Title:

Acute stress worsens the deficits in appetitive behaviors for social and sexual stimuli displayed by rats after long-term withdrawal from morphine

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Conflict of interest

Yunjing Bai, David Belin, Xigeng Zheng, Zhengkui Liu and Yue Zhang have no conflict of interest to declare.

Abstract

Rationale: Negative affective states, e.g. anhedonia, are suggested to be involved in the long-lasting motivational processes associated with relapse. Here we investigated whether anhedonic behaviors could be elicited by an acute stress in rats after protracted abstinence from morphine. **Objectives**: The behavioral responses to natural stimuli following exposure to an acute stress were examined after 14-days of withdrawal from morphine. Male rats were pretreated with either a binge-like morphine regimen or daily saline injections for 5 days. The motivation for two natural stimuli, i.e., a social stimulus (male rat) and a sexual stimulus (estrous female rat), was measured, following exposure to an acute stress (intermittent foot shock, 0.5 mA*0.5 seconds*10min; mean intershock interval: 40 seconds), under three conditions: free approach, effort- and conflict-based approach. **Results**: Foot-shock-induced stress did not influence free-approach behavior (sniffing time) towards the social or sexual stimulus. However in the effort-based approach task, the stressed morphine-withdrawn rats demonstrated an attenuated motivation to climb over a partition to approach the social stimulus while the stressed saline pre-treated rats showed an increased motivation to approach the social stimulus. When an aversive stimulus (pins) was introduced in order to induce an approach-avoidance conflict, both drug-withdrawn and drug-naïve groups exhibited a bi-modal distribution of approach behavior towards the sexual stimulus after the stress was introduced, i.e., the majority of rats had low risky appetitive behaviors but a minority of them showed rather highly "risky" approach behavior. **Conclusions**: The acute stress induces differential motivational deficits for social and sexual rewards in protracted drug-abstinent rats.

Keywords

stress, anhedonia, protracted morphine withdrawal, sexual reward, social reward, appetitive behavior

Introduction

The high rate of relapse to drug use after abstinence is a major clinical challenge in addiction (Brandon, Vidrine, & Litvin, 2007; Brownell, Marlatt, Lichtenstein, & Wilson, 1986). A wealth of evidence indicates that withdrawal from repeated exposure to drugs of abuse, such as heroin, leads to a negative affective state including anhedonia, dysphoria, irritability and anxiety (Brady, Verduin, & Tolliver, 2007; Khantzian, 1997; Koob & Le Moal, 2001). This emotional state is hypothesized to contribute to the compulsive maintenance of drug intake and relapse to drug use via the construct of negative reinforcement (Koob, 2008, 2009).

Anhedonia, which is defined as diminished pleasure or interest in responding to natural rewarding stimuli (APA, 2015), is a core feature of reward deficits (Der-Avakian & Markou, 2012) frequently reported during drug withdrawal (Bovasso, 2001; Gawin & Kleber, 1986; Hatzigiakoumis, Martinotti, Giannantonio, & Janiri, 2011; Leventhal, et al., 2008). Clinical studies have revealed that subjective reports of anhedonia correlate with drug craving in detoxified alcohol- (Martinotti, Cloninger, & Janiri, 2008; Martinotti, Nicola, et al., 2008) and opioid-dependent users (Janiri, et al., 2005; Martinotti, Cloninger, et al., 2008) while anhedonia also increases the risk of relapse in tobacco smokers (Cook, Spring, McChargue, & Doran, 2010; Leventhal, Waters, Kahler, Ray, & Sussman, 2009). Overall these observations suggest that the alteration in motivational processes observed in drug addicts under withdrawal, e.g. anhedonia, may contribute to the chronicity of the disorder by facilitating relapse.

Anhedonia, a core symptom of depression and other neuropsychiatric disorders (APA, 2015), encompasses not only the loss of the capacity to experience pleasure, but also deficits in other discrete reward-related processes interpreted as a loss of interest including reward evaluation, and blunted cost-benefit analysis as well as the loss of the ability to initiate and maintain effort-related behaviors (Der-Avakian & Markou, 2012). Similarly, impaired motivated behaviors directed towards natural rewards following drug withdrawal can take on a complex and multifaceted aspect (Bai, Li, Lv, Liu, & Zheng, 2014). One of our previous studies suggests that the anhedonia-like behaviors, as identified as deficits to engage with natural rewards (sucrose, social and sexual rewards) can be revealed, only after short-term morphine withdrawal, when more than one reward magnitude or appetitive behavioral measure is used (Bai, et al., 2014).

Clinical studies have demonstrated that although subjective anhedonic states diminish over time after the onset of detoxification from opiates or alcohol (Janiri, et al., 2005; Martinotti, Nicola, et al., 2008), anhedonia is maintained for long periods of time (Janiri, et al., 2005; Martinotti, Nicola, et al., 2008; Pozzi, et al., 2008). In contrast, in preclinical studies, anhedonia-like behaviors, including decreased intake of or instrumental responses for sucrose/sweet pellet (Barr & Phillips, 1999; Hellemans, Shaham, & Olmstead, 2002; Lieblich, Yirmiya, & Liebeskind 1991; Zhang, et al., 2007) and attenuated motivation to sexual rewarding stimulus (Barr, Fiorino, & Phillips, 1999; Nocjar & Panksepp, 2007), have often been reported after acute or short-, but not long-term withdrawal (Fiorino & Phillips, 1999; Nocjar & Panksepp, 2002, 2007).

Stress, which is an important factor contributing to drug craving in addicts and reinstatement of an extinguished instrumental response for drugs in animals (Sinha, Shaham, & Heilig, 2011), might be an important factor accounting for this inconsistency between clinical and preclinical studies. Indeed, while exposure to stress is tightly controlled in experimental animals, clinical patients face stressors on a daily basis and continue to report a shift in affective processing when encountering stressful events long after cessation of drug use (Heilig, Egli, Crabbe, & Becker, 2010). Thus, while both preclinical

and clinical studies have documented that sensitivity to stress is increased in drug dependent individuals over the course of protracted abstinence (Heilig, et al., 2010), the nature of the interaction between stress and withdrawal-induced motivational deficits after protracted abstinence from opiates remain poorly understood. Thus we investigated the influence of acute stress over motivational deficits in rats withdrawn from a binge-like morphine treatment paradigm, previously shown both to result in dependence persisting for a long time after the cessation of drug exposure (Nestler & Aghajanian, 1997), and to induce anhedonic behaviors after short-term withdrawal (Bai, et al., 2014).

For this, we measured, after protracted withdrawal, the influence of acute, shock-induced, stress, on the propensity of male Sprague Dawley rats to approach, and interact with, two natural rewards (social and sexual) of different incentive value, in three appetitive behavioral tasks with increasing cost/benefit ratio: simple, effort-based and conflict-based tests (Bai, et al., 2014).

Materials and methods

Animals and housing

Male (n = 170) and female Sprague-Dawley rats (n = 24) (Vital River Animal Center, Beijing, China) weighing 330-400g and 230-250g, respectively, at the beginning of the experiments were housed four per cage (50 × 22.5 × 30 cm) in colony rooms with a controlled temperature (22-26°C) and a reversed 12 h/12 h light/dark cycle (lights on at 21:00). Rats were allowed to habituate to the housing conditions for at least 5 days with food and water available *ad libitum*, and were gently handled daily for at least 3 days prior to the beginning of experiments. This study was approved by the International Review Board (IRB) of the Institute of Psychology, Chinese Academy of Sciences, and all experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No.85-23, revised 1985).

Drugs

Morphine hydrochloride (Qinghai Pharmaceutical Co. Ltd, Qinghai, China) was dissolved in sterile physiological saline at a final concentration of 20mg/ml.

Surgery

Females were bilaterally ovariectomized under 1% pentobarbital sodium (55 mg/kg, i.p.) anesthesia at least 2 weeks before use. Artificial estrus was induced by subcutaneous treatment with estradiol benzoate (25 μg/rat) and progesterone (1 mg/rat) about 48–52 h and 4–6 h before tests, respectively. All tests were performed between 10:00 and 20:00 during the dark phase of the cycle.

Screening of male rats for mating performance

Male rats were screened for their mating performance before being challenged in the behavioral tests reinforced by a sexual stimulus. Screening was conducted under dim light during the dark phase of the light/dark cycle. Individual male rats were placed in a carton box (60 × 50 × 40 cm height) with pine wood shaving bedding and allowed a 5-min adaptation period. A receptive female rat was then introduced and male copulatory behaviors were monitored by experienced observers. The copulation on each day ended after the rat completed its first ejaculation within 30 min. The male rats that passed the screening (those that performed ejaculation within 30 min for three consecutive days) were randomly assigned to the saline or the morphine treatment group. Ten male rats did not pass the screening and were discarded.

Binge-like morphine treatment

Male rats were pretreated twice daily for five days with intraperitoneal injections of either saline or

morphine delivered in a binge-like regimen (Bai, et al., 2014): 10, 20, 20, 40, 40, 40, 40, 40, 40, 40 mg/kg. The two doses of morphine administered on each day were at least 6 h apart. Rats were returned to their home cage immediately after each injection. All rats underwent a period of withdrawal of at least 14-days after the last saline or morphine administration.

Foot-shock stress

The paradigm of foot-shock stress used in the present study has been demonstrated previously to reinstate extinguished instrumental responding for drugs (Erb, Shaham, & Stewart, 1996; Shaham & Stewart, 1996). Foot-shocks were delivered prior to each sexual and social appetitive behavior test in four identical chambers (30.5 × 25.4 × 30.5 cm, Coulbourn Instruments, Allentown, PA, USA). Each chamber was constructed of aluminum (two side walls and ceiling) and Plexiglas (rear wall and hinged front door) and was situated in a sound-attenuating cabinet. The floor of the chamber consisted of 18 stainless-steel rods (6 mm diameter) spaced 1.5 cm apart, which were wired to a shock generator and solid-state grid scrambler (Coulbourn, H13-15) for foot shock delivery. A video camera was mounted on the ceiling of the chamber to videotape animals' behavior. On the day of behavioral testing, the rats were put into the chambers to habituate for 1 minute, and then the intermittent foot shock at variable intervals was introduced within 10 minutes (0.5 mA*0.5 seconds*10min; mean inter-shock interval: 40 seconds, range: 10-70seconds). After the stress procedure, the rats were immediately transferred to the chambers for appetitive behavior testing. The unstressed rats underwent the same procedure as the stressed rats, with the exception that the shock generators were turned off. The percent time of freezing behavior during the foot-shock stress period (within10 minutes) was quantified using automated motion-sensitive software (Coulbourn FreezeView software).

Appetitive behavior

The timeline of the experiment is described in **Fig 1.**

Apparatus

Four Open-field reward-proximity chambers made of black Plexiglas were used for assessment of the simple, effort- and conflict-based appetitive behaviors for social or sexual rewards (**Fig 2**). At one end of each of the open-field arenas (85 × 35 × 50 cm high) was mounted a wire-screen stimulus-cage (15 × 25 × 25 cm high), the front of which was made of wire mesh (1-mm wire, mesh size 10×10 mm) so that rats could approach and investigate (i.e., sniff), but not physically interact with the animals (male or estrous female rat) placed in it.

Approach-based test of motivation

Thirty-nine and forty male rats were used to test the influence of stress on free-approach behavior for social or sexual rewarding stimuli, respectively. For each reinforcer rats were divided into two treatment (vehicle vs. morphine) and stress groups (control vs. foot-shock) as follow: Saline-Control (n=10 and 12), Morphine-Control (n=9 and 9), Saline-Foot-shock (n=10 and 8), Morphine-Foot-shock (n=10 and 11) for social and sexual stimuli, respectively. On the day before appetitive behavior testing (withdrawal day 13), all rats were habituated for 15 min to the open-field arena. On the test day (withdrawal day 14), after the foot-shock stress was introduced, the rats were allowed to habituate for 10 min to the open-field arena. Then either a male, or a sexually receptive female, rat was placed in the stimulus-cage and the behavior of each test rat was videotaped for 3 hours by a camera and later analyzed using EthoVision software XT 7.1 (Noldus, The Netherlands). The open-field chamber and stimulus-cage were wiped clean with 0.1% acetic acid in water between each subject to eliminate olfactory cues. For each subject the time spent on sniffing the stimulus-cage (sniffing time, i.e.,

nose-point within the wire-screen) was automatically collected by the software as the index of social or sexual motivation.

Effort-based test of motivation

The same animals used in the approach-based test of motivation were also tested in the effort-based test with thirty-nine i.e., Saline-Control (n=10), Morphine-Control (n=9), Saline-Foot-shock (n=10), Morphine-Foot-shock (n=10), tested for a social reinforcer and forty, i.e. Saline-Control (n=12), Morphine-Control (n=9), Saline-Foot-shock (n=8), Morphine-Foot-shock (n=11) tested for the sexual reinforcer. On day 19 of withdrawal, after the foot-shock stress was introduced, all subjects were allowed to habituate for 10 min in the open-field arena equipped with a 6 cm-high, transparent, Plexiglas partition installed about 20 cm away from the wire-screen of the stimulus-cage. After this 10-min habituation period, a male or a sexually receptive female rat, which were systematically different from those used in the first test, was placed in the stimulus-cage together with some clean bedding (about 30 g) for the male rat or some female-soiled bedding previously collected from one cage that had contained three sexually receptive females during 5 days (females replaced every day) and stored in the freezer until the day of the experiment. The test rats were then given 5 min to freely approach and investigate the social or sexual reinforcer after which they were moved away from the reinforcer and the first trial of the test began with the addition of one 2 cm-high lath (transparent, Plexiglas) on the already existing 6 cm-high partition, i.e., 8 cm-high partition. Each of the twelve 4-min trials began with adding a 2 cm-high lath on the existing partition so that the heights of the partition from trial 1 to trial 12 were as follows: 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 30 cm. Each trial finished when the subject climbed over the partition to approach the reinforcer 3 times within 4 min, the subject being moved away from the stimulus-cage about 15-20 sec after climbing over the partition. If the subject climbed over the partition less than 3 times within 4 min, the test ended and the maximum height of the partition that the subject had climbed over to approach the stimulus cage was recorded as an index of motivation.

Conflict-based test of motivation

A separate cohort of male rats was used for the measure of the influence of stress on performance in a conflict-based test of motivation. Like in the other tests, vehicle or morphine-pretreated rats were randomly assigned to the control or stress group, e.g. Saline-Control (n=8 and 9), Morphine-Control (n=8 and 9), Saline-Foot-shock (n=8 and 9), Morphine-Foot-shock (n=8 and 9) for the social and sexual reinforcer, respectively. As for the other tests, on the day before testing (withdrawal day 16), all rats were habituated for 15 min in the open-field arena (without any obstacle). On the testing day (withdrawal day 17), after exposure to the foot-shock stress, rats were exposed for 10 min to the open-field arena prior to the introduction in the stimulus-cage of either a male or a sexually receptive female and associated bedding, as described previously. The test rats were then given 5 min to freely approach and investigate the social or sexual reinforcer after which they were moved away from the reinforcer and the first trial of the test began with the insertion of an obstacle on the floor of the open field, 20 cm away from the wire-screen of stimulus-cage. For the first trial the obstacle consisted of a 14 cm-wide thin board (3mm in thickness) thick with pins. With the test continuing, the obstacle became more and more difficult to surmount by means of replacing the board thick with pins in different styles and repeatedly heightening the board. According to the length of pins and average distance between pins, three types of board thick with pins were used: (a) Length - 0.5 cm, average distance - 1 cm; (b) Length - 0.8 cm, average distance - 0.5 cm; (c) Length - 2 cm, average distance - 1 cm. The board was repeatedly heightened as follows: 0, 2, 4, 7, 10, 13, 17, 21, 25, 29 cm. Thus, the

12-levels difficulties of surmounting the obstacle, i.e., 12 trials during the test were as follows: a + 0 cm, a + 2 cm, a + 4 cm, b + 4 cm, b + 7 cm, b + 10 cm, b + 13 cm, b + 17 cm, c + 17 cm, c + 21 cm, c + 25 cm, c + 29 cm. One trial was finished when the subject climbed or jumped over the obstacle 3 times within 4 min. The subject was moved away from the stimulus-cage about 15-20 sec after surmounting the obstacle. If the subject surmounted the obstacle less than 3 times within 4 min, the test ended. The amount of difficulty the subject conquered every time to approach the stimulus-cage was graded and summed up to the total score for each subject (**Table 1**).

In all experiments, the social and sexual appetitive behavior tests were always performed in the two separate chambers in order to avoid cross contamination between distinct olfactory stimuli.

Measure of morphine withdrawal on nociception and shock sensitivity

Flinch-jump test

The influence of morphine withdrawal on shock sensitivity was measured with the flinch-jump test on another cohort of male rats pre-treated either with saline (n=11) or morphine (n=11). The procedure was adjusted from Lehner and colleagues (Lehner, et al., 2010). After a 5-min period of habituation to the foot-shock chamber, the rats received foot shocks continuously. The shock titrations were continued upwards in a stepwise manner (starting at 0.05 mA, each increment: 0.05mA, shock duration: 0.5 sec). The flinch threshold was defined as the lowest shock intensity that elicited any detectable behavioral response. The vocalization threshold was defined as the lowest shock intensity that elicited vocalization by the rats. The jump threshold was defined as the lowest shock intensity that elicited simultaneous removal of at least three paws (including both hind paws) from the grid. To avoid foot damage, a cutoff of 1.0 mA was used. Using this method, the flinch, vocalization, and jump thresholds (in mA) were defined for each rat. Inter-shock interval was set to 30 s, and each animal was tested only once.

Hot-plate test

The influence of long-term morphine withdrawal on pain sensitivity was measured with a hot-plate test on another independent cohort of male rats pretreated with saline (n=10) or morphine (n=12). The apparatus, an Omnitech Analgesiometer (Omnitech Electronics, Columbus, OH) consisted of 25 ×25 cm metal hot-plate surface set at 52±0.5℃ (Anand, Coskun, Thrivikraman, Nemeroff, & Plotksy, 1999), a Plexiglas cage that fits over the hot plate, and a foot-switch operated timer. Pain thresholds were measured by the latency to nociceptive responses (limb shaking or paw licking) (Anand, et al., 1999). Latency was averaged from three trials with 15-min intervals between each trial.

Data and statistical analyses

Data are presented as mean ± SEM or single data points. For all analyses, assumptions for homogeneity of variance and normal distribution of the datasets were verified using the Levene and Kolmogorov-Smirnov tests, respectively. In case of violation of at least one of these assumptions, the statistical analyses were conducted on the data sets that were log-transformed (**Fig 4, 5A and 6C**).

The sniffing time spent on social or sexual stimuli was analyzed by four-way repeated-measures analysis of variance (ANOVAs), with “time” as the within-subjects factor and with “stimulus”, “pretreatment” and “stress” as the between-subjects factors. For the effort- and conflict-based appetitive behaviors, data were analyzed by three-way ANOVAs, with “stimulus”, “pretreatment” and “stress” as the between-subjects factors. The unconditioned freezing behavior was analyzed by two-way ANOVAs, with “pretreatment” and “foot-shock” as the between-subjects factors.

In cases of significant interactions, analyses of simple effects were performed. The measurements for

the hot-plate test and flinch-jump test were analyzed by a two-tailed *t*-test. For all analyses, significance level was set at 0.05 and the effect size is reported as partial eta square ($p\eta^2$) (Belin-Rauscent, et al., 2016; Ducret, et al., 2015).

All statistical analyses were performed using SPSS Statistics (version 16.0; SPSS Inc., Chicago, IL, USA).

Results

After 13 days of withdrawal from an escalating regimen of morphine, or vehicle, administration rats were allowed to freely investigate (e.g., sniff) either a social or sexual partner maintained in a stimulus-box located on the edge of an open field arena. As predicted, when no effort was required to investigate the partner, male rats displayed much higher interest in a sexual, e.g., a female in heat than a social, for a male rat [main effect of stimulus: $F_{1,68} = 312.96$, $P < .0001$, $p\eta^2 = 0.82$], the motivation for either stimulus being influenced neither by pretreatment [main effect of pretreatment: $F_{1,68} = 0.23$, $P > .6$, pretreatment × stimulus and pretreatment × stimulus × time interactions: $F_{1,68} = 1.27$, $P > .2$ and $F_{2,67} = 1.09$, $P > .3$] nor by acute stress [main effect of stress: $F_{1,68} = 2.03$, $P > .1$, stress × stimulus and stress × stimulus × time interactions: $F_{1,68} = 7.35$, $P < .01$ (simple-effect analysis did not reveal any significance) and $F_{2,67} = 0.92$, $P > .4$]. Similarly, acute stress did not influence differentially the interaction with a social or the sexual stimulus in vehicle or morphine pre-treated rats [stress × pretreatment and stress × pretreatement × stimulus interactions: $F_{1,68} = 0.57$, $P > .4$ and $F_{1,68} = 1.58$, $P > .2$] (**Fig 3**).

In marked contrast, drug history and stress both influenced the performance in an effort-based task in which rats had to make more and more efforts to climb over a partition of increasing height in order to approach and investigate either the social or sexual stimulus, akin to a progressive ratio of reinforcement in instrumental conditioning (Barr & Phillips, 1998). As shown in **Fig 4**, male rats still displayed higher motivation for a sexual than a social partner [main effect of stimulus: $F_{1,71} = 30.96$, $P < .001$, $p\eta^2 = 0.30$], and the motivation for the stimuli was influenced by pretreatment [main effect of pretreatment: $F_{1,71} = 7.04$, $P < .01$, $p\eta^2 = 0.09$] as well as the interaction of pretreatment with stress [pretreatment × stress and pretreatment × stress × stimulus interactions: $F_{1,71} = 3.93$, $P = 0.05$, $p\eta^2 = 0.05$ and $F_{1,71} = 8.69$, $P < .01$, $p\eta^2 = 0.11$]. The further simple-effect analyses revealed that the saline pre-treated rats were more willing to make efforts to approach a sexual than a social stimulus under the non-stress condition [$F_{1,74} = 15.05$, $P < .001$, $p\eta^2 = 0.17$] and the morphine pre-treated rats were more willing to make efforts for a sexual rather than a social stimulus after introducing an acute stress to them [$F_{1,74} = 19.33$, $P < .001$, $p\eta^2 = 0.21$]. Moreover, pretreatment and stress interactively influenced the motivation to a social but not a sexual stimulus, i.e., under a non-stress condition, the morphine pre-treated rats showed higher motivation to approach the social stimulus [simple-effect analysis: $F_{1,74} = 11.22$, $P < .01$, $p\eta^2 = 0.13$] as compared to the saline pre-treated rats, while the two pretreatment groups displayed the comparable motivation to approach the sexual stimulus [simple-effect analysis: $F_{1,74} = 0.16$, $P > .6$]. After introducing the foot shock, the morphine pre-treated rats decreased their motivation to climb over the partition to approach the social stimulus [simple-effect analysis: $F_{1,74} = 4.55$, $P < .04$, $p\eta^2 = 0.06$) and the saline pre-treated rats mildly increased their motivation to approach the social stimulus [simple-effect analysis: $F_{1,74} = 3.90$, $P = .05$, $p\eta^2 = 0.05$]. The motivation to sexual stimulus was not changed by stress either in the saline [simple-effect analysis: $F_{1,74} = 0.11$, $P > .7$] or in the morphine pre-treated rats [simple-effect analysis: $F_{1,74} = 1.12$, $P > .2$] (**Fig 4**).

We further increased the amount of difficulty that the rat needed to overcome in order to approach the

rewarding stimuli as measured under a conflict-based appetitive behavior test (**Fig 5**). In this task, due to the presence of pins on the board which height was progressively increased trial after trial, besides expending labors, rats had to take a risk of being pricked to surmount the board. Under this conflict-based task, male rats were more willing to make efforts and even take a risk to approach the sexual stimulus which was a higher-value incentive in contrast with the social stimulus [main effect of stimulus: $F_{1,60} = 58.97$, $P < .001$, $p\eta^2 = 0.50$]. The motivation to approach the social and sexual stimuli was increased by drug pretreatment [main effect of pretreatment: $F_{1,60} = 8.08$, $P < .01$, $p\eta^2 = 0.12$] but acute stress did not influence differentially the interaction with a social or the sexual stimulus in saline or morphine pre-treated rats [main effect of stress: $F_{1,60} = 0.14$, $P > .7$, stress × pretreatment and stress × pretreatment × stimulus interactions: $F_{1,60} = 0.01$, $P > .9$ and $F_{1,60} = 0.03$, $P > .8$] (**Fig 5A**). However, although no effect of acute stress was revealed by statistics, the saline and morphine pre-treated rats' approaching behaviors for sexual stimulus both demonstrated a bi-polar distribution after delivering the stress, i.e., the animals exposed to the foot-shock stress demonstrated either extremely high or extremely low approaching behaviors (**Fig 5B**).

These behavioral differences were not attributable to an influence of morphine withdrawal on nociception sensitivity or the aversiveness of foot-shocks. Thus, morphine-withdrawn rats differed from controls neither in their response to foot shocks as measured in the flinch-jump test [*t* test: Flinch: $t_{20} = -.55$, $P > .5$; Jump: $t_{20} = -.17$, $P > .8$; Vocalization: $t_{20} = .24$, $P > .8$] (**Fig 6A**) nor in their nociception sensitivity as measured in a hot-plate test [Latency to nociceptive responses: *t* test: $t_{20} = .17$, $P > .8$] (**Fig 6B**). Yet, the intensity of foot-shocks used as an acute stress produced a clearly fear response, but of similar magnitude in saline- and morphine pre-treated rats which displayed much more freezing behaviors than the control rats [main effect of foot-shock: $F_{1,63} = 161.88$, $P < .0001$, $p\eta^2 = .72$, pretreatment: $F_{1,63} = 0.03$, $P > .8$ and foot-shock × pretreatment interaction: $F_{1,63} = 0.49$, $P > .4$] (**Fig 6C**).

Discussion

Most preclinical studies have shown that the decreases both in preparatory and consummatory responses for natural rewards are often observed shortly after cessation of drug exposure (Bai, et al., 2014; Barr, et al., 1999; Barr & Phillips, 1999; Cui, Ren, Wu, Han, & Cui, 2004; Der-Avakian & Markou, 2010; Nocjar & Panksepp, 2007; Zhang, et al., 2007), and dramatically subside over time with abstinence even to the point that animals eventually become more sensitive to natural rewards over protracted periods of withdrawal (Fiorino & Phillips, 1999; Nocjar & Panksepp, 2002, 2007). However, during the protracted abstinence phase, small, normally insignificant, challenges can provoke negative affect (Sinha & Li, 2007), drug craving and relapse in addicts (Sinha, et al., 2011). Although this increased sensitivity to stressful life events has long been recognized clinically, the circumstances and psychological mechanisms by which stress influences affective states are quite limited (Heilig, et al., 2010).

After at least 14-days of withdrawal from morphine, we did not find anhedonic behavior for social or sexual stimulus in morphine pre-treated rats which were tested under three conditions. For instance, the decrease in time spent investigating social or sexual stimuli or effort-based approach to a social stimulus in the free approach- and effort-based tests, respectively, that was found in short-term morphine-withdrawn rats (Bai, et al., 2014) was no longer observed after long-term withdrawal (**Fig 3** and **4**). In contrast, morphine-withdrawn animals were more willing to overcome obstacles or take risks in order to approach social and sexual stimuli, as revealed by their higher performance in effort- and conflict-based tests (**Fig 4** and **5**). This suggests that morphine treatment resulted in motivational

sensitization to natural rewards after protracted abstinence. The cross-sensitization to natural reward developed during protracted drug abstinence period probably represents a compensatory mechanism within the brain reward system (Kelley & Berridge, 2002).

Under a simple testing condition, i.e., free approach-based test, stress did not significantly influence the appetitive motivation displayed by the rats to either social or sexual reinforcers (**Fig 3**). However, when effort was required (Salamone, Yohn, Lopez-Cruz, San Miguel, & Correa, 2016; Treadway & Zald, 2011), the motivation to a social stimulus was influenced by stress, i.e., the morphine-withdrawn rats displayed a significant decrease in their willingness to climb over the partition to approach the social stimulus, whereas the same stressful stimulus increased motivation for a social reward in saline pre-treated rats (**Fig 4**). These differences were not due to an influence of drug treatment over the aversiveness of foot-shocks as saline and morphine pre-treated rats did not differ in their freezing response to the presentation of shocks of same duration and intensity as the one used in the appetitive experiment.

So far, there is no clear conclusion whether stress can induce adaptive or maladaptive social responses. But some studies in humans suggest that stress can increase beneficial affiliative interactions between men (Buchanan & Preston, 2014; von Dawans, Fischbacher, Kirschbaum, Fehr, & Heinrichs, 2012). And moderate stress can increase social support-seeking behavior in male rat cagemates (Muroy, Long, Kaufer, & Kirby, 2016). It seems that in this study the foot-shock stress induced adaptive social responses in the drug-naïve rats which increased their approach behaviors for social mates. Nevertheless, the sensitized social appetitive behavior in the morphine-withdrawn rats, one of adaptive natural reward-seeking behaviors developed during protracted drug abstinence, was disrupted by the foot-shock stress, suggesting that acute stress could reduce the appetitive motivation to social reward in morphine-abstinent subjects.

The conflict-based test is not only an effort-based but also a risk-related task since it is designed to introduce a conflict between avoiding aversive stimuli (pins) and approaching rewarding stimuli. When a relatively low-incentive reward (social stimulus) was presented, all male rats avoided the aversive stimulus, thereby greatly decreasing their approach towards the reinforcer (**Fig 5A**). When a high-incentive, sexual, stimulus, was used, male rats persisted in their approach behavior despite the presence of the aversive stimulus, and more so did the morphine-withdrawn rats which displayed more approach behaviors than the saline pre-treated rats, in agreement with our previous study (Bai, et al., 2014). Hence, performance in this conflict task reflects not only facets of motivation (baseline performance is dependent on the motivational value of the reinforcer) but also inhibitory control, or rather, perseverative responding, under a cost/benefit computation condition. This persistent appetitive approach to the reinforcer observed in morphine pre-treated rats could not be attributed to a lower sensitivity to the aversiveness of the pins on the board since the nociception thresholds of the animals as measured in the flinch-jump and hot-plate test were not influenced by drug pretreatment. As it cannot be accounted for either by an effort-related process (Salamone, et al., 2016), this persistence may instead reflect long-term deficits in inhibitory control in morphine pre-treated rats (Jentsch & Pennington, 2014; Jentsch & Taylor, 1999) observed only in a subset of the population. Indeed, even if, at the population level foot-shock stress seemed not to influence conflict-based appetitive approach performance towards a sexual stimulus in the saline or morphine pre-treated rats, their approach behaviors actually exhibited a pattern of bi-modal distribution (Belin, Berson, Balado, Piazza, & Deroche-Gamonet, 2011) (**Fig 5B**), revealing that only a subset of each group of rats exposed to stress did indeed persist in approaching the sexual stimulus in spite of the aversiveness of the pins they had to

cross.

Thus, these data suggest that, regardless the influence of drug history on the overall performance in the task this apparent highly risky appetitive behaviors exhibited by a minority of rats could be due to stress-induced impairment of inhibitory control rather than to an increased motivation towards a sexual stimulus since foot-shock stress did not increase sexual motivation in male rats in the free approach-based test (**Fig 3**), triggering quite the opposite effect actually (**Fig 5B**).

A few preclinical studies have shown that acute stress can disrupt risk assessment behavior in mice (Quartermain, Stone, & Charbonneau, 1996; Stone, Rhee, & Quartermain, 1996) and clinical studies also show that risk-taking behavior, which is related to inhibitory control (Morein-Zamir & Robbins, 2015), is affected by acute stress only in a subset of individuals. For instance, acute psychosocial stress leads to a higher rate of risk taking only in cortisol responders in the pure gain domain of an economic decision making task (Buckert, Schwieren, Kudielka, & Fiebach, 2014) and increases risk behavior only in adolescents with high social anxiety (Reynolds, et al., 2013). These inter-individual differences in perseverative responding exacerbated by stress warrant further investigations, but they resonate well with the recent evidence that inter-individual differences in impulse control predict a differential vulnerability to develop perseverative compulsive responses in the face of an aversive situation, as measured as the persistence of cocaine self-administration in the face of punishment (Belin, Mar, Dalley, Robbins, & Everitt, 2008) or in a schedule-induced polydipsia procedure (Ansquer, et al., 2014).

In summary, the anhedonic symptoms observed during short-term withdrawal from morphine were found here dramatically to diminish after long-term abstinence, in agreement with previous reports (Fiorino & Phillips, 1999; Nocjar & Panksepp, 2002, 2007). Similarly, the present study revealed that, when challenged with the effort-based tasks, rats withdrawn from morphine for a protracted period of time exhibited an increased motivation to approach both social and sexual stimuli. Importantly, this study further demonstrates that an acute stress in these long-term drug-abstinent rats, otherwise more motivated to approach natural rewards, triggers an anhedonia-like state. Thus acute stress not only decreased motivation for a sexual stimulus both in drug-withdrawn and drug-naïve rats, but it selectively decreased the interest for social reward in the former. Moreover, this study also revealed inter-individual differences of behavioral performance in response to acute stress under the conflict-based task which reflected at least two psychological constructs, i.e., motivational anhedonia-like response in majority of rats and impaired inhibitory control of behavioral approach in a minority of rats.

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Figure and table captions

Fig 1 The timeline of the experiment.

Fig 2 Social or sexual appetitive behavior tests in male rats. The open-field chamber with a stimulus-cage holding a male or estrous female rat could be adapted to different appetitive behavior testing tasks. **(A)** The subjects could freely approach and investigate the incentive rat inside the stimulus-cage during the simple appetitive behavior test. **(B)** The subjects had to expend labors, i.e. climb over a continuously heightened partition, to approach the stimulus-cage during the effort-based appetitive behavior test. **(C)** The subjects had to surmount a dangerous obstacle, i.e. climb over a continuously heightened board thick with pins, to approach the stimulus-cage during the conflict-based appetitive behavior test.

Table 1 Conflict-based appetitive behavior test

Fig 3 Effect of the foot-shock stress on appetitive motivations to social and sexual rewarding stimuli in male rats during the simple appetitive behavior test. The 3-hour test (1 hour/block) was performed on day 14 of withdrawal from morphine. The time spent on sniffing the stimulus-cage holding a male or an estrous female rat was displayed. Values are mean ± SEM.

Fig 4 Effect of the foot-shock stress on appetitive motivations to social and sexual rewarding stimuli in male rats during the effort-based appetitive behavior test. The test was performed on day 19 of morphine withdrawal. The maximum heights of the partition that animals were willing to climb over to approach the stimulus-cage holding a male or an estrous female rat were displayed. The statistical analyses were conducted on data that were log transformed. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $^{\#}P = 0.05$. Values are mean ± SEM.

Fig 5 Effect of the foot-shock stress on conflict-based appetitive behaviors for social and sexual rewarding stimuli in male rats. The scores for approaching behavior for a male rat or an estrous female rat were displayed **(A)**. Values are mean ± SEM. The scores for conflict-based appetitive behavior for a male rat or an estrous female rat were shown in single points **(B)**.

Fig 6 The fearful responses to foot shock and shock/nociception sensitivity in male rats after 14-days withdrawal from morphine were demonstrated. The lowest shock intensity that elicited flinch, jump and vocalization in male rats was presented as shock threshold **(A)**. The pain threshold of rats was defined as the latency to nociceptive responses in hot-plate test **(B)**. Unconditioned freezing behavior of rats during the foot-shock stress period was shown as percent time of freezing behavior (The statistical analyses were conducted on data that were log transformed) **(C)**. $****P < 0.0001$, relative to the unstressed groups. Values are mean ± SEM.