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ORIGINAL INVESTIGATION

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Social stress is as effective as physical stress in reinstating morphine-induced place preference in mice

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Abstract *Rationale:* Relapse to drug-seeking in abstinent heroin addicts and reinstatement in experimental animals are observed when exposed to drug-associated stimuli or cues, the drug itself, and stressful events. It has been shown that footshock-induced stress increases the rewarding effects of opiates, delays extinction, and induces the reinstatement of drug-seeking. However, the effects of social stress on the reinstatement of opiate-seeking after extinction has not been studied. *Objectives:* The role of physical (restraint and tail pinch) and social (social defeat) stressors on the reinstatement of morphine-induced conditioned place preference (CPP) was evaluated. *Methods:* Adult male OF1 mice were conditioned with 10, 20, or 40 mg/kg of morphine or saline. Only morphine-conditioned animals acquired CPP. All mice underwent extinction sessions until the CPP was extinguished. Then, the effects of physical or social stress on the reinstatement of CPP were evaluated. Morphine- and saline-conditioned animals were exposed to the respective stressor or control stress condition immediately or 15 min before reinstatement tests. In experiment 1, animals underwent restraint for 15 min. In experiment 2, animals were exposed to tail pinch or placed in a cage without any manipulation for 15 min. In experiment 3, animals performed an agonistic encounter with an isolated or anosmic mouse or were placed in a cage without any social contact or manipulation. *Results:* Restraint, tail pinch, and social defeat in an agonistic encounter with an isolated mouse produce the reinstatement of CPP in morphine-conditioned animals. *Conclusions:* These data demonstrate that social stress is as effective as physical stress in reinstating morphine-seeking.

Keywords Morphine · Conditioned place preference · Reinstatement · Stress · Restraint · Tail pinch · Social defeat · Mice

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Introduction

Drug addiction can be considered as a chronic, recurrent brain disease characterized by relapse. The high rate of relapse to opioid use after detoxification is a major clinical problem and remains the primary challenge in treating drug abuse. Intense drug craving and relapse to drug-using behavior are seen in abstinent heroin addicts when, after many years of withdrawal, they are confronted with environmental stimuli previously associated with drug-taking behavior, the drug itself, or stress (de Wit 1996; O'Brien 1997). Drug craving is a subjective feeling experienced by human drug addicts that motivates them to seek drugs and can produce relapse (O'Brien 1997). It is very difficult to directly evaluate craving in laboratory animals, but it is possible to measure relapse directly if, after the acquisition and subsequent extinction of a particular behavioral response (for example, pressing a lever or developing a preference for a place), a laboratory animal reinitiates this response, which is often referred to as reinstatement (Carroll and Comer 1996). This recovery of the learned response seems to reflect the reinduction of craving, leading to drug-seeking following a period of extinction of drug use.

The animal model mainly used to study relapse to drug-seeking is the extinction-reinstatement model of the intravenous self-administration paradigm. In this proce-

dure, laboratory animals are trained to make a response, such as pressing a lever, to self-administer a drug, and after the extinction of that behavior, the ability of several stimuli to reinstate the response is determined. The paradigm of conditioned place preference (CPP) has also been used recently to study the relapse phenomenon in animals. In this procedure, animals are first trained to acquire a CPP, and afterwards, they undergo a process of extinction of this preference. It has been observed that the same stimuli that reinstate self-administration are capable of inducing the reinstatement of the CPP. The most important environmental events that may lead to reinstatement are re-exposure to the drug itself, presentation of drug-associated stimuli or cues, and exposure to a stressful event (for a review, see Shalev et al. 2002; Shaham et al. 2003; Weiss 2005).

Stress is known to increase the rewarding effects of opiates (Will et al. 1998, 2004; Der-Avakian et al. 2005) and has an established role in relapse (Sinha 2001; Lu et al. 2003). Using the self-administration paradigm, it has been reported that stress can be even more effective in reinstating drug-seeking behavior than re-exposure to drugs (Shaham et al. 1997a). A brief presentation of intermittent footshock reinstates heroin-seeking in rats (Shaham and Stewart 1995a,b; Shaham 1996; Shaham et al. 1998, 2000a,b; Shalev et al. 2000a,b, 2001a). Similarly, using the CPP reinstatement procedure, it was reported that intermittent footshock delays the extinction of morphine CPP (Lu et al. 2000), reinstates morphine-seeking after extinction (Der-Avakian et al. 2001; Wang et al. 2001), and reactivates morphine-seeking following drug-free periods in rats that were not exposed to extinction conditions (Lu et al. 2000; Wang et al. 2000–2002). Much less is known about the effect of stressors other than footshock on the reinstatement of opiate-seeking. Using the self-administration paradigm, it has been demonstrated that acute food deprivation (Shalev et al. 2000a, 2001b) and the administration of CRF (Shaham et al. 1997a), but not restraint stress (Shalev et al. 2000a,b), reinstate opioid-seeking in rats.

Psychological distress can be considered as a marker for the return to illicit drug use in abstinent subjects (Flynn et al. 2004). In humans, emotional stressors are primary activators of stress response, and subordination stress (social defeat) is an important factor that may lead to psychopathological changes (Bjorkqvist 2001). Thus, social defeat in rodents could be considered as a stressor with essential ethological relevance (Tornatzky and Miczek 1993), which more closely mimics real-life situations occurring in humans. After being defeated, mice show profound physiological and behavioral changes (de Groot et al. 1999; Lumley et al. 1999; Keeney et al. 2001; Griebel et al. 2002). Although it has been demonstrated that morphine-induced CPP is influenced by both the experience of defeat and the effects of defeat on social status, which suggests that social stress could attenuate the rewarding effects of morphine (Coventry et al. 1997), the effects of social defeat on the reinstatement of morphine-seeking have not been studied.

To our knowledge, the present work represents the first study aimed at investigating the effects of different types of stressors on the reinstatement of morphine-seeking in mice. For this purpose, the reinstatement model of CPP was used, in which animals are trained to associate a distinctive environment with the rewarding effects of morphine so that the drug-associated contextual stimuli acquire conditioned incentive properties, thus causing animals to spend more time in the morphine-paired environment, indicating the acquisition of a CPP. Then, animals undergo extinction sessions until the time spent in the drug-paired compartment decreases to the level before conditioning. The reinstatement of this previously extinguished CPP is evaluated after the respective experimental treatment (stress exposure). The specific objectives of this study were: (1) to evaluate the effects of two physical stressors, restraint and tail pinch, on the reinstatement of morphine-induced CPP; (2) to determine the effects of a social stressor (social defeat) on the reinstatement of morphine-induced CPP; and (3) to compare the effects of both physical and social stressors on the reinstatement of morphine-seeking.

Materials and methods

Subjects A total of 398 male mice of the OF1 strain that were 42 days of age (Charles River, Barcelona, Spain) were used. Of these mice, 334 were housed in groups of four in plastic cages (25 × 25 × 14.5 cm) for 10 days (308 animals used for CPP) or for 1 month (26 animals used as nonaggressive opponents in the test of social interaction) before experiments. Animals used for CPP were handled for 5 min a day (caressing the back of the mouse, grasping it by the tail or by the neck and turning it to imitate the movement performed when injecting) for 2 days before the preconditioning phase to reduce their stress levels in response to experimental manipulations. Moreover, they were housed in groups because isolation increases baseline stress and can alter the acquisition of a CPP. Sixty-four mice (used as aggressive opponents) were housed individually in plastic cages (23 × 13.5 × 13 cm) for a month before experiments to induce heightened aggression (Rodríguez-Arias et al. 1998). All animals lived in the vivarium under constant temperature (21±2°C), a reversed light schedule (white lights on: 1930–0730 hours), and food and water available ad libitum. Procedures involving mice and their care were conducted in conformity with national, regional, and local laws and regulations, which are in accordance with the European Communities Council Directives (86/609/EEC, 24 November 1986).

Apparatus For CPP, eight identical computerized (MONPRE 2Z software, CIBERTEC, SA, Spain) Plexiglas boxes with two equal size compartments (30.7 cm length × 31.5 cm width × 34.5 cm height) separated by a gray central area (13.8 cm length × 31.5 cm width × 34.5 cm height) were used. The compartments have different-colored walls (black vs white) and also distinct floor textures (fine grid in

the black compartment and wide grid in the white one). Four infrared light beams in each compartment and six in the central area allowed the recording of the position of the animal and its crossings from one compartment to the other.

Drugs Animals were injected intraperitoneally with morphine (Laboratorios Alcaliber, Madrid, Spain) in a volume of 0.01 ml/g. Control groups were injected with physiological saline (NaCl 0.9%), also used to dissolve the drugs.

Procedure of CPP

Acquisition The place conditioning, consisting of three phases, was carried out during the dark cycle following a procedure unbiased in terms of initial spontaneous preference (Manzanedo et al. 2001). During the first phase, or preconditioning (Pre-C), mice were given access to both compartments of the apparatus for 900 s a day for 3 days. On day 3, the time spent in each compartment was recorded. Ten animals showing strong unconditioned aversion (<33% of session time) or preference (>67%) for any compartment were discarded. In each group, half of the animals received the drug or vehicle in one compartment and the other half received it in the other one. After assigning the compartments, an ANOVA showed that there were no significant differences between time spent in the drug-paired and the vehicle-paired compartments during the Pre-C phase. In the second phase (conditioning), which lasted 4 days, 12 groups of animals were conditioned with 40 mg/kg of morphine (157 animals, n=8–13 per group), three groups with 20 mg/kg (36 animals, n=12), three groups with 10 mg/kg (36 animals, n=12), and six groups with physiological saline (48 animals, n=8). Animals conditioned with 40 mg/kg of morphine received an injection of saline before being confined to the vehicle-paired compartment for 1 h, and after an interval of 4 h, received morphine immediately before confinement in the drug-paired compartment for 1 h. Animals conditioned with 10 or 20 mg/kg of morphine only spent 30 min in each compartment after the respective injections. Control animals received saline before being confined for 1 h to both compartments. The central area was not used and was blocked by guillotine doors. During the third phase or postconditioning (Post-C), on day 8 the guillotine doors separating the two compartments were removed and the time spent by the untreated mice in each compartment was recorded for 900 s. The difference between the time spent in the drug-paired compartment in the Post-C and that spent in the Pre-C is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates aversion. The dose of 40 mg/kg was selected on the basis of previous studies that demonstrated that animals presented a more stable and robust CPP when they received a high dose of morphine (Ribeiro Do Couto et al. 2003, 2005). Moreover, the CPP induced with lower doses of morphine and a shorter exposure to the conditioning compartment was also evaluated. A different group of animals (n=12) was conditioned with 40 mg/kg of morphine and tested for

Table 1 Experimental groups used in each experiment according to the treatment during conditioning, the stress received, and the time of reinstatement test after stress

Groups	Treatment conditioning (n)	Type of stressor	Reinstatement test
Experiment 1	Saline (8)	Restraint	Immediate
	Saline (8)		Delayed
	Morphine 10 (12)		Immediate
	Morphine 20 (12)		Immediate
	Morphine 40 (8)		Immediate
	Morphine 40 (8)		Delayed
Experiment 2	Saline (8)	Tail pinch	Immediate
	Saline (8)	Tail pinch	Delayed
	Morphine 10 (12)	Tail pinch	Immediate
	Morphine 20 (12)	Tail pinch	Immediate
	Morphine 40 (12)	Tail pinch	Immediate
	Morphine 40 (12)	Tail pinch	Delayed
	Morphine 40 (8)	Non stress	Immediate
	Morphine 40 (8)	Non stress	Delayed
Experiment 3	Saline (8)	Social defeat	Immediate
	Saline (8)	Social defeat	Delayed
	Morphine 10 (12)	Social defeat	Immediate
	Morphine 20 (12)	Social defeat	Immediate
	Morphine 40 (12)	Social defeat	Immediate
	Morphine 40 (12)	Social defeat	Delayed
	Morphine 40 (8)	Agonistic interaction	Immediate
	Morphine 40 (8)	Agonistic interaction	Delayed
	Morphine 40 (8)	Non stress	Immediate
	Morphine 40 (8)	Non stress	Delayed

the presence of abstinence symptoms 24 h after the last administration (time of Post-C test). No significant withdrawal symptoms (paw tremor, jumping, and body tremor) were observed.

Extinction After the Post-C test, those animals conditioned with morphine that presented a weak CPP expression (<60 s) were discarded (n=5, 4, and 7 animals in experiments 1, 2 and 3, respectively; n=16). It was decided that the initial CPP had to be readily apparent in these groups to study acquisition, extinction, and reinstatement. Animals underwent a daily extinction session, which consisted of their placement in the apparatus for 900 s until the time spent in the drug-paired compartment was similar to that of Pre-C. Thus, in each group, all the animals received the same number of extinction sessions, independently of their individual scores, because the criterion of extinction was a lack of significant differences with respect to Pre-C values. Only one extinction session was performed on saline-conditioned groups to confirm the lack of CPP.

Reinstatement On the day following the last extinction session, the effects of the different stressors were evaluated. The tests of reinstatement were the same as for Post-C (free ambulation for 900 s), except that the animals were tested immediately or 15 min after the administration of the respective stress protocol. All the stress procedures were performed in the vivarium, and, when the reinstatement test was delayed, animals were returned to the home cages and waited for 15 min.

Experiment 1: effects of restraint on the reinstatement of morphine-induced CPP To evaluate the effects of acute immobilization stress, six groups of animals were used (see Table 1). On the Post-C day, only animals conditioned with morphine presented CPP. Subsequently, all groups underwent the extinction sessions (3–10 sessions), and 24 h after extinction, animals were submitted to immobilization-induced stress for 15 min. Restraint is a powerful stressor which is widely used in many studies (Patel et al. 2005; for a review see Lu et al. 2003). To induce restraint, when mice spontaneously passed into a cylindrical glass tube (4 cm in diameter and 10 cm in length, with holes 0.5 cm in diameter to permit respiration), two test tubes 0.5 cm in diameter were carefully introduced underneath the animal thus reducing the size of the tube to 3 cm so that it was impossible for the animal to turn. Immediately or 15 min after restraint, the reinstatement test was performed on the animal.

Experiment 2: effects of tail pinch on morphine-induced CPP Eight groups of animals were used in experiment 2 (see Table 1). On the Post-C day, only those animals treated with morphine during conditioning presented CPP. Subsequently, all groups underwent the extinction sessions (3–9 sessions), and 24 h after extinction, animals were submitted to a modified tail-pinch schedule to evaluate the effects of this physical/tactile stressor on reinstatement. Although the term "tail pinch" originally referred to a momentary application of pressure (Antelman et al. 1975), now this term refers to continuous pressure (Marinelli et al. 2004) that induces a stress response in rats (Brake et al. 2004; Marinelli et al. 2004). Animals received a single session of tail pinch, in which a plastic clothespin (making a pressure of 800 g) was fastened on the tail (at 1–1.5 cm from the body) of each animal for 15 min, during which time it was placed in a transparent plastic cage (23 × 13.5 × 13 cm). Mice occasionally attempted to remove the clothespin but without success. No tissue damage was observed. Immediately or 15 min after tail pinch, the reinstatement test was performed on the animals. With the aim of demonstrating the lack of effects of the procedure itself, two additional groups underwent the same procedure, but without tail pinch.

Experiment 3: effects of an agonistic encounter on the reinstatement of morphine-induced CPP To study the effects of an agonistic encounter with a different result for the experimental mouse (victory or defeat) on the reinstatement of morphine CPP, ten groups of animals were used (see Table 1). On the Post-C day, only animals treated with morphine during conditioning presented CPP. Subsequently, all groups underwent the extinction sessions (3–10 sessions), and 24 h after extinction, animals underwent an agonistic encounter for 15 min in a neutral transparent plastic cage (23 × 13.5 × 13 cm). To evaluate the effects on reinstatement of defeat in a social interaction, which can be considered a type of social stress (Ginsburg and Allee 1942; Koolhaas et al. 1997; Keeney et al. 2001; Griebel et al. 2002; Yap et al. 2005), the mice underwent an agonistic encounter with an aggressive opponent (of equal age and body weight), which had previous fighting experience and had been previously screened for a high level of aggression. Experimental mice presented avoidance/flee and defensive/submissive behaviors after suffering aggression (threat and attack) from an opponent. The criterion used to define an animal as defeated was the assumption of a specific posture of defeat, as characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al. 1982; Rodríguez-Arias et al. 1998). All defeated mice experienced similar levels of aggression because attack behavior from the opponent initiated immediately after seeing the experimental mouse (latency<30 s). Immediately or 15 min after social defeat, the reinstatement test was performed on the animals. With the aim of demonstrating the lack of effects of the procedure itself, two additional groups underwent an agonistic encounter in which the experimental animals were not defeated, because the opponents were grouped animals which were made temporarily anosmic by intranasal lavage with 4% zinc sulfate solution 1 day before testing (Smoothy et al. 1986); these animals elicit attack but never initiate it, and no aggressive behaviors were observed. Moreover, to demonstrate the lack of effects of exposure to the neutral cage used for the agonistic encounter without an opponent mouse, another two groups were used.

Assessment of corticosterone concentrations Seven groups of animals (n=7) were conditioned with morphine and, after extinction, were submitted to restraint (two groups), tail pinch (two groups), social defeat (two groups), or no stress (control group). Animals belonging to one group of each stressor and to the control group were decapitated immediately after stress, while animals belonging to the other groups were decapitated 30 min after stress. Two time points were chosen because it has been previously demonstrated in rats that plasma corticosterone immediately after stress is not a reflection of the actual intensity of the stressor and of ACTH release due to the saturation of the adrenocortical synthesis, unless the poststress period is also studied, in which case more severe stressors are characterized by a slower return to basal hormone levels (García et al. 2000; Márquez et al. 2002). A blood sample of each animal was collected and centrifuged at 1,000×g for 15 min at 4°C, obtaining the aliquots which were maintained in carbonic ice until the measurement of corticosterone in the sample by radioimmunoassay (RIA). Corticosterone RIA used 125I-carboximethyloxime-tyrosine-methyl ester (ICN-Biolink 2000, Barcelona, Spain), synthetic corticosterone (Sigma) as the standard and an antibody raised in rabbits against corticosterone–carbox-

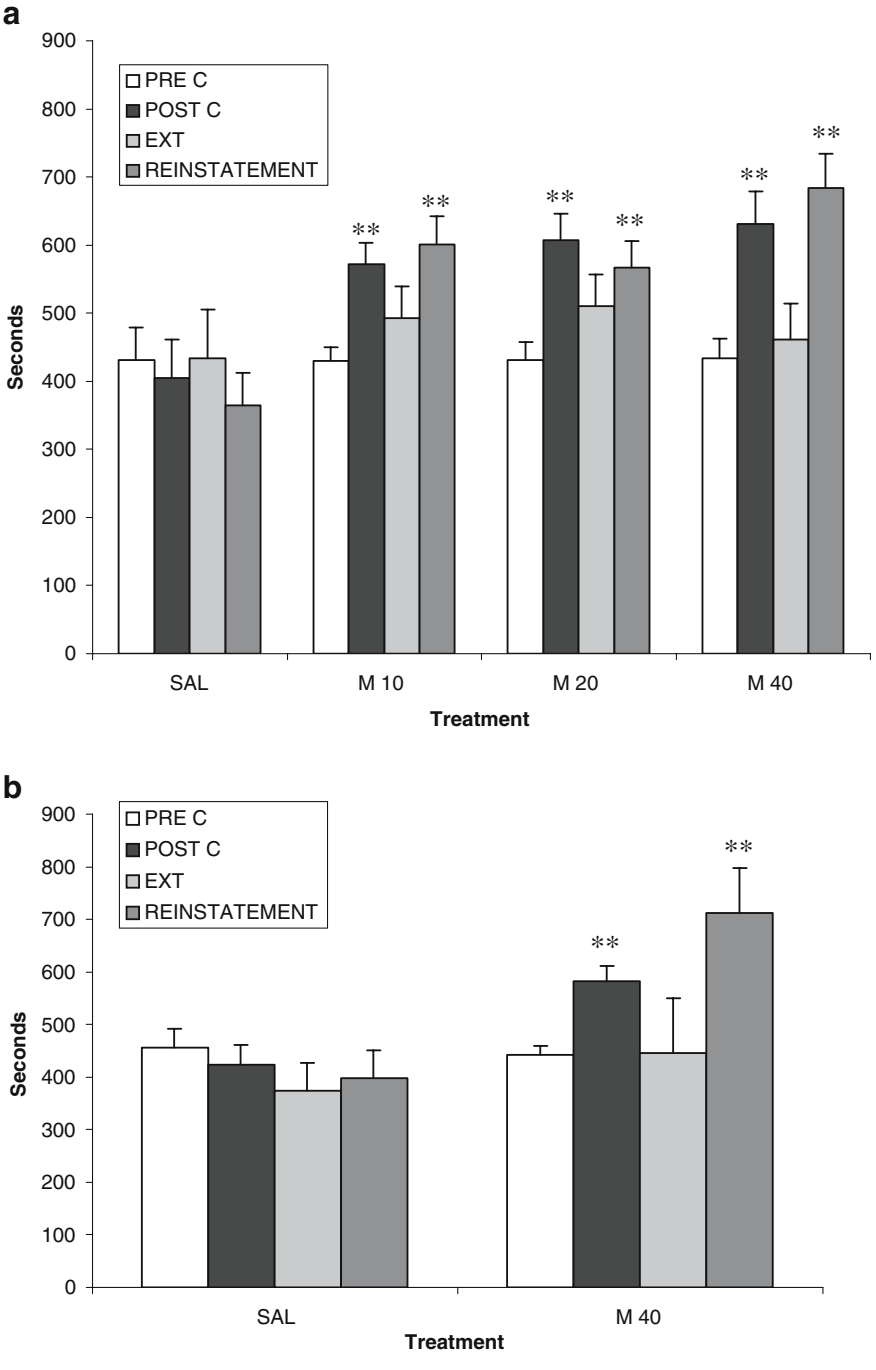

Fig. 1 Acquisition, extinction, and reinstatement of morphine-induced CPP after exposure to restraint-induced stress. *Bars* represent the mean (±SEM) time spent in the drug-paired compartment before conditioning sessions (*white bars*), after conditioning sessions (*black bars*), in the last extinction session (*light gray bars*), and in the reinstatement test (*dark gray bars*). **a** Immediate test reinstatement. The test of reinstatement was performed immediately after restraint in the following groups during conditioning: saline (*SAL*) and 10, 20, and 40 mg/kg morphine (*M10*, *M20*, and *M40*, respectively). **b** Delayed test reinstatement. The test of reinstatement was performed 15 min after restraint in the following groups during conditioning: saline (*SAL*) and 40 mg/kg morphine (*M40*). *Double asterisks* represent $p<0.01$, significant difference in the time spent in Pre-C vs Post-C sessions or reinstatement tests (Newman–Keuls post hoc comparison)

imethyloxime–bovine serum albumin, kindly provided by Dr. Makara (Institute of Experimental Medicine, Budapest, Hungary). The RIA protocol recommended by Bagdy and Makara (1995) was followed.

Statistical analysis In each experiment, the times spent in the drug-paired compartment during Pre- and Post-C, the last extinction session, and the reinstatement tests were analyzed with a mixed two-way ANOVA, with a between-subjects variable Treatment with six (experiment 1), eight (experiment 2), or ten (experiment 3) levels (groups of stressor procedure) and a within-subjects variable Days with four levels (Pre-C, Post-C, Extinction, and Reinstatement). Data of corticosterone measurement were analyzed with a one-way ANOVA, with a variable Treatment with seven levels (groups of stress). In all cases, post hoc comparisons were performed with Newman–Keuls tests.

Results

Experiment 1: effects of restraint on the reinstatement of morphine-induced CPP The results obtained in experiment 1 are represented in Fig. 1. The ANOVA revealed a significant effect of the variables Treatment [$F(5,47)=5.060$; $p<0.001$] and Days [$F(3,141)=12.474$; $p<0.0001$] and the interaction Treatment×Days [$F(15,141)=3.039$; $p<0.001$]. Post hoc comparisons showed a lack of differences between groups in Pre-C and Extinction, while in Post-C, the groups receiving morphine exhibited an increase in the time spent in the drug-paired compartment in comparison to those conditioned with saline ($p<0.01$). Similarly, in Reinstatement the groups treated with morphine and exposed to immobilization showed an increase in the time spent in the drug-paired compartment in comparison to those treated with saline and exposed to stress ($p<0.01$).

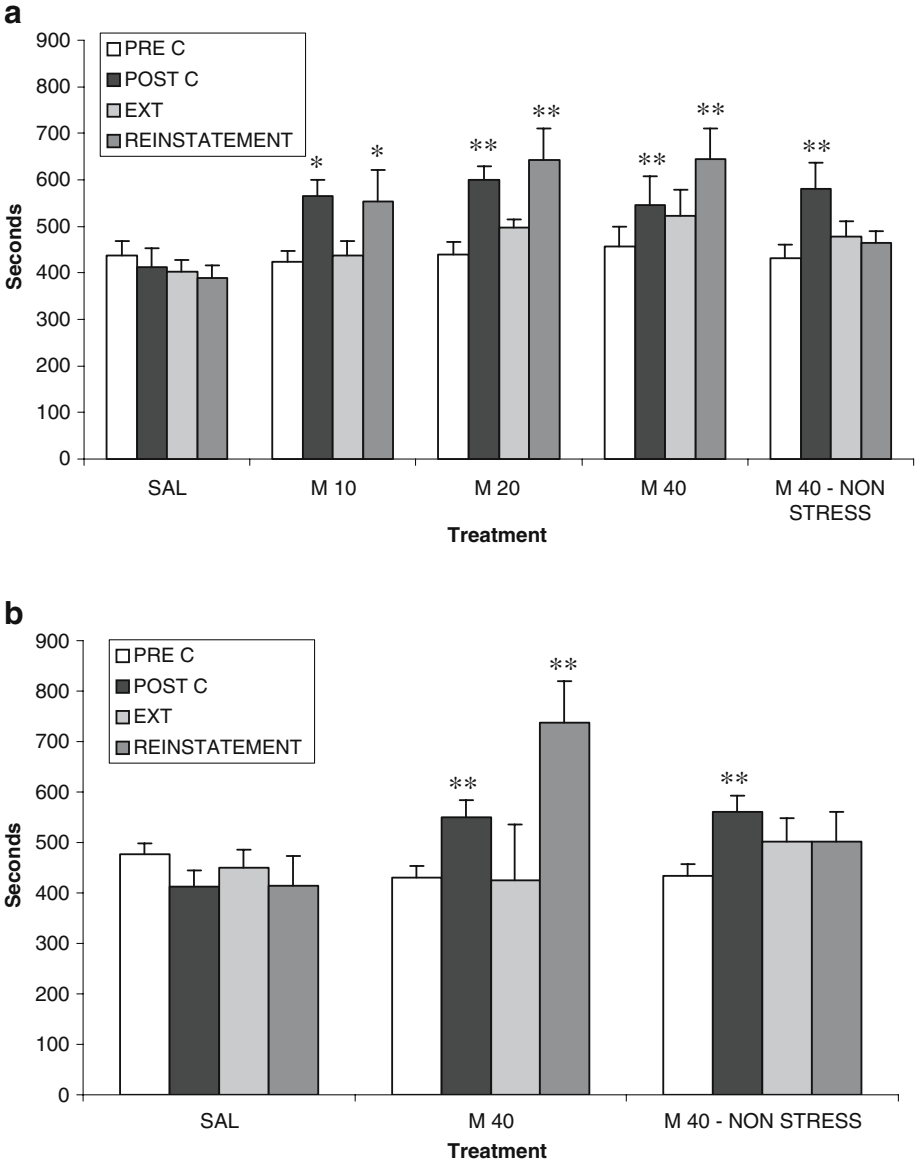

Fig. 2 Acquisition, extinction, and reinstatement of morphine-induced CPP after exposure to tail pinch-induced stress. *Bars* represent the mean (±SEM) time spent in the drug-paired compartment before conditioning sessions (*white bars*), after conditioning sessions (*black bars*), in the last extinction session (*light gray bars*), and in the reinstatement test (*dark gray bars*). **a** Immediate test reinstatement. The test of reinstatement was performed immediately after tail pinch in the following groups during conditioning: saline (*SAL*) and 10, 20, and 40 mg/kg morphine (*M10*, *M20*, and *M40*). An additional group was conditioned with 40 mg/kg of morphine and exposed to the cage without tail pinch immediately before reinstatement test (*M40non stress*). **b** Delayed test reinstatement. The test of reinstatement was performed 15 min after tail pinch in the following groups during conditioning: saline (*SAL*) and 40 mg/kg morphine (*M40*). An additional group was conditioned with 40 mg/kg of morphine and exposed to the cage without tail pinch 15 min before reinstatement test (*M40 non stress*). *Double asterisks* represent $p<0.01$ and *asteriks* represent $p<0.05$; significant difference in the time spent in Pre-C vs Post-C session or reinstatement tests (Newman–Keuls post hoc comparison)

Experiment 2: effects of tail pinch on the reinstatement of morphine-induced CPP The ANOVA revealed a significant effect of the variables Treatment [$F(7,66)=2.339$; $p<0.03$] and Days [$F(3,198)=14.153$; $p<0.0001$] and the interaction Treatment×Days [$F(21,198)=3.188$; $p<0.0001$]. Post hoc comparisons revealed a lack of difference between groups in Pre-C and Extinction, while in Post-C the groups receiving morphine exhibited an increase in the time spent in the drug-paired compartment in comparison to those conditioned with saline ($p<0.05$). In Reinstatement, the groups treated with 20 or 40 mg/kg of morphine and exposed to tail pinch showed an increase in the time spent in the drug-paired compartment in comparison to those treated with saline and exposed to stress ($p<0.01$) (Fig. 2).

Experiment 3: effects of an agonistic encounter on the reinstatement of morphine-induced CPP The results obtained in experiment 3 are represented in Figs. 3 and 4. The ANOVA revealed a significant effect of the variables Treatment [$F(9,89)=2.509$; $p<0.01$] and Days [$F(3,267)=17.067$; $p<0.0001$] and the interaction Treatment×Days [$F(27,267)=2.417$; $p<0.001$]. Post hoc comparisons showed a lack of difference between groups in Pre-C and Extinction, while in Post-C the groups receiving morphine spent more time in the drug-paired compartment than those conditioned with saline ($p<0.05$). In Reinstatement, the groups conditioned with 40 mg/kg of morphine that had been defeated exhibited an increase in the time spent in the drug-paired compartment with respect to the groups conditioned with saline or conditioned with morphine but without social defeat ($p<0.05$). The groups conditioned with 10 and 20 mg/kg of morphine that had been defeated also exhibited an increase in the time spent in the drug-paired compartment with respect to those conditioned with saline ($p<0.05$).

Corticosterone concentrations (see Table 2) The ANOVA revealed that the variable Treatment was significant [$F(6,43)=27.813$; $p<0.0001$]. All the stressed groups presented higher corticosterone levels than the control

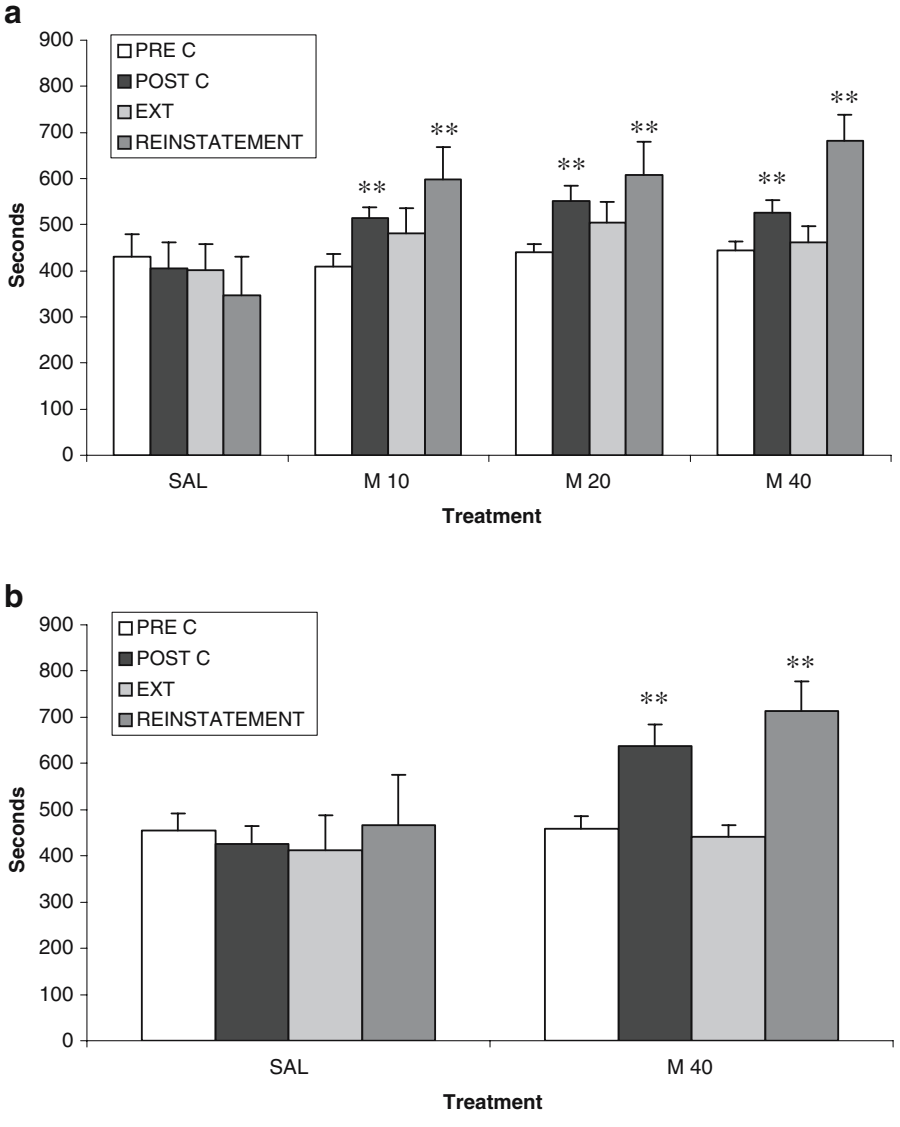

Fig. 3 Acquisition, extinction, and reinstatement of morphine-induced CPP after exposure to social defeat. *Bars* represent the mean (±SEM) time spent in the drug-paired compartment before conditioning sessions (*white bars*), after conditioning sessions (*black bars*), in the last extinction session (*light gray bars*), and in the reinstatement test (*dark gray bars*). **a** Immediate test reinstatement. The test of reinstatement was performed immediately after defeat in the following groups during conditioning: saline (*SAL*), 10, 20, and 40 mg/kg morphine (*M10*, *M20*, and *M40*). **b** Delayed test reinstatement. The test of reinstatement was performed 15 min after defeat in the following groups during conditioning: saline (*SAL*) and 40 mg/kg morphine (*M40*). *Double asterisks* represent $p<0.01$; significant difference in the time spent in Pre-C vs Post-C session or reinstatement tests (Newman–Keuls post hoc comparison)

group ($p<0.01$). Moreover, animals exposed to social defeat presented higher levels of corticosterone than animals exposed to restraint ($p<0.05$) or tail pinch ($p<0.01$) when evaluated 30 min after the finalization of stress exposure.

Discussion

The present work demonstrates that exposure to social stress is as effective as exposure to physical stress in inducing the reinstatement of a previously extinguished morphine-induced CPP. The administration of morphine induces CPP that can be extinguished by daily exposure to the apparatus of place conditioning without any treatment. Physical stressors such as restraint or tail pinch administered immediately or 15 min before reinstatement tests are capable of reinstating CPP after extinction. Similarly, psychological stress, such as defeat in a social interaction, suffered immediately or 15 min before reinstatement tests produces a renovation of the previously extinguished morphine-induced CPP.

With respect to the effects of exposure to physical stressors on reinstatement, the results obtained in the present study using restraint and tail pinch as stressors are generally in agreement with those reported previously using another physical stressor, such as footshock. It has been observed that a brief presentation of intermittent footshock reinstates drug-seeking in rats previously trained to self-administer heroin (Shaham and Stewart 1995a,b; Shaham 1996; Shaham et al. 1998, 2000a,b; Shalev et al. 2000a,b, 2001a) and in rats with an extinguished morphine-induced CPP (Lu et al. 2000; Der-Avakian et al. 2001; Wang et al. 2000–2002). This footshock stress-

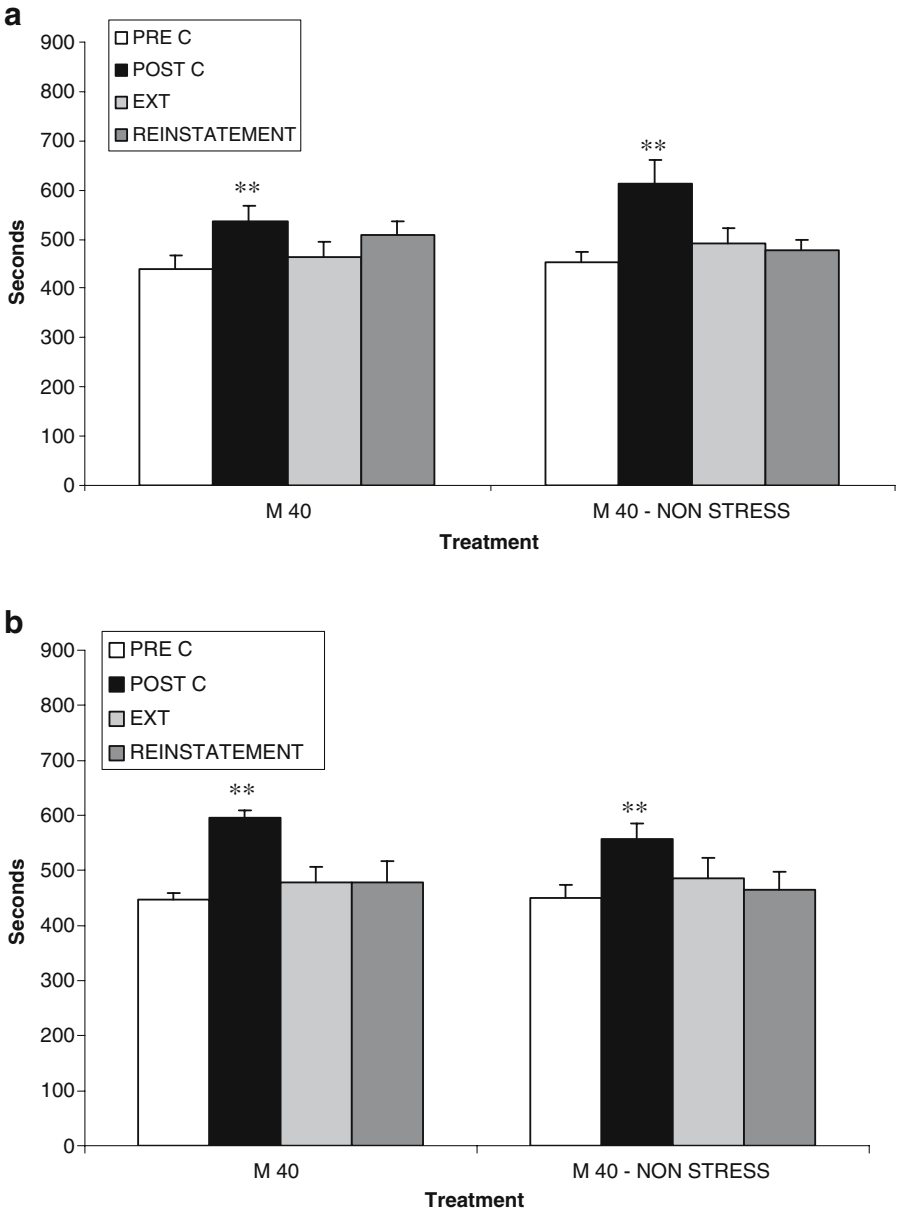

Fig. 4 Acquisition, extinction, and lack of reinstatement of morphine-induced CPP after exposure to a nonaggressive social interaction. *Bars* represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment before conditioning sessions (*white bars*), after conditioning sessions (*black bars*), in the last extinction session (*light gray bars*), and in the reinstatement test (*dark gray bars*). **a** Immediate test reinstatement. The test of reinstatement was performed immediately after the social encounter in the following groups during conditioning: saline (*SAL*) and 40 mg/kg morphine (*M40*). An additional group was conditioned with 40 mg/kg of morphine and exposed to the cage without an opponent immediately before the reinstatement test (*M40 non stress*). **b** Delayed test reinstatement. The test of reinstatement was performed 15 min after the social encounter in the following groups during conditioning: saline (*SAL*) and 40 mg/kg morphine (*M40*). An additional group was conditioned with 40 mg/kg of morphine and exposed to the cage without an opponent 15 min before the reinstatement test (*M40 non stress*). *Double asterisks* represent $p<0.01$, significant difference in the time spent in Pre-C vs Post-C session (Newman–Keuls post hoc comparison)

Table 2 Corticosterone levels after stress exposure

Groups	Treatment conditioning	Type of stressor	Obtention of blood sample	Mean (±SEM)
Control	Morphine 40	Non stress	Immediate	0.18 (0.04)
Restraint 0	Morphine 40	Restraint	Immediate	19.89 (1.38)
Restraint 30	Morphine 40	Restraint	Delayed	7.81 (1.18)
Tail pinch 0	Morphine 40	Tail pinch	Immediate	17.05 (2.11)
Tail pinch 30	Morphine 40	Tail pinch	Delayed	6.09 (1.09)
Defeat 0	Morphine 40	Defeat	Immediate	18.54 (1.46)
Defeat 30	Morphine 40	Defeat	Delayed	12.83 (1.47)

Groups of treatment: All groups were conditioned with 40 mg/kg of morphine and underwent extinction sessions. Control represents animals without stress, Restraint 0 and 30 represent animals exposed to restraint for 15 min and killed immediately or 30 min after the finalization of restraint to obtain blood sample, Tail pinch 0 and 30 represent animals exposed to tail pinch for 15 min and killed immediately or 30 min after the finalization of stressor to obtain blood sample, Defeat 0 and 30 represent animals exposed to social defeat in an agonistic encounter with an aggressive opponent for 15 min and killed immediately or 30 min after the finalization of the agonistic encounter to obtain blood sample. Mean (±SEM) of corticosterone level in μg/100 ml

induced reactivation of morphine CPP is attenuated by nonselective and selective corticotrophin-releasing factor type 1 (CRF1) antagonists (Lu et al. 2000), by clonidine infusion into the bed nucleus of the stria terminalis (Wang et al. 2001), and by lesions of the central nucleus of the amygdala (Wang et al. 2002) and the ventral noradrenergic bundle (Wang et al. 2001). On the other hand, one study using the self-administration paradigm has observed that footshock and restraint stress has no effect on the reinstatement of heroin-seeking in rats (Shalev et al. 2000a,b). The authors stated that these stressors are ineffective because they were given outside the self-administration environment and suggested that, in heroin-trained rats, the effects of stress on reinstatement are context- and time-dependent (Shalev et al. 2000a,b). Our results do not support this hypothesis because restraint and tail pinch were administered in a different environment from place conditioning and with two temporal intervals before the test (0 and 15 min), but they produced a clear reinstatement of morphine-induced CPP. The reinstating effects of restraint stress have also been observed using a cocaine-induced CPP (Sanchez et al. 2003). The results obtained in the present study using different physical stressors and mice as experimental subjects confirm and extend the idea that exposure to physical stress produces a reinstatement of opiate-seeking.

Because the most common stressors in humans are of a psychological or social nature (Bjorkqvist 2001), the use of a social conflict between members of the same species to induce stress supposes an advantage over animal models using aversive physical stimuli that are not relevant to situations that humans encounter in everyday life. Thus, the main finding of the present work is that exposure to emotional or social stress, such as defeat in a social interaction, is highly effective in reinstating morphine-seeking. An agonistic encounter with an anosmic nonaggressive mouse does not produce the reinstatement of CPP, which suggests that a social interaction without an experience of defeat does not produce stress and could be rewarding if experimental animals experience a victory. Similarly, it has been shown that the state of arousal induced by exposure to an appetitive stimulus (receptive female) has no effect on the reinstatement of heroin-seeking, indicating that arousal per se cannot account for the effect of stress on reinstatement (Shaham et al. 1997b). Neither does exposure to the neutral cage itself reinstate morphine-induced CPP, which is in agreement with other reports that exposure to a novel environment has no effect on the reinstatement of self-administration (Shalev et al. 2000a,b). Thus, only animals that experienced defeat in a social interaction with a conspecific presented a reinstatement of morphine-induced CPP, which suggests that social stress is as effective as physical stress in reinstating drug-seeking. Because social defeat stress induces the activation of the mesocorticolimbic dopamine (DA) system (Tidey and Miczek 1996) and increases the expression of μ-opioid receptor mRNA in the rat ventral tegmental area (VTA) (Nikulina et al. 1999), both systems could be involved in social stress-induced reinstatement.

Several mechanisms have been proposed to explain the effect of footshock stress on relapse to drug-seeking (Shalev et al. 2002) that could also explain why the exposure to physical or social stress reinstates morphine-induced CPP, as observed in the present study. Firstly, it has been suggested that activation of the mesocorticolimbic DA system by stressors underlies their effect on reinstatement (Shaham and Stewart 1995a,b), and an increase in the extracellular levels of DA in the nucleus accumbens and medial prefrontal cortex has been observed in animals exposed to different stressors such as footshock (Sorg and Kalivas 1991), tail-shock (Abercrombie et al. 1989), and social defeat (Tidey and Miczek 1996). However, the fact that footshock induces less DA release than drug priming (Shaham and Stewart 1996) but can be a more effective stimulus for reinstatement (Shaham 1996), and the pharmacological and anatomical dissociation between footshock- and drug-induced reinstatement (Shaham et al. 2000a), run against this hypothesis. Secondly, it has been hypothesized that stressors induce a withdrawal-like state that leads to relapse (Whitehead 1974). However, experimental results do not support this idea because withdrawal does not reinstate heroin-seeking and footshock can reinstate drug-seeking in animals receiving a maintenance dose of heroin (Shaham and Stewart 1995a,b; Shaham et al.

1996). One recent study demonstrates that only morphine-conditioned withdrawal reinstates morphine CPP, which may be a stress-driven effect (Lu et al. 2005). Thirdly, it has also been suggested that stress may provoke relapse by interfering with neuronal inhibitory processes, which inhibits responding when reinforcers are not available (Highfield et al. 2000). In support of this idea, it has been observed that footshock stress increases resistance to extinction, and footshock's reinstating effects depend on the manipulation of the medial septum, a brain area involved in response inhibition (Highfield et al. 2000). Finally, it has been hypothesized that repeated exposure to drug might sensitize or induce neuroadaptations in brain systems involved in the stress response. However, changes in sensitivity in brain systems involved in footshock stress-induced opioid reinstatement in response to the stressor are yet to be reported. A recent study, however, demonstrated a cocaine-induced neuroadaptation specific to stress-induced reinstatement: footshock stress increased the CRF levels in the VTA, and blockade of CRF receptors in this area attenuated the stress-induced reinstatement of cocaine-seeking (Wang et al. 2005). Most of the behavioral functions for neuroadaptations (increased expression of the brain-derived neurotrophic factor, recruitment of mitogen-activated protein kinase signal transduction, increases in glutamate receptors, etc.) have been demonstrated for cocaine-induced reinstatement, although these molecular changes are promising targets to explore with respect to a more general role in relapse (Weiss 2005).

The phenomenon of reinstatement of learned behaviors after extinction was firstly described by Pavlov in his classical conditioning studies with dogs (Pavlov 1927). The two main models used to study reinstatement, self-administration and CPP, evaluate different aspects of drug addiction. Self-administration involves an operant conditioning, models drug-taking behavior, and evaluates the primary rewarding properties of drugs, while CPP involves a Pavlovian conditioning, models cue-elicited conditioning that motivates drug-taking behavior, and assesses the incentive value of drug-associated cues (secondary reinforcing effects of drugs) to maintain addictive behaviors and the memory of this learning. The recovery of CPP after extinction is a classical conditioning model of reinstatement and this renovation of the approach behavior to the drug-associated context could be seen as an occasion for drug-taking behavior to be engendered (Bardo and Bevins 2000). It is well known that environmental cues associated with drug experience play a critical role in maintaining drug-taking behavior and in relapse after extinction. The main limitations of this model focus on the facts that the drug is given by the experimenter and that, generally, total drug exposure is low. Moreover, because this model does not evaluate the primary reinforcing effects of drugs, its relevance to compulsive and chronic drug use is limited. In future studies, it would be necessary to complement the results given here by using the self-administration paradigm to more clearly demonstrate that social defeat-stress produces the reinstatement of drug-seeking.

In conclusion, the present study demonstrates that exposure to social stress produces the same effects as physical (restraint and tail pinch) stressors on the reinstatement of morphine-induced CPP. Mechanisms involved in these effects remain to be elucidated in future studies.

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