ORIGINAL INVESTIGATION

Effect of baclofen on morphine-induced conditioned place preference, extinction, and stress-induced reinstatement in chronically stressed mice

Shanshan Meng · Wuxing Quan · Xu Qi · Zhiqiang Su · Shanshan Yang

Abstract

Rationale and Objective A stress-induced increase in excitability can result from a reduction in inhibitory neurotransmission. Modulation of gamma-aminobutyric acid (GABA)ergic transmission is an effective treatment for drug seeking and relapse. This study investigated whether baclofen, a $GABA_B$ receptor agonist, had an impact on morphine-induced conditioned place preference (CPP), extinction, and stress-induced relapse in chronically stressed mice.

Methods Chronic stress was induced by restraining mice for 2 h for seven consecutive days. We first investigated whether chronic stress influenced morphine-induced CPP, extinction, and stress-induced relapse in the stressed mice. Next, we investigated whether three different doses of baclofen influenced chronic stress as measured by the expression of morphine-induced CPP. We chose the most effective dose for subsequent extinction and reinstatement experiments. Reinstatement of morphine-induced CPP was induced by a 6-min forced swim stress. Locomotor activity was also measured for each test.

Results Chronic stress facilitated the expression of morphine-induced CPP and prolonged extinction time. Forced swim stress primed the reinstatement of morphine-induced CPP in mice. Baclofen treatment affected the impact of chronic stress on different phases of morphine-induced CPP.

Conclusions Our results showed that baclofen antagonized the effects of chronic stress on morphine-induced CPP. These findings suggest the potential clinical utility of $GABA_B$ receptor-positive modulators as an anti-addiction agent in people suffering from chronic stress.

Keywords Addiction · Morphine · Baclofen · Restraint stress · Forced swim stress · Dose · Schedule · Conditioned place preference · Reinstatement · Extinction

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Introduction

Drug addiction is a global public health concern with major economic consequences. While moderately effective treatments are available for some addictions, high relapse rates still occur. Human studies (Gawin and Kleber 1986; Wallace 1989) and experiments with animal models (Barsy et al. 2011; Grakalic et al. 2006; Kreibich et al. 2009) have identified stress as a critical factor in the drug addiction process, including triggering relapse. Extensive literature has attributed actions in the limbic system that in turn influence motivational, emotional, and affective behaviors, as reinforcing the effects of drug abuse (Koob 1992; Martin et al. 2008; Nielsen et al. 2012; Rezayof et al. 2002). All of these findings suggest that treatment aimed at reducing stress could be therapeutically effective for drug addiction.

Electrophysiological experiments indicate that stress or high levels of corticosteroids induce activation of the amygdala, increasing the excitability of principal neurons (Duvarci and Pare 2007; Roozendaal et al. 2009). This increase in excitability can also be the result of stress-induced reduction in inhibitory neurotransmission, which has been reported in the amygdala (Davis et al. 1994; Feng et al. 2013) and in the hypothalamus (Verkuyl et al. 2005). In

addition, gamma-aminobutyric acid (GABA) is the dominating inhibitory neurotransmitter in the central nervous system (CNS), and modulation of GABAergic transmission was effective against drug seeking and relapse (de Guglielmo et al. 2012; Fattore et al. 2009; Kemppainen et al. 2012; Pan et al. 2012; Parker et al. 2011). Recent studies have focused on the role of $GABA_B$ receptors in regulating abuse of drugs such as alcohol (Leggio et al. 2012), nicotine (Fattore et al. 2009), morphine (Heinrichs et al. 2010), and cocaine (Halbout et al. 2011).

Baclofen is a lipophilic analog of GABA with high affinity for the bicuculline-insensitive $GABA_B$ receptor (Bowery 1989). Imaging studies indicate that baclofen blunts the limbic activation associated with visual drug cues in drug-addicted humans (Brebner et al. 2002). Baclofen also inhibited the expression of many psychostimulant-induced behaviors in rodents including conditioned place preference (CPP) (Li et al. 2001) and self-administration (Brebner et al. 2005; Weerts et al. 2007). Meantime, it has been reported that stress can induce opiate and psychostimulant reinforcement (Kabbaj et al. 2001; Kreibich et al. 2009; Rozeske et al. 2012). However, little is known about whether baclofen blocks drug addiction facilitated by stress.

This study examined if baclofen, a $GABA_B$ receptor agonist, pharmacologically inhibited morphine-induced CPP and stress-induced relapse in chronically stressed mice. We used a CPP and extinction/reinstatement model of morphine-seeking behavior in mice conditioned to morphine. As baclofen can have muscle relaxant and sedative effects, it was tested at doses known to affect operant response but not spontaneous locomotor activity (Fattore et al. 2002; Spano et al. 2007; Tabaeizadeh et al. 2013).

Methods and materials

Animals

Three hundred male adult BALB/c mice aged 5–8 weeks were obtained from the Laboratory Animal Center of the Fourth Military Medical University (FMMU). The animals were housed in plastic boxes in groups of four with food and water available ad libitum in a colony room with controlled temperature (24±2 °C), humidity (50–60 %), and a 12/12-h light/dark cycle. Mice were allowed to adapt to laboratory conditions for at least 1 week before the procedure. Experiments were carried out during the light phase of the cycle. At the beginning of each experiment, there were 10 new mice in each group, and the use of animals was reviewed and approved by the Institutional Ethical Committee of the FMMU. In experiment 1, two mice from the stressed and

non-stressed groups treated with morphine were excluded after post-conditioning (Post-C) 8d because of failure to express morphine preference, and one mice from non-stressed group treated with morphine was excluded after Extinction 10d test because of extinction failure. In experiment 5, one mice from the non-stressed group treated with morphine was excluded after Post-C 7d, and one mice from stressed group treated with morphine was excluded after Extinction 10d test. The whole experiments complied with the current laws of China.

Drugs

(RS)-Baclofen (Tocris Cookson, Bristol, UK) and morphine (Shenyang, China) dosages, and the subcutaneous (s.c.) route of administration (Feng et al. 2011; Heinrichs et al. 2010) were selected based on previous studies showing efficacy in altering drug reward. Morphine (1 and 3 mg/kg) was used in experiment 2, and 10 mg/kg morphine was used in other experiments. Baclofen (BL) was administered in 0.612, 1.25, or 2.5 mg/kg (s.c.). All drugs were dissolved in a volume of saline corresponding to 0.1 ml/10 g body weight.

Chronic stress

Chronic stress was induced by restraining the mice in well-ventilated Perspex restraining tubes for 2 h on seven consecutive days, during which the animals were not physically compressed or experience pain (Chotiwat and Harris 2006). After restraint, mice returned to their home cages and given food and water ad libitum. Non-restrained controls were placed in a Perspex cage in the same experimental room and experienced a similar degree of daily handling during the restraining period.

Conditioned place preference apparatus and procedure

CPP was performed in a CPP apparatus with a computer equipped with an automonitoring system (Jiliang, Shanghai, China). It consists of two compartments, identical in size (30×30×40 cm each), separated by a platform with the sides painted white and black, respectively. General experimental protocol was adapted from previous studies, with some modifications. The whole protocol had six different phases: pretreatment, pre-conditioning test, conditioning, post-conditioning test, extinction, and reinstatement test; the total duration of each phase was disparate because of the different treatments, and the details were shown in Fig. 1. On each test day, the activity of each animal was recorded and measured to determine the time spent on each side of the experimental box when the guillotine doors were raised.
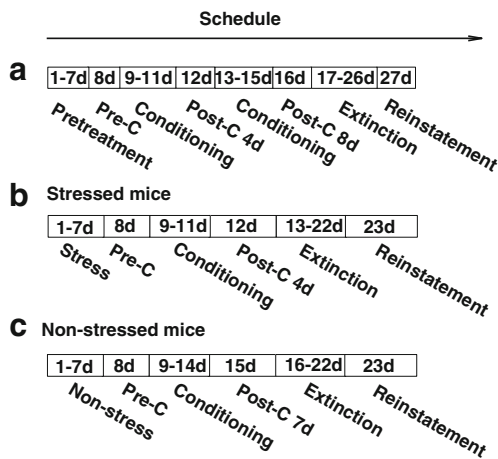
Fig. 1 Experimental design. **a** This schedule was used for experiments 1–2. On days 1–7, stressed mice underwent 2 h of restraint daily, and non-stressed mice experienced a similar degree of daily handling but were not restrained; then in experiment 1, all the mice were tested on days 8 (Pre-C), 12 (Post-C 4d), 16 (Post-C 8d), 17 (Extinction 1), 20 (Extinction 4), 23 (Extinction 7), and 24 (Reinstatement); in experiment 2, all the mice were tested on days 8 (Pre-C), 12 (Post-C 4d), and 16 (Post-C 8d). **b** This schedule was used for the stressed mice in experiments 3–5. Stressed mice underwent 2 h of restraint daily on days 1–7, and then in experiment 2 were tested on days 8 (Pre-C) and 12 (Post-C 4d). In experiment 3, stressed mice were tested on days 8 (Pre-C), 12 (Post-C 4d), and 16 (Extinction 4). In experiment 4, stressed mice were tested on days 8 (Pre-C), 12 (Post-C 4d), 22 (Extinction 10), and 23 (Reinstatement). **c** This schedule was used for the non-stressed mice in experiment 4. After 7 days handling, mice were tested on days 8 (Pre-C), 15 (Post-C 7d), 22 (Extinction 7), and 23 (Reinstatement). During all test periods, doors were raised, and mice were allowed to explore compartments. Time spent on each side was measured. *C*, conditioning; *d*, day

Pre-conditioning

After receiving the chronic stress, control and restraint mice were placed in one of the two compartments and allowed access to explore both environments for 15 min individually. Time spent in each compartment was automatically measured by the PC software and the initial preference or aversion for one side of the two compartments was determined. Animals spending more than 10 min in a specific compartment were excluded from the study. All mice showed a moderate preference for the black compartment (501.6±14.3 s, $P<0.05$ vs. the time spent in the white side, Student's *t* test).

Conditioning

During this period, the doors were closed. Animals received two s.c. injections per day of either morphine (morning, starting at 8:00 a.m.) or saline (afternoon, starting at 2:00 p.m.) before placed in the white or black boxes for 30 min. Then, sessions started immediately. Mice in the control group were injected with saline twice per day and placed in either a black or a white compartment. After each

session, the subjects returned to their own home cages. In the experiment 3, each mouse in baclofen-treated groups received an additional injection with baclofen (s.c.) 30 min before morphine treatment.

Post-conditioning

At 24 h after the last morphine treatment, doors were opened so that animals had access to both compartments for 15 min. The time spent in each compartment was measured. Only animals displaying robust morphine CPP (i.e., an increase in preference for the drug-paired compartment ≥100 s compared to the pre-conditioning test) were used in the subsequent phase of the study.

Extinction

At 24 h after the post-conditioning test, mice were given extinction training daily. The procedure was similar to the pre-conditioning test. The amount of time that mice spent in each chamber was determined. Only animals meeting the criterion of not more than ±20 % of time spent in the drug-paired compartment during the extinction phase compared to the pre-conditioning test was used for the next phase. In experiment 4, each mouse in the baclofen-treated group received a single injection with baclofen (s.c.) immediately after extinction training.

Stress-induced reinstatement

After extinction, animals were divided into saline-treated and baclofen-treated groups. All mice underwent forced swim stress (FS) as described (Briand et al. 2010). Briefly, mice were placed for 6 min in plastic cylinders (23 cm height×14 cm diameter) containing 23–25 °C water that were 10 cm deep. Following FS, mice were returned to their home cages 20 min prior to the reinstatement test. Drug or saline was injected 30 min before FS.

Locomotor activity

On each test day, the total distance traveled by each mouse in 15 min was defined as locomotor activity and determined in the same apparatus as the CPP procedures. Total distance traveled in both sides of the apparatus was analyzed using the automonitoring system.

Statistical analysis

All data were expressed as means±standard error of the mean (SEM). Statistical significance was determined by repeated measures ANOVA with post hoc multiple comparisons using the Newman–Keuls method. Student's *t* test and

one-way ANOVA were used if necessary. In all cases, $P < 0.05$ was considered statistically significant. Calculations were performed using the SPSS 16.0 statistical package.

Results

Experiment 1: effects of stress on the expression, extinction, and reinstatement of morphine-induced conditioned place preference

We first determined whether chronic stress from 2 h of daily restraint for 7 days affected the expression, extinction, and reinstatement of 10 mg/kg morphine-induced CPP. A repeated measures ANOVA [between-subjects factors: stress condition (stress, non-stress), drug (morphine, saline); within-subjects factor: conditioning day (pre-conditioning (Pre-C), Post-C 4d, Post-C 8d)] was used. The analysis revealed a significant effect of stress condition [$F(1, 36) = 4.98$, $P < 0.05$], drug [$F(1, 36) = 21.43$, $P < 0.01$], and interaction of stress condition, drug, and conditioning day [$F(2, 72) = 32.54$, $P < 0.01$]. A Newman–Keuls test showed that only the stressed mice treated with morphine spent significantly more time on the drug-paired side after three pairing sessions ($P < 0.01$, Fig. 2a). These results suggested that after three pairing sessions only the stressed mice acquired morphine-induced CPP. Further test found that the non-stressed mice also expressed morphine-induced CPP after three more pairing sessions ($P < 0.01$, Fig. 2a). These results indicated that the failure on the first test day in these mice was due to a lacking of pairing time. These data demonstrated that chronic stress facilitated the expression of morphine-induced CPP.

Next, the effect of chronic stress on the extinction of morphine-induced CPP was investigated. Time spent on the morphine-paired side showed significant differences between extinction tests (Extinction 1d, 4d, 7d, 10d), compared to Pre-C test [repeated measures ANOVA, $F(1, 34) = 9.61$, $P < 0.01$, and significant effects of test, $F(4, 136) = 3.25$, $P < 0.05$; stress condition, $F(1, 34) = 4.53$, $P < 0.05$; drug, $F(1, 34) = 4.71$, $P < 0.05$; and interaction of stress condition, drug, and test, $F(4, 136) = 3.05$, $P < 0.05$; Fig. 2a]. The Newman–Keuls test revealed that stressed mice spent significantly greater amounts of time in the morphine-paired chamber at extinction day 7 compared to Pre-C test. This did not happen in the non-stressed mice or in stressed mice conditioned with saline ($P < 0.05$, Fig. 2a). Moreover, similar to the mice in other groups, no significant CPP was induced in the stressed mice conditioned with morphine after 10 extinction trainings. These data suggested that chronic stress prolonged the extinction time of morphine-induced CPP in mice.

It is unknown whether acute FS induced the reinstatement of stress-facilitated morphine-induced CPP in mice. A repeated measures ANOVA [between-subjects factors: stress condition (stress, non-stress), drug (morphine, saline); within-subjects factor: test (extinction 10d, reinstatement)] analysis showed significant effects of drug [$F(1, 32) = 13.21$, $P < 0.01$], and test [$F(1, 32) = 9.65$, $P < 0.01$], and a statistical interaction of drug and test, but no effect of stress condition. Further, Newman–Keuls test found that both stressed and non-stressed mice treated with morphine spent significantly more time in the drug-paired side after primed with 6-min FS, compared to mice in the Extinction 10d test ($P < 0.01$, Fig. 2a). This result was not seen for saline-treated mice.

Locomotor activity was also measured. The repeated measures ANOVA [between-subjects factors: stress
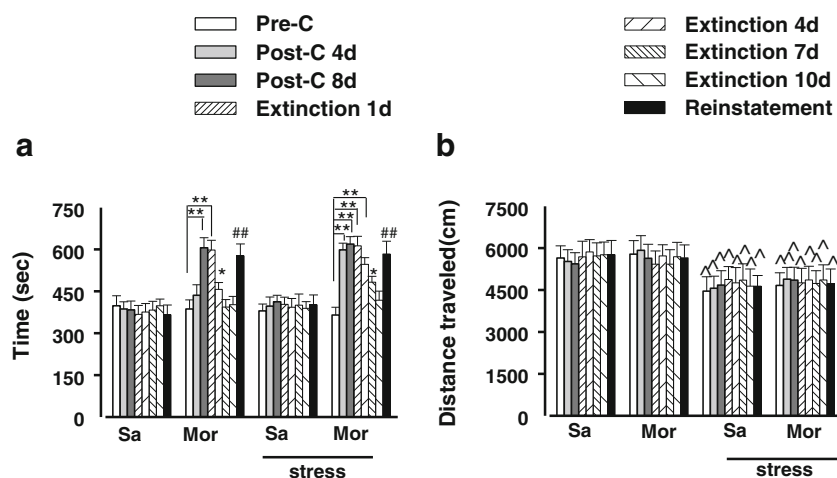

Fig. 2 Effect of stress on expression, extinction, and reinstatement of morphine-induced conditioned place preference. Animals were conditioned with 10 mg/kg morphine, and all groups were tested on the schedule described in Fig. 1. On reinstatement day, mice underwent 6 min of forced-swim stress and were returned to home cages for 20 min before testing.

Data are means±SEM and are as follows: **a** time in seconds spent in the drug-paired compartment; **b** distance traveled in millimeters during the test period. $N = 10$ mice per group. *$P < 0.05$, **$P < 0.01$ vs. pre-conditioning test (Pre-C). ##$P < 0.01$ vs. the last extinction test. ^$P < 0.05$ vs. the corresponding test in non-stressed mice. *Sa*, saline; *Mor*, morphine

condition (stress, non-stress), drug (morphine, saline); within-subjects factor: test (all the test days)] analysis revealed a significant effects of stress condition [$F(1, 32)=33.21$, $P<0.01$], but no effect of drug and test. Newman–Keuls test showed that no groups showed significant differences in distance traveled in the two chambers during any test period (Fig. 2b). However, a significant decrease of the distance traveled was found in the stressed mice, compared with the non-stressed mice ($P<0.05$). These results suggested that chronic stress reduced the locomotor activity of the mice.

Experiment 2: 1 and 3 mg/kg morphine induced morphine preference in the stressed mice after three pairing sessions

To further confirm the effect of chronic stress on expression of morphine preference, two lower doses of morphine, 1 and 3 mg/kg, were used. A repeated measures ANOVA [between-subjects factors: stress condition (stress, non-stress), drug (saline, 1 mg/kg morphine, 3 mg/kg morphine); within-subjects factor: conditioning day (Pre-C, Post-C 4d, Post-C 8d)] revealed a significant effect of stress condition [$F(1, 54)=5.78$, $P<0.05$], drug [$F(2, 54)=12.34$, $P<0.01$], and interaction of stress condition, drug, and conditioning day [$F(2, 108)=21.56$, $P<0.01$]. A Newman–Keuls test showed only the stressed mice conditioned with 1 mg/kg, and 3 mg/kg morphine spent significantly more time on the drug-paired side after three pairing sessions (1 mg/kg morphine, $P<0.05$; 3 mg/kg morphine, $P<0.01$, Fig. 3a). After seven pairing sessions, more place preference also was found in the non-stressed mice conditioned with 3 mg/kg morphine ($P<0.01$, Fig. 3a). All of these data suggested that chronic stress enhanced the sensitivity to morphine reward in mice.

Moreover, a significant effect of stress condition on locomotor activity was found [$F(1, 54)=6.51$, $P<0.05$], but no effect of drug and test. Newman–Keuls test showed a significant decrease of the distance traveled in the stressed mice, compared with the non-stressed mice (Fig. 3b).

Experiment 3: baclofen blocked morphine-induced CPP facilitated by chronic stress

In the following experiments, 10 mg/kg morphine was used because of the more preference in drug-paired compartment after three conditioning (1 mg/kg, 489.2±23.6 s; 3 mg/kg, 545.8±30.5 s; 10 mg/kg, 598±23.5 s). Since chronic stress accelerated the expression of morphine-induced CPP, we then determined whether BL treatment could inhibit this effect. Three different doses of BL (0.612, 1.25, and 2.5 mg/kg) were chosen based on the previous study (Fattore et al. 2009). After chronic stress for 1 week, the mice were trained as the CPP procedure. As shown in Fig. 4a, mice that received morphine for 3 days spent more time in the white compartment, showing a significant CPP for morphine ($P<0.01$ vs. pre-conditioning).
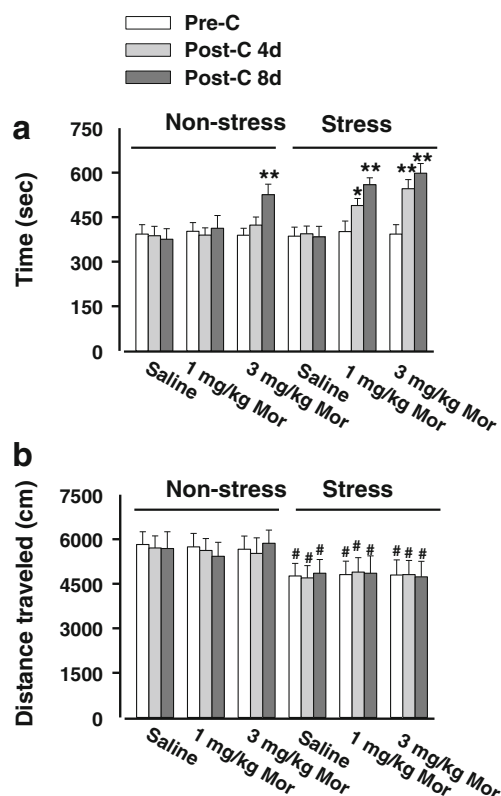

Fig. 3 Effect of two lower doses of morphine on conditioned place preference in the stressed mice. Morphine (1 and 3 mg/kg) was chosen here to induce morphine preference, all the mice were tested only on days 8 (Pre-C), 12 (Post-C 4d), and 16 (Post-C 8d) on the schedule of Fig. 1a. Data are means±SEM and are as follows: **a** time in seconds spent in the drug-paired compartment; **b** distance traveled in millimeters during the test period. $N=10$ mice per group. *$P<0.05$, **$P<0.01$ vs. pre-conditioning test (Pre-C). #$P<0.05$ vs. the corresponding test in non-stressed mice. *Mor*, morphine

A one-way ANOVA analysis [between-subjects factors: drug (saline, BL 0.612, BL 1.25, BL 2.5, morphine, morphine+BL 0.612, morphine+BL 1.25, morphine+BL 2.5)] revealed that time spent in the drug-paired side showed no significant difference in all the saline-treated groups after three conditioning sessions (Fig. 4a). However, in the morphine-treated groups, all three doses of BL, injected 30 min before morphine treatment, significantly decreased the time in the morphine-paired chamber [BL 0.612, $F(3, 36)=3.56$, $P<0.05$; BL 1.25, $F(3, 36)=7.26$, $P<0.01$; BL 2.5, $F(3, 36)=9.26$, $P<0.01$; $n=10$ in each group, Fig. 4a], compared to the mice treated only with morphine. Moreover, 1.25 and 2.5 mg/kg BL treatment significantly inhibited morphine-induce CPP, but 0.612 BL did not ($P<0.01$ vs. pre-conditioning). These data demonstrated that 1.25 and 2.5 mg/kg baclofen blocked morphine-induced CPP facilitated by chronic stress. BL (2.5 mg/kg) was chosen in the following experiments.

In addition, although chronic stress reduced the distance traveled by mice in the chambers (Fig. 2b), saline-paired, morphine-paired, or BL treatment did not show significant
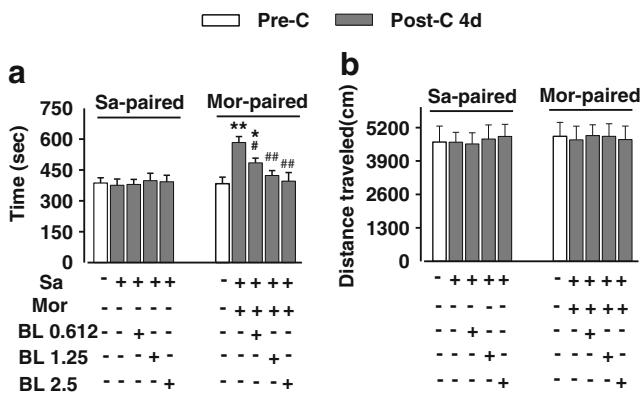
Fig. 4 Effects of baclofen on morphine-induced conditioned place preference. All mice underwent restraint stress for 1 week and were tested on days 8 and 12. Three doses of baclofen were used: 0.612, 1.25, and 2.5 mg/kg. +, mice treated with drug or vehicle; − not treated. Data are means±SEM and are as follows: **a** chronic stress as time in seconds spent in the drug-paired compartment; **b** locomotor activity as distance traveled in millimeters during the test period. N=10 mice per group. **$P<0.01$ vs. pre-conditioning test (Pre-C). #$P<0.05$, ##$P<0.01$ vs. group injected with morphine only. *BL*, baclofen; *Sa*, saline; *Mor*, morphine
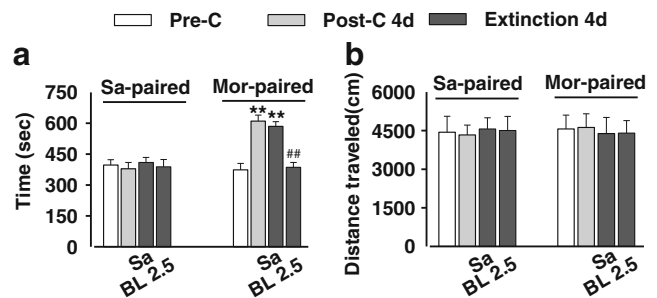

Fig. 5 Effects of baclofen on extinction of morphine-induced CPP and locomotor activity in stressed mice. After completing CPP training, extinction was from days 13 to 16. Baclofen was injected (2.5 mg/kg, s.c.) immediately after each extinction training session. Data are means±SEM and are as follows: **a** time (in seconds) spent in the drug-paired compartment; **b** distance traveled (in millimeters) during the test period. N=10 mice per group. **$P<0.01$ vs. pre-conditioning test (Pre-C). ##$P<0.01$ vs. post-conditioning test (Pre-C 4d). *BL*, baclofen; *Sa*, saline; *Mor*, morphine

difference in the distance (Fig. 4b), suggesting that the effect of BL was not due to a change in locomotor activity of mice after three conditioning sessions.

Experiment 4: baclofen enhanced extinction of morphine-induced CPP facilitated by chronic stress

In light of the above finding, chronic stress not only impacted the expression of morphine-induced CPP but also slowed down the extinction, and BL blocked the expression. Therefore, we wondered whether BL would facilitate the extinction of morphine-induced CPP. Mice that completed CPP training were administered with systemic saline or 2.5 mg/kg BL immediately after each extinction training session. The repeated measures ANOVA [between-subjects factors: morphine (morphine, saline), drug (BL, saline); within-subjects factor: test (Pre-C, Post-C 4d, Extinction 4d)] analysis revealed a significant effects of morphine [$F_{(1, 36)}=10.54$, $P<0.01$] and BL [$F_{(1, 36)}=4.17$, $P<0.05$]; the interaction of morphine and BL was also found [$F_{(1, 36)}=7.54$, $P<0.01$]. Newman–Keuls test revealed that mice receiving saline injection showed no chamber preferences. However, after three morphine conditioning sessions, mice expressed place preferences ($P<0.01$ vs. pre-conditioning, Fig. 5a). Importantly, in morphine-conditioned mice, the time spent in the drug-paired side was significantly different between the saline-treated and 2.5 mg/kg baclofen-treated groups after four extinction trainings ($P<0.01$, Fig. 5a). No morphine preference was seen in the baclofen-treated group. These data suggested that 2.5 mg/kg baclofen promoted the extinction of morphine-induced CPP facilitated by chronic stress. Similar to previous

experiments, 2.5 mg/kg baclofen did not affect the distance traveled by mice (Fig. 5b).

Experiment 5: baclofen prevents stress-induced reinstatement of morphine place preference in stressed and non-stressed mice

It has been reported that BL inhibited drug-induced reinstatement of extinguished nicotine-seeking behavior and nicotine place preference in rodents (Fattore et al. 2009). But it is unknown whether BL could prevent stress-induced reinstatement of morphine place preference. Based on the results of experiment 1, here the non-stressed mice received 6 morphine-pairings and 7 extinction trainings, and the stressed mice received 3 morphine-pairings and 10 extinction trainings. Then, 2.5 mg/kg BL were injected to the mice 30 min before the FS. Repeated measures ANOVA showed a significant effect of drug [$F_{(1, 37)}=7.85$, $P<0.01$] and test [$F_{(2, 74)}=5.32$, $P<0.01$] in non-stressed mice, and a significant effect of drug [$F_{(1, 37)}=8.02$, $P<0.01$] and test [$F_{(2, 74)}=6.21$, $P<0.01$] in stressed mice. Newman–Keuls test revealed that stressed mice which acquired morphine-induced CPP did not show chamber preferences after 10 extinction trainings ($P<0.01$, Fig. 6c), and non-stressed mice also did not after 7 extinction trainings ($P<0.01$, Fig. 6a). BL injection 30 min before FS significantly prevented the reinstatement of morphine place preference both in stressed (one-way ANOVA, $F_{(3, 36)}=6.74$, $P<0.01$; Newman–Keuls test, $P<0.01$, Fig. 6c) and non-stressed mice (one-way ANOVA, $F_{(3, 36)}=5.35$, $P<0.01$; Newman–Keuls test, $P<0.01$, Fig. 6a), but saline injection did not. Moreover, baclofen treatment did not reduce the distance traveled by mice (Fig. 6b, d), compared with saline injection. Acute FS also did not change distance traveled compared with the previous day.
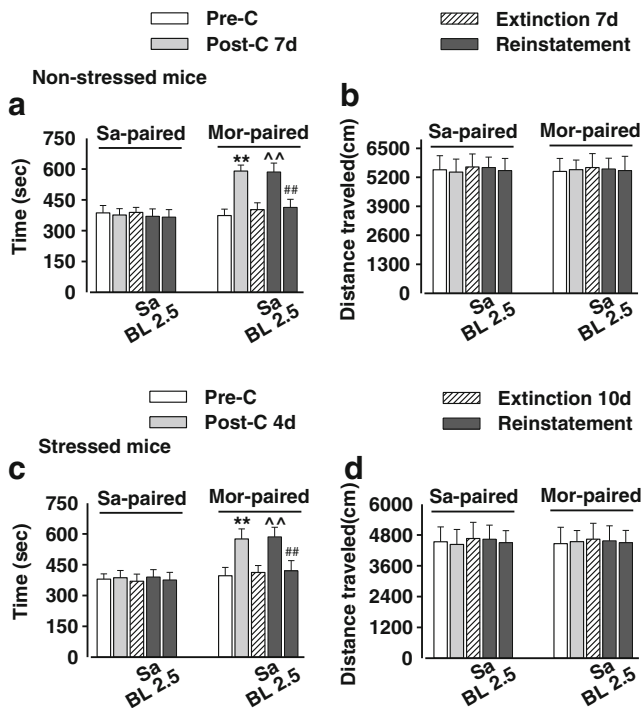
Fig. 6 Effects of baclofen on forced-swim-induced reinstatement of morphine place preference in stressed and non-stressed mice. **a**, **b** Non-stressed mice were tested on days 8, 16, 23, and 24 (non-stressed schedule). **c**, **d** Stressed mice were tested on days 8, 12, 22, and 23 (stressed schedule). Baclofen was injected (2.5 mg/kg, s.c.) 30 min before forced swim stress. Data are means±SEM and are as follows: **a**, **c** time (in seconds) spent in the drug-paired compartment; **b**, **d**. distance traveled (in millimeters) during the test period. $N=10$ mice per group. $**P<0.01$ vs. pre-conditioning test (Pre-C). $^{\wedge\wedge}P<0.01$ vs. the last extinction test. $^{\#\#}P<0.01$ vs. the group injected only with morphine. *BL*, baclofen; *Sa*, saline; *Mor*, morphine

Discussion

There has been considerable interest in potential interactions between exposure to stressors and addiction to drugs. In the present study, 2 h of restraint stress for seven consecutive days facilitated the expression of morphine-induced place preference and inhibited extinction. In addition, we also found 6-min forced swim stress-reinstated morphine-induced CPP in the stressed and non-stressed mice. Baclofen, a GABA$_B$ receptor agonist, effectively antagonized the impact of chronic stress on morphine addiction, but did not affect the locomotor activity. Our results suggested the clinical utility possibility of baclofen in maintaining morphine addicts in a drug-free state.

A great number of studies demonstrate that stress potentiates the reinforcement effects of drugs of abuse (Lu et al. 2003; Rozeske et al. 2011), but the key mechanisms are still little known. Some evidence supports increased activity in reward-related brain structures after chronic stress (Correll et al. 2005; Perrotti et al. 2004). Recently, it has been reported that chronic stress causes amygdala hyperexcitability in

rodents (Rosenkranz et al. 2010). GABA is the dominating inhibitory neurotransmitter in the CNS, and modulation of GABAergic transmission has been demonstrated to be effective on affecting drug seeking and relapse (de Guglielmo et al. 2012; Fattore et al. 2009; Kemppainen et al. 2012; Pan et al. 2012; Parker et al. 2011). All of these imply that augmentation of GABAergic transmission can attenuate the hyperexcitability caused by chronic stress, further reverse the vulnerability to drug addiction.

GABA$_B$ receptors have been identified in the CNS, where they provided important inhibitory control of post-synaptic excitability and presynaptic transmitter release. Post-synaptically located GABA$_B$ receptors activated potassium conductances, thereby hyperpolarizing neurons (Gahwiler and Brown 1985; Kaupmann et al. 1998), while pre-synaptically located GABA$_B$ receptors inhibited transmitter release by inhibiting activation of voltage-gated calcium channels (Mintz and Bean 1993; Scholz and Miller 1991). In other words, baclofen treatment can enhance the inhibitory transmission in the CNS, which may be the underlying mechanism that baclofen injection after the chronic stress blocked the facilitation of morphine-induced CPP by chronic stress according to our findings.

The morphine-induced preference in the stressed mice observed in the current study was highly resistant to extinction; repeated re-exposure to the chambers over seven extinction cycles did not extinguish the morphine-induced preference in stressed mice, although it did affect non-stressed mice. We proposed that this likely reflected the increased rewarding effects of morphine and the strength of the learned association between morphine-reward and the morphine-paired context after stress. Baclofen administration immediately after chamber re-exposure inhibited the preference for the morphine-paired chamber after seven extinction cycles in the stressed mice. Motor activity was not significantly altered by repeated baclofen treatment. These findings indicated that augmenting GABA$_B$ receptor signaling facilitated the extinction of morphine-induced CPP in the stressed mice.

Previous studies support memory acquisition and extinction can be altered by GABAergic drugs. For example, baclofen enhanced extinction of opiate and methamphetamine-induced CPP (Heinrichs et al. 2010; Voigt et al. 2011). Systemic baclofen enhanced the rate and extent of extinction of conditioned passive avoidance in mice which were demonstrated learned helplessness (Dubrovina and Zinov'ev 2008). Chronic stress and re-exposure to cues associated with morphine increased neuronal activity which may reflect hyperresponsive glutamatergic neurotransmission (Bell et al. 2000; Hotsenpiller et al. 2001; Rosenkranz et al. 2010). GABA$_B$ receptors inhibited glutamatergic neurotransmission (Lei and McBain 2003; Porter and Nieves 2004). Thus, GABA$_B$ receptor activation is a possible mechanism by which baclofen may promote the extinction of morphine-induced CPP.

Recently, GABA receptors were investigated for possible involvement in the resumption of drug use after a drug-free period. A growing body of studies show the efficacy of baclofen in reducing reinstatement of cocaine-seeking induced by either a drug priming (Campbell et al. 1999; Weerts et al. 2007) or a drug-associated cue (Filip and Frankowska 2007), for example, drug-induced reinstatement of extinguished nicotine-seeking behavior and nicotine place preference in rodents (Fattore et al. 2009), as well as cue-induced reinstatement of alcohol-seeking behavior in alcohol-preferring lines of rats (Maccioni et al. 2008). For opiates, our results were consistent with Spano et al. (2007) who reported that the GABA$_B$ receptor agonist baclofen prevented heroin-induced reinstatement of heroin-seeking behavior in rats.

Both in the stressed and non-stressed mice, baclofen blocked the effect of forced swim stress on reinstatement of morphine-induced CPP. This finding suggested that the inhibitory effect of baclofen on reinstatement was due to influence on acute stress. Some evidence suggests that corticotropin-releasing factor (CRF) was highly co-expressed with GABAergic neurons in the amygdala (Day et al. 1999; Veinante et al. 1997). CRF has received extensive attention as a mediator in maintaining addicted state and constitutes a prototype for critical important neuropeptide for addictive processes (Heilig and Koob 2007; Koob and Volkow 2010; Koob and Zorrilla 2010) and has been implicated in neuroendocrine and behavioral responses to stress (Britton et al. 1982; Koob and Bloom 1985). CRF was activated during stress-induced drug reinstatement, where it facilitated relapse and increases anxiety during acute and chronic withdrawal (Koob 1999; Shaham et al. 1995). In addition, stress-induced reinstatement of drug-related responding in animal models appeared to be dependent on the activation of both CRF and norepinephrine in elements of the extended amygdala (Shaham et al. 2000), and significantly more CRF mRNA was found in the stress-related brain regions in rats after exposed to 30 min of restraint (Babb et al. 2013). Baclofen treatment before the acute stress enhanced GABAergic transmission in the amygdala at both pre-and post-synaptic sites, blunting the CRF-mediated effect, then prevented the reinstatement of morphine-induced CPP, which was similar to the mechanism of binge ethanol consumption (Lowery-Gionta et al. 2012).

Although it was reported that exposure to chronic restraint led to increased novelty-induced locomotor activity (Marin et al. 2007), and restraint stress induced depressive-like behavior in the forced swim test and memory impairment in the object recognition test, without altering locomotor activity (Budni et al. 2013), Kohler et al. found that chronic stress rat group showed a significant decrease in motor activity (Bowman et al. 2002; Kohler and Rauca 1992), which is consistent with our results. As of this writing, no clear explanation exists for these differences. However, it may be possible that the age (young vs. adult animals), species used (mouse vs. rat), and test procedures (open filed vs. CPP) contribute to these differences. Reduced locomotor activity was also observed in rats after chronic forced swim stress alone, which induced depressive-like behavior.

Some evidence showed that baclofen treatment reduced morphine maintenance response of self-administration (SA) in rats (Ramshini et al. 2013; Yoon et al. 2007), and chronic immobilization stress induced enhancement of opioid SA (Shaham 1993). However, there are few studies about the effect of baclofen on stress-induced reinforcement of morphine SA, which should get more attention.

Taken together, our results suggest a powerful effect of baclofen on blocking the facilitated expression of morphine-induced CPP by chronic stress, promoting the extinction, and preventing stress-induced reinstatement of morphine preference. Our findings could have implications for the development of addiction therapies. Looking to the future, further studies are required to investigate the molecular mechanisms underlying the effect of baclofen, in order to find more positive allosteric modulators of GABA$_B$ receptors, which are able to selectively modify the GABA$_B$ receptor function without side effects.

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