9% sterile saline for a subcutaneous injection in a volume of 1.0 ml/kg.

Surgery

All rats were anesthetized by inhalation of 1–3% isoflurane in oxygen and prepared with Silastic catheters, surgically implanted in the jugular vein. The catheter was passed subcutaneously to a guide cannula (Plastics One, Roanoke, VA) and mounted on the animal's head. Immediately after catheter implantation, rats were implanted with ICSS stimulating electrodes (11 mm in length) into the posterior lateral hypothalamus (anteroposteriorly: -0.5 mm from bregma; mediolaterally: ± 1.7 mm; dorsoventrally: 8.3 mm from dura) according to the atlas of Pellegrino et al. (1979). Four indentations were made in the skull to accommodate screws that, together with the application of dental acrylic, held the cannula and electrode in place.

Apparatus

ICSS training and testing took place in sixteen Plexiglas operant chambers (25 $\times$ 31 $\times$ 24 cm) (Med Associates, St. Albans, VT) as described in detail previously (Kenny and Markou, 2006). Heroin self-administration took place in twelve Plexiglas operant cages (Med Associates), as described previously (Chen et al., 2006). The catheter fittings on the animals' skulls were connected to polyethylene tubing contained inside a protective metal spring (tether) that was suspended into the operant chamber from a liquid swivel attached to a balance arm. Drug was delivered by syringe pump (Razel, St. Albans, VT) as described previously (Caine and Koob, 1993), using a 30 ml syringe delivering 0.1 ml infusions over 1 s. Each operant chamber contained a single retractable lever that extended approximately one inch into the chamber. After completion of a fixed ratio 1 (FR1) schedule of reinforcement, a 28 V white stimulus light located above the lever signaled the delivery of a drug infusion, and remained active for a 20 s time out (TO) period, during which responses were recorded but had no scheduled consequences. Nose-poke access to food and water was available throughout the 23 h session, including the drug TO periods.

Intracranial self-stimulation (ICSS) threshold procedure

Animals were trained to respond according to a modification of the discrete-trial current-threshold procedure (Kornetsky and Esposito, 1979), described in detail previously (Markou and Koob, 1992; Kenny and Markou, 2006). Briefly, ICSS current levels were varied in alternating descending and ascending series in 5 μA steps. In each testing session, four alternating descending/ascending series were presented. The threshold for each series was defined as the midpoint between two consecutive current intensities that yielded "positive scores" (animals responded for at least two of the three trials) and two consecutive current intensities that yielded "negative scores" (animals did not respond for two or more of the three trials). The overall threshold of the session was defined as the mean of the thresholds for the four individual series. Each testing session was ~ 30 min in duration.

Heroin self-administration procedure

Animals were placed into the operant chambers and trained to nosepoke (FR1) for food (45 mg chow pellets; Bio-Serve, Frenchtown, NJ) and water (100 μl aliquots) in 1 or 23 h sessions (Chen et al., 2006). After 5 d of food and water training, animals were allowed to lever press for heroin (20 $\mu\text{g/kg}$ /0.1 ml infusion) on a FR1 20 s TO schedule during 1 or 23 h daily sessions. All heroin self-administration sessions commenced with the lever being extended, after which time the completion of each FR1 resulted in an injection signaled immediately by a cue light, which remained lit for a 20 s TO period, during which responses were recorded but not reinforced. Drug, food, and water responses were recorded by an IBM (White Plains, NY) PC-compatible computer. A "chew pad" (i.e., duct tape wrapped around the center of a ribbon-shaped metal spring) was placed on the self-administration chamber floor during 23 h sessions to prevent rats chewing on drug lines.

Testing procedures

Experiment 1: development of heroin dependence and brain reward function. To investigate potential alterations in the activity of brain reward systems associated with development of heroin dependence, rats ($n = 15$) demonstrating stable ICSS thresholds (defined as $\leq 10\%$ variation in thresholds over three consecutive days) were permitted to intravenously self-administer heroin (20 μg per infusion) during 0 h (control rats; $n = 5$), 1 h ($n = 5$) or 23 h ($n = 5$) sessions. ICSS thresholds were assessed immediately after each self-administration session in 23 h rats, during the 60 min period when heroin was not available for self-administration. Reward thresholds were assessed in control rats at time-points similar to the 23 h rats. Thresholds were assessed immediately before and after heroin self-administration sessions in 1 h rats. Previously, our laboratories have shown that 23 h daily access to heroin self-administration induced heroin dependence-like behavioral alterations in rats, including physical signs of opiate withdrawal and motivational signs of withdrawal such as reduced food intake (Chen et al., 2006).

Experiment 2: conditioned withdrawal and heroin intake. Next, we examined the potential for previously neutral stimuli repeatedly paired with precipitated opiate withdrawal to motivate heroin intake. On the preconditioning day, a second cohort of 1 h ($n = 4$) and 23 h ($n = 12$) rats were injected with saline 30 min into their daily self-administration session, and heroin intake was monitored during the next 30 min. The next day, a compound stimulus consisting of a light (100 pulses per minute) and tone (~ 60 dB constant tone) was initiated in the self-administration chambers. Rats were immediately injected with a low dose of naloxone (30 $\mu\text{g/kg}$) instead of saline, and heroin intake was monitored during the next 30 min (after which cues were deactivated). Previously, this naloxone dose was shown to precipitate affective signs of opiate withdrawal, such as elevations of ICSS thresholds and a conditioned place aversion, but not physical signs of withdrawal in opiate dependent rats (Schulteis et al., 1994). The above cue-injection pairing procedure was repeated for four consecutive conditioning days. On the test day, 1 and 23 h rats were presented with the light/tone stimulus and then injected with saline, and postinjection heroin intake was monitored for 30 min. To demonstrate that any cue-induced alteration of heroin intake on the test day in 23 h rats was directly related to cue-withdrawal pairings and not secondary to intrinsic actions of conditioning stimuli per se, we also examined heroin

intake in a separate group of 23 h rats (unpaired rats; $n = 5$) injected with saline instead of naloxone during the four conditioning sessions. Importantly, these unpaired rats also received naloxone (30 $\mu\text{g/kg}$) injections 2–3 h after saline-cue pairings to ensure that their withdrawal experience matched that of the “paired” 23 h rats above while avoiding the withdrawal–cue pairing.

Experiment 3: conditioned withdrawal and brain reward function. Finally, we investigated the alterations in hedonic processing that may underlie naloxone and cue-evoked increases in drug intake in 1 and 23 h rats. Specifically, we examined the effects of naloxone on reward thresholds in 1 and 23 h rats and investigated whether these effects were susceptible to classical conditioning. On day 25 of heroin access, control and 23 h rats (same rats as those described in experiment 1 above) had post-heroin thresholds assessed as usual. Rats were then injected with saline (preconditioning day), and postinjection thresholds were assessed. The following day, control and 23 h rats had baseline thresholds assessed. A compound stimulus (same as above) was then initiated in the ICSS chambers. Previously, we have shown that this compound stimulus is hedonically neutral, with no intrinsic actions on reward thresholds in control or opiate-dependent rats (Kenny and Markou, 2005). Control and 23 h rats were then injected with naloxone (30 $\mu\text{g/kg}$) instead of saline. Immediately after naloxone injection, control and 23 h rats were placed back into the ICSS chambers where the compound stimulus was presented throughout the entire 30 min postinjection ICSS session. This cue–injection conditioning procedure was performed for four consecutive days. On the test day, which occurred on the day immediately after the fourth and final cue–injection pairing, control and 23 h rats had baseline thresholds assessed as usual, were then presented with the light/tone stimulus and injected with saline, and postinjection thresholds were assessed. A similar experimental design was used for 1 h rats. However, 1 h rats were injected with saline or naloxone (according to experimental design) immediately after the self-administration session but before post-heroin threshold assessment. In this manner, the effects of naloxone and conditioned stimuli on heroin-induced lowering of reward thresholds could also be assessed.

Statistical analyses

During the escalation phase of the experiment, the number of heroin infusions consumed by 1 and 23 h rats was analyzed by two-way repeated measures ANOVA. Mean absolute ICSS thresholds ($\pm\text{SEM}$) are presented for each experiment in Results. During the escalation phase, percentage change from baseline reward thresholds for all rats was calculated by expressing the heroin-influenced threshold scores as a percentage of baseline thresholds, minus 100. Baseline reward thresholds were the mean of the thresholds obtained on the 3 d immediately before the first heroin self-administration session. Percentage change in reward thresholds for control and 23 h rats were analyzed by individual one-way ANOVAs. Pre-heroin and post-heroin reward thresholds for 1 h rats were analyzed by two-way repeated-measures ANOVA. To assess conditioning effects on heroin consumption, the number of heroin infusions self-administered during the 30 min postsaline injection period on the preconditioning day, and the 30 min postnaloxone injection period on the four conditioning sessions, were analyzed by individual two-way repeated-measures ANOVA for 1 h rats and 23 h paired and unpaired rats. Heroin intake on the test day was compared with intake on the preconditioning day by paired t test for each group. Because baseline

Figure 1. Unlimited daily access to heroin escalated heroin intake and decreased the excitability of brain reward systems. *a*, Heroin intake ($\pm\text{SEM}$; 20 μg per infusion) in rats during limited (1 h) or unlimited (23 h) self-administration sessions. *** $p < 0.001$, main effect of access (1 or 23 h), two-way repeated measures ANOVA. *b*, Percentage change from baseline ICSS thresholds ($\pm\text{SEM}$) in control rats, which remained heroin naive for the duration of the experiment and had ICSS thresholds assessed at the same time points as the 23 h rats. *c*, Percentage change from baseline ICSS thresholds ($\pm\text{SEM}$) in 1 h rats. Daily post-thresholds ($\pm\text{SEM}$), assessed immediately after each heroin self-administration session, were lowered compared with prethresholds ($\pm\text{SEM}$), assessed immediately before each self-administration session in 1 h rats. * $p < 0.05$, main effect of heroin on reward thresholds, two-way repeated measures ANOVA. *d*, Percentage change from baseline ICSS thresholds ($\pm\text{SEM}$) in 23 h rats. Reward thresholds, assessed immediately after each daily 23 h self-administration session, became progressively more elevated as exposure to self-administered heroin increased across sessions. * $p < 0.05$, main effect of heroin on reward thresholds, two-way repeated measures ANOVA.

heroin intake differed between paired and unpaired 23 h rats throughout the entire study, changes in absolute heroin intake on the test day between these groups could not be directly compared. Instead, percentage change from baseline heroin intake on the test day for paired and unpaired 23 h rats was compared by t test. Percentage change from baseline heroin intake on the test day was calculated for paired and unpaired 23 h rats by expressing intake on the test day as a percentage of intake on the preconditioning day, minus 100. To assess conditioning effects on reward thresholds, the percentage change from baseline reward thresholds for control and 23 h rats was calculated by expressing the postsaline or postnaloxone threshold scores as a percentage of daily (preinjection) thresholds, minus 100. The percentage change from baseline reward thresholds for 1 h rats during conditioning sessions was calculated by expressing postsaline or postnaloxone threshold scores as a percentage of baseline thresholds, minus 100. Baseline reward thresholds were again the mean of the thresholds obtained on the 3 d immediately before the first heroin self-administration session. Threshold data were analyzed as outlined above for the heroin self-administration data during the conditioning sessions. After statistically significant effects in the ANOVAs, *post hoc* comparisons among means were conducted with Fisher's least significant difference test as appropriate.

Results

Experiment 1: development of heroin dependence and brain reward function

Heroin intake remained stable across self-administration sessions in 1 h (nondependent) rats ($F_{(4,18)} = 1.1$; not significant (NS)) (Fig. 1*a*). Similarly, baseline reward thresholds in 1 h rats, assessed before each self-administration session, remained stable and unaltered across days ($F_{(4,18)} = 1.0$; NS) (Fig. 1*c*). However, post-heroin reward thresholds were significantly lowered com-

Figure 2. Conditioned heroin withdrawal motivated heroin intake. **a**, Heroin intake in 1 h rats for 30 min after saline injection on the preconditioning day (saline), after naloxone (30 μ g/kg) injection and cue presentation during the four conditioning sessions (naloxone + cues), and on the test day (saline + cues). Naloxone injection and cue presentation significantly increased heroin intake in 1 h rats. $*p < 0.05$, main effect of naloxone/cues compared with intake observed after saline injection. However, cue presentation and vehicle (saline) administration on the test day did not alter heroin intake in 1 h rats. **b**, Heroin intake in unpaired 23 h rats injected with saline instead of naloxone during conditioning sessions. Cue presentation and vehicle did not alter heroin intake in unpaired 23 h rats during the conditioning sessions, or on the test day. **c**, Heroin intake in paired 23 h rats. Naloxone injection and cue presentation significantly increased heroin intake in 23 h rats during the naloxone/cue pairings. $***p < 0.001$, main effect of naloxone/cues compared with intake observed after saline injection. Importantly, cue presentation and vehicle on the test day significantly increased heroin intake in paired 23 h rats. $*p < 0.05$, paired t test compared with intake on the saline day. **d**, Percentage change from baseline heroin intake on the test day was significantly increased in paired 23 h rats compared with unpaired 23 h rats. $*p < 0.05$, t test compared with percentage change in intake on the saline day. Data are mean $\pm$ SEM.

pared with daily preheroin thresholds in 1 h rats ($F_{(4,18)} = 2.1$; $p < 0.01$) (Fig. 1c). Reward thresholds remained stable and unaltered for the duration of the experiment in control rats ($F_{(4,23)} = 0.5$; NS) (Fig. 1b). In contrast, thresholds became gradually elevated across sessions in 23 h (dependent) rats ($F_{(4,24)} = 1.6$; $p < 0.05$) (Fig. 1d). Similarly, heroin intake in 23 h rats also progressively escalated across self-administration sessions ($F_{(4,23)} = 11.9$; $p < 0.001$) (Fig. 1a).

Experiment 2: conditioned withdrawal and heroin intake

As seen in Figure 2a, naloxone increased heroin intake in 1 h rats on each of the four conditioning days ($F_{(3,4)} = 4.1$; $p < 0.05$). Importantly, cue presentation with vehicle (saline) did not alter drug intake on the test day in 1 h rats (Fig. 2a), indicating that this action of naloxone was not susceptible to classical conditioning processes. In 23 h rats, naloxone increased heroin intake during the four conditioning days ($F_{(4,44)} = 24.7$; $p < 0.001$) (Fig. 2b). The magnitude by which naloxone increased heroin intake was greater in 23 h rats than in 1 h rats ($F_{(1,14)} = 8.5$; $p < 0.05$, main effect of access, two-way ANOVA). Most importantly, cue presentation with vehicle significantly increased heroin intake in 23 h rats on the test day compared with heroin intake on the preconditioning day ($p < 0.05$, paired t test) (Fig. 2b). Importantly, a cue-induced increase in heroin intake was not observed in the unpaired 23 h rats that received saline injections instead of naloxone during the conditioning sessions (Fig. 2c). Finally, cue presentation with vehicle significantly increased the percentage

of baseline heroin intake in paired 23 h rats on the test day compared with percentage of baseline heroin intake in unpaired 23 h rats ($p < 0.05$, t test) (Fig. 2d).

Experiment 3: conditioned withdrawal and brain reward function

In control rats, postinjection reward thresholds remained stable and unaltered compared with baseline thresholds on the preconditioning day, during the 4 d of cue-injection pairings and on the test day (Fig. 3a). In 1 h rats, saline injection had no effects on heroin-induced lowering of reward thresholds on the preconditioning day. However, during the four conditioning sessions, naloxone reversed heroin-induced lowering of reward thresholds above baseline (before heroin) levels (Fig. 3b). On the test day, cue presentation with vehicle did not alter heroin-induced lowering of reward thresholds in 1 h rats. In 23 h rats, postsaline thresholds were unchanged compared with their already elevated baseline levels on the preconditioning day (Fig. 3c). During the conditioning sessions, naloxone elevated postinjection reward thresholds in 23 h rats (i.e., precipitated withdrawal) above their elevated levels ($F_{(4,4)} = 3.6$; $p < 0.05$) (Fig. 3c). Interestingly, there was a nonsignificant trend for the magnitude by which naloxone elevated reward thresholds to progressively increase over the four conditioning days (Fig. 3c). Most importantly, on the test day withdrawal-associated cues and vehicle significantly elevated reward thresholds in 23 h rats ($p < 0.05$) (Fig. 3c).

Discussion

Development of compulsive opiate intake may depend not only on the positive reinforcing and hedonic actions of opiates, but also on avoidance and escape from the aversive consequences of opiate withdrawal (Ahmed et al., 2002; Kenny et al., 2003b; Koob and Le Moal, 2005). However, the motivational significance of opiate withdrawal in general, and in driving opiate consumption in particular, has remained a controversial issue for many years. Indeed, it has been argued that withdrawal powerfully motivates drug intake (Wikler and Pescor, 1967; Koob and Le Moal, 1997, 2001; Kenny and Markou, 2005), perhaps by increasing the appetitive value of drugs (Hutcheson et al., 2001), whereas others have argued that withdrawal plays no role whatsoever in modulating drug intake (Stewart and Wise, 1992; Robinson and Berridge, 2003). These contrasting views likely arise from the fact that, in most previous studies investigating the role of withdrawal in motivating drug intake, animals were made opiate dependent through nonvolitional (experimenter-administered) delivery of opiates, or had limited histories of opiate self-administration that may not have resulted in dependence (Stewart and Wise, 1992). In contrast, rats in the present study became opiate dependent through excessive volitional opiate consumption during unlimited (23 h) daily self-administration sessions, with potentially many opportunities to associate the effects of volitional heroin intake on the activity in brain reward systems. Furthermore, in the present study, withdrawal was precipitated with a relatively low naloxone dose (0.03 mg/kg) unlikely to otherwise disrupt operant performance. This contrasts with previous studies in which withdrawal has been precipitated with relatively high doses of opioid receptor antagonists (e.g., Stewart and Wise, 1992). With low naloxone doses, it was anticipated that rats could more readily overcome precipitated withdrawal through further drug consumption and therefore better associate drug intake with alleviation of withdrawal (Carrera et al., 1999; Hutcheson et al., 2001).

Figure 3. Conditioned heroin withdrawal decreased the excitability of brain reward systems. *a*, Percentage change from baseline ICSS thresholds ($\pm$ SEM) in control rats after saline injection on the preconditioning day (saline), during the four cue/injection pairings (naloxone + cues), and on the test day (saline + cues). *b*, Percentage change from baseline ICSS thresholds ($\pm$ SEM) in 1 h rats. Saline did not alter the lowering of reward thresholds observed after heroin intake during 1 h self-administration sessions. However, naloxone (30 μ g/kg) administration and cue presentation reversed heroin-induced lowering of reward thresholds. $*p < 0.05$, main effect of naloxone/cues compared with thresholds observed after saline injection, one-way repeated measures ANOVA. On the test day, cue presentation and vehicle did not alter the lowering of reward thresholds observed after heroin intake during 1 h self-administration sessions. *c*, Percentage change from baseline ICSS thresholds ($\pm$ SEM) in 23 h rats. Saline did not alter the already elevated baseline reward thresholds in 23 h rats. However, naloxone (30 μ g/kg) administration and cue presentation raised reward thresholds above their already elevated baseline levels in 23 h rats. $*p < 0.05$, main effect of naloxone/cues compared with thresholds observed after saline injection, one-way repeated measures ANOVA. On the test day, cue presentation and vehicle significantly elevated reward thresholds in 23 h rats. $*p < 0.05$, paired *t* test compared with thresholds on the saline day.

In 1 h rats, baseline ICSS thresholds assessed before each daily self-administration session remained stable and unaltered across days, whereas heroin self-administration significantly lowered ICSS thresholds. Importantly, in a very recent study we demonstrated that ICSS thresholds remained stable and unaltered in control rats without access to heroin or any drug of abuse when thresholds were assessed at the same time-points as the 1 h rats in the present study (Kenny and Markou, 2006). Indeed, a major advantage of the ICSS procedure is that rats demonstrate little satiation to ICSS during extended testing sessions (Olds, 1958; Annau et al., 1974), and ICSS thresholds can be assessed multiple times each day without significant alterations in thresholds across daily sessions (Markou and Koob, 1991; Kenny et al., 2003a). Furthermore, similar lowering of ICSS thresholds has been observed across a broad range of experimenter-administered heroin doses (Hubner and Kornetsky, 1992). Thus, the daily lowering of reward thresholds observed in 1 h rats most likely reflects transiently increased reward sensitivity through heroin-induced amplification of reward signals in the brain. Importantly, this limited daily access to heroin did not induce persistent alterations in the baseline sensitivity of brain reward systems. In 23 h rats, excessive heroin consumption induced a progressive elevation in daily ICSS thresholds. Thus, excessive heroin consumption in 23 h rats decreased baseline sensitivity of brain reward systems, and this evolving reward deficit motivated an escalating pattern of heroin intake, further decreasing reward sensitivity. Consistent with this hypothesis, we have shown previously that extended (6 h) daily access to cocaine self-administration also persistently decreased brain reward function and propelled an escalation of cocaine intake in rats (Ahmed et al., 2002). Overall, the present data demonstrate that the quantities of heroin consumed by rats with unlimited daily access to the drug induced profound decreases in the excitability of reward systems, likely a crucial factor that drives escalation of heroin intake and the transition to heroin dependence. Furthermore, these data suggest that as opiate dependence develops, a new source of motivation emerges in which heroin is consumed not only for its acute reward-facilitating effects, but also to counter the persistent deficits in the sensitivity of reward systems. Hence, the emergence of a negative reinforcement dimension to heroin self-administration, in conjunction with the positive reinforcing effects of heroin, likely contributes to the compulsive drug intake so characteristic of human addicts.

In 1 h rats, naloxone significantly increased heroin intake and reversed the stimulatory effects of heroin on brain reward systems, but did not reveal any long-lasting adaptations in reward pathways (i.e., did not precipitate withdrawal). Interestingly, naloxone-induced increases in heroin intake and reversal of heroin-induced lowering of reward thresholds were not susceptible to classical conditioning in 1 h rats. Based on these observations, it is likely that naloxone-induced increases in heroin self-administration in 1 h rats represents a compensatory response to overcome reversal of heroin-induced stimulation of brain reward systems. Indeed, similar compensatory increases in drug intake were observed in cocaine or amphetamine self-administering rats treated with doses of dopamine receptor antagonists that attenuated the reward-facilitating effects of these drugs (Yokel and Wise, 1975; Ettenberg et al., 1982). In 23 h rats, naloxone increased heroin intake, and the magnitude by which intake was increased was far greater than that observed in 1 h rats. In addition, naloxone also elevated postinjection reward thresholds in 23 h rats (i.e., precipitated withdrawal) above their already elevated levels. Interestingly, there was a nonsignificant trend for the magnitude by which naloxone elevated reward thresholds to progres-

sively increase over the four conditioning days. This observation likely reflects a progressive increase in the magnitude of the conditioned withdrawal effect with repeated cue-withdrawal pairings (Schultheis et al., 2004; Kenny and Markou, 2005). Most importantly, on the test day, withdrawal-associated cues elevated reward thresholds and provoked significant increases in heroin consumption in 23 h rats. Recently, we demonstrated that in unpaired opiate-dependent rats, cue presentation and saline administration had no effects on ICSS thresholds during conditioning sessions or on the test day (Kenny and Markou, 2005). Thus, the cue-induced elevations of ICSS thresholds observed in paired 23 h rats in the present study were most likely related to cue-withdrawal pairings and were not secondary to intrinsic actions of conditioning stimuli on ICSS thresholds in opiate-dependent rats per se. Thus, naloxone-paired conditioned stimuli attain negative affective valence in heroin-dependent 23 h rats and thereby induce a decrease in the sensitivity of brain reward systems mimicking that observed during unconditioned withdrawal. Moreover, conditioned reward deficits served as a source of motivation that triggered heroin consumption in 23 h rats. These data suggest that naloxone induced two distinct sources of motivation to consume heroin: in nondependent 1 h rats, heroin intake was increased to counter the antagonism of the pharmacological effects of heroin by naloxone. In dependent 23 h rats, heroin consumption may have been triggered to alleviate withdrawal-associated reward deficits. Importantly, only this second source of motivation was susceptible to classical conditioning. Together, these findings provide compelling evidence that conditioned stimuli predicting the onset of withdrawal-associated reward deficits motivate drug intake. These data are also consistent with previous observations demonstrating that withdrawal-associated cues produced an aversive conditioned withdrawal state in opiate-dependent rats (Wikler and Pescor, 1967; Kenny and Markou, 2005), nonhuman primates (Goldberg et al., 1969), and human opiate addicts (O'Brien et al., 1977), and with anecdotal reports that addicts experience withdrawal symptoms and drug craving when encountering drug-related environmental stimuli (Wikler, 1973).

The demonstration that previously neutral stimuli, when repeatedly paired with opiate withdrawal, can produce drug seeking in dependent but not nondependent rats, and that the state elicited by these conditioned stimuli is a negative emotional state as reflected in decreased reward function, is relevant to our current views on the etiology of addiction. Addiction has long been hypothesized to be a neuroadaptive process. This neuroadaptation was originally thought to be reflected in the development of physical drug dependence (Himmelsbach, 1943). However, this position fell out of favor with the discovery that classes of drugs such as psychomotor stimulants could serve as positive reinforcers without development of physical dependence, culminating in the subsequent focus on the role of positive reinforcing effects of drugs of abuse (Wise and Bozarth, 1987) and the sensitization of the neural mechanisms guiding positive reinforcement (Robinson and Berridge, 1993) in addiction. However, accumulating evidence suggests that there is indeed a “dark side” of addiction, in which increases in drug self-administration are motivated by negative emotional states (Koob and Le Moal, 2005). This view is supported by data demonstrating that all drugs of abuse, including the psychomotor stimulants, elicit deficits in brain reward sensitivity during withdrawal distinct from physical withdrawal signs (Markou and Koob, 1991; Schultheis et al., 1995; Epping-Jordan et al., 1998; Ahmed et al., 2002; Kenny et al., 2003b). The present study extends this conceptual framework by showing that

such negative emotional states can impart motivational power for drug seeking to previously neutral stimuli, and supports the hypothesis that craving for drugs is elicited not only by the memory of the pleasurable effects of drugs (de Wit and Stewart, 1981; Arroyo et al., 1998; Shaham et al., 2003), but also by the memory of the aversive effects of drug abstinence (Wikler, 1973).

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