Modulation of stress- and cocaine prime-induced reinstatement of conditioned place preference after memory extinction through dopamine D3 receptor

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Abstract

Accumulating evidence indicates that dopamine (DA) D3 receptor (DAD3R) antagonists appear highly promising in attenuating cocaine reward and relapse in preclinical models of addiction. In the present study, we investigated the effects of the selective DAD3R antagonist SB-277011-A on the reinstatement of cocaine-induced conditioned place preference (CPP) produced by a priming dose of cocaine, by social defeat stress and by two kinds of physiological stressors (restraint and tail pinch) in male adult mice. We also explored reinstatement-related plasma corticosterone levels (as marker of stress response) and the effects of blocking DAD3R. Administration of SB-277011-A (24 or 48 mg/kg i.p.) did not modify conditioned reinstatement of cocaine seeking triggered by cocaine prime. By contrast, we found that the vulnerability to reinstatement of the CPP of defeated animals that have undergone CPP extinction was abolished by the DAD3R antagonist (24 mg/kg) given 30 min before the test session. Reactivation of the CPP response produced by physiological stress stimuli was also attenuated by SB-277011-A (48 mg/kg i.p.). On the other hand, the blockade of DAD3R significantly prevented the increased corticosterone release during reinstatement of cocaine-induced CPP that was seen in social defeated animals, in mice suffering physiological stress and after cocaine prime. Present results demonstrate a modulation by DAD3R of the reactivation of the incentive value of cocaine-associated cues induced by social and physiological stress stimuli, which was associated to a glucocorticoid-dependent mechanism. Our results also point to a possible potential therapeutic use of selective DAD3R antagonists for the prevention of stress-induced cocaine-seeking and relapse.

Keywords

Dopamine receptor 3 subtype (DAD3R); Conditioned place preference (CPP); Cocaine addiction; Reinstatement; Stress; Corticosterone.

1. Introduction

The repeated cycles of cessation of consumption and relapse remain the major clinical concern in treating cocaine addiction. Cocaine relapse can be triggered by multiple factors, including conditioned cues that act as reminders of the drug experience, and stress which contribute to the reinstatement of cocaine seeking behavior and cocaine-conditioned place preference (CPP) (Koob and Volkow, 2010, Perry et al. , 2014, Venniro et al. , 2016).

Memory extinction has received much attention for its potential utility in the development of treatments for several neuropsychiatric disorders, including addiction (Milton, 2013). Thus, drug memories extinction therapy has been developed to inhibit the motivational effect of drug cues to prevent relapse. Extinction is an active process that recruits a new learning process and forms a new memory to temporally suppress previously conditioned responding rather than erasing acquired memory traces (Lattal et al. , 2006). Accordingly, evidence from several studies in humans and rodents have shown that the effectiveness of extinction training is limited and drug relapse can be influenced by a number of factors, including acute exposure to drugs of abuse (drug-induced reinstatement), stress and environmental cues that have previously been associated with drug use in a Pavlovian manner (Milton, 2013).

The reinstatement of drug craving or seeking by priming injections of the abused drug is a robust phenomenon in both humans and laboratory animals. On the other hand, traumatic experiences and social stress, such as aggression, may contribute to relapse/reinstatement of drug seeking behavior (Flynn et al. , 2004, Ribeiro Do Couto et al. , 2006). A great number of studies have shown the importance of dopaminergic projection from ventral tegmental area (VTA) to the

nucleus accumbens (NAc) in drug-induced reinstatement. Systemic injection of DA receptors antagonists tends to attenuate or block drug-induced rewards and incentive motivation and attenuate reinstatement induced by priming injections of the drug (Volkow et al. , 2017). Despite this considerable body of knowledge, there had been little progress in bringing novel and effective treatment of addiction in clinic. Recently, the DAD3 receptor (DAD3R) has gained attention as a clinical target for the treatment of addiction-related behavior (Ashok et al. , 2017, Galaj et al. , 2016). Although its role in cocaine actions remains somewhat uncertain and conflicting findings have been reported when using DAD3R knockout mice (Kong et al. , 2011, Song et al. , 2013), it has been proposed that DAD3R may control the effects of those factors which trigger relapse, such as a priming injection of the drug, stress and drug cues (Ashby et al. , 2015, Epstein et al. , 2006) In the present work we have evaluated the role of DAD3R on the reinstatement of previously extinguished cocaine-induced conditioned place preference triggered by i) a priming dose of cocaine; ii) a brief episode of social defeat stress; we have adopted the social defeat paradigm that profoundly alters the motivation for social interaction in rodents (Avgustinovich and Kovalenko, 2005, Berton et al. , 2006) and iii) exposure to physiological stressors, such as restraint and tail pinch. For that, we investigated the effect of acute administration of the selective DAD3R antagonist, SB-277011-A on the reinstatement of cocaine-induced CPP produced by a priming dose of cocaine and by social and physiological stress. Since stressful events have profound effects on learning and memory, can promote drug abuse and trigger relapse (Finsterwald and Alberini, 2014), and the role of D3DAR signaling on stress and cocaine-induced rises in corticosterone have not been extensively explored, we also have measured the

reinstatement-related plasma corticosterone levels (as marker of stress response) and the effects of blocking DAD3R on corticosterone release.

2. Materials and Methods

2.1. Animals

All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/UE), and were approved by the local Committees for animal research (Comité de Ética y Experimentación Animal; CEEA; RD 53/2013). Protocols were designed to minimize the number of experimental animals and to minimize their suffering. Male C57BL/6 mice (n=121 at the beginning of the study; Charles River, Laboratories France) initially aging 6 weeks were maintained on arrival in a room with controlled temperature (22 ± 2°C) and humidity (50 ± 10%), with free access to water and food. Animals were adapted to a reversed 12 h light-dark cycle (lights off: 08:00 h – 20:00 h) for 7 days before the beginning of the experiments. Mice were housed in groups of four in plastic cages (25 L × 25 W × 14,5 H cm) for 10 days (n=91; used for CPP; n=8 used for basal plasma corticosterone levels measurement) or for 1 month (n=8; used as nonaggressive opponents in the test of social interaction). Animals utilized for CPP experiment were handled 5 min daily for 5 days before the beginning of the experiments. The mice used as aggressive opponents (n=22) were housed individually in plastic cages (23 L × 13,5 W × 13 H cm) for a month before experiments to induce heightened aggression (Rodriguez-Arias et al. , 1998).

2.2. Drugs and reagents

Cocaine HCl (Alcaliber, Madrid, Spain) was dissolved in sterile saline (NaCl 0.9%) and SB-277011-A (N-[trans-4-[cyano-3,4-dihydro-2(1H)-isoquinolinyl)ethyl]cyclohexyl]-4-quinolinecarboxamide dihydrochloride; Tocris,

St. Louis, MO, USA) was dissolved in deionized distilled water (vehicle) and all injections were administered intraperitoneally (i.p.) in a volume of 0.01 ml/g body weight.

2.3. Conditioned Place Preference Paradigm

Briefly, the conditioned place preference apparatus (Panlab, Barcelona, Spain) used to induce a reliable preference consists in a box with two equally sized chambers (20 L × 18 W × 25 H cm) interconnected by a rectangular corridor (20L x 7W x 25H cm). Distinctive visual and tactile cues distinguish the compartments: the motifs painted on the walls (either black dots or grey stripes), the floor coloring (black or grey) and the floor texture (smooth or rough). The sensory cues combination that produces a balanced choice are for walls and floor coloring and texture, respectively: (A) black dots, black smooth floor; (B) grey stripes, grey rough floor. Transparent walls are also used to minimize the time the animal spent in the corridor. The weight transducer technology and PPCWIN software allows the detection and analysis of animal position throughout the test and the number of entries in each compartment. The CPP experimental protocol consists of three distinct phases: a preconditioning phase, a conditioning phase, and a testing phase. We used a counterbalanced unbiased procedure in terms of initial spontaneous preference.

During the first phase, or preconditioning (Pre-C), mice were given access to both compartments of the apparatus for 900 s and the time spent in each one was recorded. Animals showing strong unconditioned aversion (<33% of session time) or preference (>67%) for any compartment were discarded (n=3). After assigning the compartments, a Student’s t-test showed that there were no

significant differences between the time spent in the cocaine-paired and the saline-paired compartments during the pre-C phase. 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 (Fig. 1A-D).

In the second phase (conditioning), guillotine doors blocked access from both chambers to the central corridor. Eight groups of animals were conditioned with 25 mg/kg of cocaine (88 animals, n=8–15 per group). On days 1 and 3, animals received an injection of cocaine (25 mg/kg i.p.) before being confined to the drug-paired compartment for 30 min and, after an interval of 4 h, received saline immediately before confinement in the vehicle-paired compartment for 30 min. The dose of 25 mg/kg of cocaine was selected on the basis of previous studies which demonstrated that it induces a robust CPP (Maldonado et al. , 2007, Rodriguez-Arias et al. , 2017). On days 2 and 4, animals received an injection of saline before being confined to the vehicle-paired compartment for 30 min and, after an interval of 4 h, received cocaine immediately before confinement in the drug-paired compartment for 30 min (Fig. 1A-D). One animal was discarded in this phase.

The third phase or post-conditioning (Post- C) (Fig. 1A-D) was conducted on day 5, exactly as in the preconditioning phase (free entree to each chamber for 900 s). Eighty-seven animals underwent twice a week (in no consecutive days) extinction sessions for 7-8 weeks (depending on the group), which was conducted exactly as in the pre-C and the post-C tests (free access to each compartment for 900 s). The criterion of extinction was a lack of significant differences (Student´s *t* test) in the time spent by each group in the drug-associated chamber during the extinction (ext)-test with regard to that in the Pre-

C test. Thus, all the animals in each group underwent the same number of extinction sessions, independently of their individual scores. Once achieved the criterion (Pre-C=ext), a new session was performed 48 h later to confirm extinction (Fig. 1A-D).

Two days after extinction was ratified, mice were subjected to different kind of stressful stimuli or were administered with a cocaine prime (12.5 mg/kg i.p.) and then tested to evaluate the reinstatement of the cocaine-induced CPP (Fig. 1A-D). The dose of cocaine prime was chosen as it has been previously shown that it elicits CPP reactivation in male mice that have undergone extinction of cocaine-induced CPP (Rodriguez-Arias et al. , 2009). The reinstatement (reinst-)test was similar to the pre-C, post-C and ext-tests, that is, free entree to each chamber for 900 s. All procedures for reinstatement were performed in a separated room from where the place preference equipment is maintained, which constituted then a non-contingent place to that of the previous conditioning injections.

2.4. Experimental groups

Experiment 1: effect of DAD3R antagonism on the reinstatement of cocaine-induced CPP evoked by a cocaine prime

In order to provoke the reinstatement of the cocaine-induced CPP, animals were injected with a cocaine prime (12,5 mg/kg) and 15 min later were subjected to the reinst-test. A group of control animals were administered with a saline prime instead of cocaine to confirm that the reinstatement of the drug-induced CPP was due to the cocaine. The possible involvement of DAD3R in the reinstatement of the cocaine-induced CPP evoked by a cocaine prime was

studied by means of the administration of a single dose of a DAD3R antagonist, SB-277011-A (24 or 48 mg/kg) 30 min before the cocaine prime. Control mice received a vehicle injection instead of the DAD3R antagonist (Fig. 1A).

Experiment 2: effect of DAD3R antagonism on the reinstatement of the cocaine-induced CPP evoked by social stress

The effects of social defeat, which can be considered a type of social stress (Miczek et al. , 2011, Ribeiro Do Couto et al. , 2009), on reinstatement of cocaine-induced CPP was assessed. For that, mice underwent an antagonistic encounter with an aggressive opponent (of equal age and body weight) that had been individually housed, had previous fighting experience and had been previously screened for an elevated level of aggressive behavior. Experimental mice exhibited avoidance/flee and defensive/submission behaviors after suffering the aggressive behavior (threat and attack) of the opponent. Defeated mice always exhibit this extreme form of upright submissive behavior (Rodriguez-Arias, et al., 1998). This encounter lasted 15 min and took place in a neutral transparent plastic cage (23 L × 13,5 W × 13 H cm). The criterion used to define an animal as defeated was the assumption of a specific posture of defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al. , 1982). All defeated mice experienced similar levels of aggression because of the attack behaviors from the opponent, which were initiated immediately after seeing the experimental mouse (latency<30 s). No behavioral sequelae were seen in controls or aggressive mice. Immediately after social defeat, the reinstatement test was performed. With the aim of demonstrating the lack of effects of the procedure itself, an additional group underwent an agonistic/nonaggressive social encounter with a conspecific

mouse that was previously grouped (Ribeiro Do Couto, Aguilar, 2009). Since this type of opponent never initiates attack, experimental mice do not suffer the experience of defeat. So, this type of agonistic encounter can be view as a normal social interaction between two conspecific animals with a similar low level of aggressive behavior, and no aggression was observed during these encounters. This encounter lasted 15 min and took place in a neutral transparent plastic cage (23 L × 13,5 W × 13 H cm).

To evaluate the implication of DAD3R in the reactivation of the cocaine-induced CPP evoked by social stress, a single dose of SB-277011-A (12 or 24 mg/kg) was injected 30 min before the antagonistic encounter. Control mice were injected with vehicle instead of the antagonist (Fig. 1B).

Experiment 3: effect of the blockade of DAD3R on the reinstatement of cocaine-induced CPP elicited by restraint

The reinstatement of the cocaine-induced CPP was also evoked by acute restraint stress. For that, mice were placed for 15 min in plastic cylindrical restrainers (2.5 cm diameter × 11.5 cm length) with a hole in one of the ends to allow normal breathing and were subjected to the reinst-test. The possible involvement of DAD3R in the reactivation of the cocaine-induced CPP evoked by restraint was studied by means of the administration of a single dose of the DAD3R antagonist, SB-277011-A (24 or 48 mg/kg) 30 min before the immobilization. Control mice received a vehicle injection instead of the DAD3R antagonist (Fig. 1C).

Experiment 4: effect of DAD3R antagonism on the reinstatement of cocaine-induced CPP elicited by tail pinch

Tail pinch procedure was performed to elicit the reactivation of the cocaine-induced CPP. For that, mice were put in a plastic cage (25 H × 25 W × 14,5 H cm) and a binder clip (7 mm wide, and its inner section was oval with 8 mm wide and 13 mm height) was placed in the last third of the tip of the tail for 15 min (clamping force, 10 Newton). After taking off the clip, mice were subjected to the reinst-test. The implication of DAD3R in the reactivation of the cocaine-induced CPP evoked by this stimulus was studied by means of the administration of a single dose of SB-277011-A (24 or 48 mg/kg) 30 min before the tail pinch. Control mice received a vehicle injection instead of the DAD3R antagonist (Fig. 1D).

Doses of 12 and 24 mg/kg of the selective D3 antagonist SB-277011-A were selected based on previous studies from other authors, in which similar doses were evaluated in rats or mice in the CPP paradigm or drug self-administration (Xi et al 2004; Xi et al. 2005; Ashby et al. 2015; Cervo et al. 2007; Kalhed et al. 2010, Int J Neuropsychopharmacol 13: 181-190; Le Foll et al. 2002; Gilbert et al. 2005, Sinapse 57: 17-28). As the dose of 24 mg/kg was effective in reducing the reinstatement of extinguished cocaine-induced CPP elicited by psychosocial stress but failed to block reinstatement induced by physiological stressors (restraint or tail pinch) or cocaine-priming, a higher dose of SB-277011-A (48 mg/kg) was used.

2.5. Corticosterone Measurements

Immediately after the reinstatement test, all groups of mice were sacrificed by cervical dislocation and trunk blood samples were collected and centrifuged (4000 rpm, 15 min, 4ºC) to separate the plasma, which was maintained at -80ºC until the measurement of corticosterone levels (Martin et al. , 2011). Basal corticosterone levels were obtained from a separate set of animals that did not receive any treatment and that were not tested for CPP. Plasma corticosterone concentrations were quantified using commercially available kits for mice (125 I-corticosterone radioimmunoassay; MP Biomedicals, USA). The sensitivity of the assay was 7.7 ng/ml.

2.6. Data collection and statistical analysis

Data were recorded automatically by PPCWIN software (Panlab, Barcelona, Spain). As these data were collected by computer, blinding to experimental group was not required. The data are expressed as the mean ± SEM. For the CPP experiments, the statistical analysis was performed using one-way ANOVA with repeated measures followed by multiple comparisons testing using the Tukey post-hoc test to determine specific group differences. Plasma corticosterone levels were analyzed using one-way ANOVA followed by the Tukey *post-hoc* test. Correlations between different parameters were assessed using the Pearson's correlation coefficient. Differences with a $p<0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

Conditioned place preference (CPP) is a behavioral paradigm widely used to study the rewarding properties of addictive substances (Tzschentke, 2007). In consequence, we used cocaine-induced CPP to determine to what extent DAD3R is involved in regulating the reinstatement of extinguished cocaine-induced place preference after exposure to a cocaine prime or to different kind of stressful stimuli.

3.1. DAD3R antagonism with SB-277011-A did not blockade the reinstatement of cocaine-induced CPP by a cocaine prime

One-way ANOVA with repeated measures revealed significant differences between treatments for all the series of animals [$F(1.9,13) = 13.00$, $p=0.0010$ for veh + saline; $F(1.4,17) = 10.00$, $p=0.0027$ for veh + cocaine prime; $F(1.7,17) = 17.00$, $p=0.0001$ for SB 24 + coc prime; $F(1.7,10) = 57.00$, $p<0.0001$ for SB 48 + coc prime]. As it can be seen in Fig. 2A-D, Tukey's *post hoc* test showed that, after the conditioning period, mice spent more time in the cocaine-associated chamber during the post-C test than during the pre-C test. As expected, the extinction sessions significantly diminished the time that mice spent in the drug-paired compartment during the post-ext test regarding the post-C test (Fig. 2A-D).

In order to induce the reinstatement of cocaine-induced CPP, a cocaine prime (12.5 mg/kg i.p.) was administered 15 min before the test for reinstatement. As it can be seen in Fig. 2B, the time spent in the cocaine-paired chamber during the post-reinst test increased significantly when compared with those in the pre-C and post-ext tests. By contrast, mice that received a saline prime instead of

cocaine did not augment the time spent in the drug-associated compartment during the post-reinst test, which was significantly lower than the post-C time (Fig. 2A).

To study whether DAD3R is implicated in the reinstatement of cocaine-induced CPP promoted by a cocaine prime, we administered two different doses (24 and 48 mg/kg, i.p.) of SB-277011-A, a selective antagonist of DAD3R, 30 min before the priming injection. Our data showed that the blockade of DAD3R with any of the two doses of the antagonist did not inhibit the reinstatement of cocaine-induced CPP evoked by a drug prime, given that the animals spent significantly more time in the cocaine-conditioned chamber during the post-reinst test than during the pre-C and post-ext tests (Fig. 2C,D).

We evaluated as well if the blockade of DAD3R affected the number of entries to both chambers during the post-reinst test. One-way ANOVA showed significant differences between groups for the number of total entries to both saline- and cocaine-paired chambers [$F(3,35) = 5.07$, $p=0.0051$]. As it can be observed in Fig. 2E, mice that received a cocaine prime increased the number of total entries to both compartments regarding the pre-C test compared with the animals that were injected with a saline prime, although this enhancement was not statistically significant. The animals that received the lower dose of SB-277011-A showed similar number of total entries with regard to the cocaine-primed group. However, the number of total entries of animals injected with 48 mg/kg of SB-277011-A was significantly lower when compared with the animals that received vehicle instead of the antagonist before the cocaine prime and with the mice that were injected with a lower dose of SB-277011-A before the cocaine prime. Our data revealed the same pattern of entries in the saline- and cocaine-

paired chambers (Figs. 2F,G). Reinstatement of cocaine-induced CPP of the animals that were injected with vehicle before receiving a cocaine prime was further demonstrated by the fact that their post-reinst CPP-score was significantly correlated with the diminished number of entries in the saline-paired compartment (r=-0.5668, p=0.0434; Pearson's correlation; Fig. 2H).

3.2. SB-277011-A antagonized the reinstatement of cocaine-induced CPP by social stress

One-way ANOVA with repeated measures revealed significant differences between treatments for all the series of animals [F (1.678,15.10) = 16.17, p=0,0003 for veh + not defeat; F (2.159,32.39) = 30.46, p<0,0001 for veh + social defeat; F (1.300,16.90) = 12.22, p=0,0016 for SB 12 + social defeat; F (2.287,32.02) = 15.99, p<0.0001 for SB 24 + social defeat]. *Tukey post hoc* test revealed that mice spent significantly more time in the cocaine-paired chamber in the test after the conditioning sessions than during the pre-C test and that, in the course of the post-ext test, the time spent by the animals in the drug-associated chamber was statistically lower than during the post-C test (Fig. 3A-D).

When animals were subjected to an episode of social stress and subsequently tested for the reinstatement of the cocaine-induced CPP, we found that they spent significantly more time in the drug-paired compartment when compared with the post-ext and pre-C tests (Fig. 3B). On the contrary, non-defeated mice did not increase the seconds spent in the drug-associated chamber during the post-reinst test regarding the pre-C and the post-ext tests (Fig. 3A).

The administration of 12 mg/kg of SB-277011-A 30 min before the social defeat episode did not impede the reinstatement of the cocaine-induced CPP, given that mice augmented significantly the seconds spent in the cocaine-paired compartment during the post-reinst test when compared with the pre-C and post-ext tests (Fig. 3C). In contrast, a higher dose of the DAD3R antagonist (24 mg/kg) did block the reinstatement of the cocaine-induced CPP by social stress. Tukey *post hoc* test showed that mice injected with 24 mg/kg of SB-277011-A before the antagonistic encounter did not augment the time passed in the drug-paired chamber during the post-reinst test with regard to the post-ext and pre-C tests. Additionally, this time was significantly lower than that in the post-C test (Fig. 3D).

When we studied the number of entries of animals during the post-reinst test, one-way ANOVA revealed significant differences between experimental groups for the number of total entries [$F(3,49) = 19.36$, $p<0,0001$]. Additionally, the *post hoc* test showed that socially stressed mice did statistically ($p<0.001$) augment the number of total entries to both compartments regarding the non-defeated animals. Additionally, either 12 or 24 mg/kg of SB-277011-A significantly diminished the increase in the number of total entries during the post-reinst test (Fig. 3E). The number of entries to the saline- and cocaine-paired chambers followed the same pattern than the number of total entries, but we did not find significant differences between groups (Fig. 3F,G). We did find significant correlations between the reinstatement CPP-score of socially stressed mice, which received the DAD3R antagonist or its vehicle, and the number of total entries ($r=0.4140$, $p=0.0058$; Pearson's correlation; Fig. 3H), the number of entries to the cocaine-paired chamber ($r=0.4586$, $p=0.0020$; Pearson's

correlation; Fig. 3I) and the number of entries to the saline-associated chamber (r=0.3338, p=0.0287; Pearson's correlation; Fig. 3J).

Administration of SB-277011-A did not alter the responses of defeated animals (avoidance/flee and defensive/submission behaviors after suffering the aggressive behavior, upright submissive position, limp forepaws, upwardly angled head, and retracted).

3.3. The blockade of DAD3R antagonized the reinstatement of cocaine-induced CPP by physiological stress

One-way ANOVA with repeated measures revealed significant differences between treatments for all the series of animals [$F(1.5,13) = 24.00$, $p=0.0001$ for veh + restraint; $F(1.6,19) = 8.30$, $p=0.0042$ for SB 24 + restraint; $F(1.8,31) = 27.00$, $p<0.0001$ for SB 48 + restraint; $F(1.7,22) = 24.00$, $p<0.0001$ for veh + tail pinch; $F(1.8,23) = 15.00$, $p<0.0001$ for SB 24 + tail pinch; $F(1.8,14) = 8.50$, $p=0.0045$ for SB 48 + tail pinch]. As it was observed in the previous experiments, Tukey's *post hoc* test revealed that mice spent significantly more time in the cocaine-paired compartment during the post-C test in comparison with the pre-C test, and that the time spent in the drug-associated compartment diminished significantly during the post-ext test (Fig. 4A-C and 5A-C).

In order to evoke the reinstatement of cocaine-induced CPP, animals were exposed to two different physical stressors, restraint and tail pinch. *Post hoc* test showed that, after 30 min of restraint, animals significantly increased the time spent in the drug-associated chamber during the post-reinst test compared with the post-ext and pre-C test (Fig. 4A). Similarly, animals exposed to the tail pinch

spent significantly more time in the cocaine-paired compartment during the post-reinst test with regard to the post-ext and pre-C tests (Fig. 5A).

Two different doses of the DAD3R antagonist were administered before exposure to physiological stressors to study the ability of SB-277011-A to prevent the reinstatement of cocaine-induced CPP. The administration of the lower dose of this drug (24 mg/kg) did not impede the significant increase in the time spent by physically stressed mice in the cocaine-associated chamber during the post-reinst test when compared with the pre-C and post-ext tests (Figs. 4B and 5B). However, when 48 mg/kg of SB-277011-A were administered before the exposure to the physical stressors, we did observe a significant diminution of the time spent by mice in the drug-paired chamber during the post-reinst test regarding those in the post-C and in the pre-C tests (Figs. 4C and 5C).

When we evaluated the effect of SB-277011-A on the number of entries of physically stressed animals to both compartments and to the cocaine- and saline-paired chambers separately, one-way ANOVA revealed significant differences between experimental groups for the number of total entries [$F(2,38) = 23.28$, $p<0.0001$ for restraint; $F(2,34) = 5.03$, $p=0.0122$ for tail pinch], the number of entries to the cocaine-paired chamber [$F(2,38) = 22.69$, $p<0.0001$ for restraint; $F(2,34) = 3.61$, $p=0.0380$ for tail pinch] and for the number of entries to the saline-associated chamber [$F(2,38) = 15.10$, $p<0.0001$ for restraint; $F(2,34) = 5.53$, $p=0.0083$].

Additionally, we found that the DAD3R antagonist decrease all of them in a dose-dependent manner. As can be seen in the Figs. 4D-F, the administration of 24 mg/kg of SB-277011-A to restrained mice significantly decreased the number of total entries, the number of entries to the cocaine-associated chamber

and to the saline-paired chamber regarding the animals that received the vehicle instead the antagonist. The injection of the higher dose of the drug to the immobilized animals also diminished significantly the number of total entries regarding the control group and the group that received 24 mg/kg of SB-277011-A, the number of entries to the cocaine-paired chamber in comparison with the vehicle-administered group and the group injected with 24 mg/kg of SB-277011-A, and the number of entries to the saline-paired chamber regarding the control group. Additionally, in restrained animals we found significant correlations between the post-reinst CPP-score and the number of total entries ($r=0.3894$, $p=0.0130$; Pearson's correlation; Fig. 4G), the number of entries to the drug-conditioned compartment ($r=0.3779$, $p=0.0162$; Pearson's correlation; Fig. 4H), and the number of entries to the saline-paired chamber ($r=0.3609$, $p=0.0222$; Pearson's correlation; Fig. 4I).

DAD3R antagonist also decreased the number of total entries and the number of entries to both saline- and cocaine-associated compartments in a dose-dependent manner (Fig 5D-E). The administration of 24 mg/kg of SB-277011-A to mice subjected to tail pinch significantly decreased the number of entries to the saline-paired chamber regarding the animals that received the vehicle instead the antagonist. The injection of the higher dose of the drug to the animals subjected to tail pinch diminished significantly the number of total entries, the number of entries to the cocaine-paired chamber and the number of entries to the saline-paired chamber regarding the control group. No significant correlations were observed between the post-reinst CPP-score of animals that were subjected to tail pinch and their number of total entries (Fig. 5G), the number

of entries to the cocaine-associated chamber (Fig. 5H), and the number of entries to the saline-paired chamber (Fig. 5I).

3.4 DAD3R mediated the increase in corticosterone during the reinstatement of cocaine-induced CPP

One-way ANOVA showed significant differences in the corticosterone plasma concentration for all the sets of animals [$F(4,33) = 31.00$, $p<0.0001$ for cocaine prime set of mice; $F(4,45) = 38.00$, $p<0.0001$ for social defeat set of animals; $F(3,43) = 84.00$, $p<0.0001$ for restraint set of mice; $F(3,39) = 67.00$, $p<0.0001$ for tail pinch set of animals]. When we measured the plasma levels of corticosterone in animals that received a cocaine prime or were subjected to social or physiological stress before the post-reinst test, we found a significant enhancement in comparison with its basal levels in control animals (Fig. 6A,F,K,P). Additionally, a saline injection and an agonistic encounter without defeat (controls) also increased significantly the plasma concentration of the hormone (Fig. 6A,F). However, the levels of corticosterone after the post-reinst test in animals that received a cocaine prime were higher than those in saline-primed animals (Fig. 6A).

We administered different doses of SB-277011-A to study the implication of DAD3R in the release of corticosterone during the reinstatement of cocaine-induced CPP. We found that 12 mg/kg of the antagonist did not blocked the augment in corticosterone release after the post-reinst test either in social defeated animals, which showed statistically higher corticosterone levels than the control groups (Fig. 6F). However, 24 mg/kg of SB-277011-A antagonized the increase in corticosterone levels after the post-reinst test in mice primed with cocaine or subjected to social or physiological stress, which showed significantly

lower corticosterone levels than the same mice receiving vehicle instead of the DAD3R antagonist (Fig. 6A,F,K,P). Nonetheless, despite the administration of 24 mg/kg of the antagonist, corticosterone plasma levels of restrained animals after the post-reinst test were significantly higher than the control group (Fig. 6K). The administration of 48 mg/kg of SB-277011-A before the cocaine prime or the exposure to a physiological stressor significantly antagonized the increase in corticosterone release observed after the post-reinst test (Fig. 6A,K,P).

In addition, we found significant correlations between corticosterone plasma levels and the post-reinst CPP-score of animals socially stressed that received an injection of the DAD3R antagonist or its vehicle ($r=0.3410$, $p=0.0484$; Pearson's correlation; Fig. 6G), the animals that were administered with SB-277011-A or its vehicle before restraint ($r=0.3755$, $p=0.0185$; Pearson's correlation; Fig. 6L) and the mice that were injected with the DAD3R antagonist or its vehicle before tail pinch ($r=0.33592$, $p=0.0341$; Pearson's correlation; Fig. 6Q). Nonetheless, the correlation between the corticosterone plasma concentration and the post-reinst CPP-score of cocaine-primed animals was not significant (Fig. 6B)

Blood corticosterone concentration of socially stressed mice receiving any dose of antagonist or its vehicle also correlated significantly ($r=0.3563$, $p=0.0454$; Pearson's correlation; Fig. 6I) with the number of entries to the cocaine-conditioned compartment, although the correlations with the number of total entries (Fig. 6H) and the number of entries to the saline-paired chamber (Fig. 6J) were not significant. In addition, the plasma corticosterone levels of physically stressed mice that were administered with the DAD3R antagonist or with its vehicle correlated significantly with the number of total entries ($r=0.6566$,

$p<0.0001$ for restraint; $r=0.4692$, $p=0.0045$ for tail pinch; Pearson's correlation; Fig. 6M,R), the number of entries to the drug-paired chamber ($r=0.6569$, $p<0.0001$ for restraint; $r=0.4041$, $p=0.0161$ for tail pinch; Pearson's correlation; Fig. 6N,S) and the number of entries to the saline-associated compartment ($r=0.5837$, $p<0.0001$ for restraint; $r=0.4920$. $p=0.0027$ for tail pinch; Pearson's correlation; Fig. 6O,T).

4. Discussion

In the present study we have used the CPP paradigm for studying the involvement of DAD3R in the reinstatement of cocaine seeking behaviors induced by the drug prime and by different kind of stressors. In addition, the response of the HPA axis (as a marker of stress response) to the DAD3R blockade was determined. Here, we describe a previously little known role for DAD3R in reinstatement of cocaine-associated memories induced by psychosocial stress. We found that acute injection of the selective DAD3R antagonist, SB-277011-A significantly reduced cocaine-induced CPP reactivation induced in defeated animals that had extinguished the CPP response. Similar results were obtained when using physiological stressors, such as restraint and tail pinch. These results indicate that dopaminergic neurotransmission via DAD3R stimulation is critical for stress-induced cocaine reinstatement of animals that have undergone CPP extinction. By contrast, SB-277011-A did not alter the expression of the cocaine-primed reinstatement.

4.1. DAD3R is critical for the reinstatement of cocaine-induced CPP triggered by social and physiological stressors

According to previous studies (Tzschentke, 2007), we found that the pairing of mice with cocaine and specific visual and tactile cues produced a robust CPP. Through repeated sessions of extinction training (exposure to the previously drug-paired context without drug treatment), the place preference was extinguished, in agreement with studies reported previously (Milton and Everitt, 2012, Ribeiro Do Couto et al., 2009, Torregrossa et al., 2011). Extinction of drug memories is widely accepted to involve new learning that inhibits or overrides

initial learning rather than forgetting. Unfortunately, extinguishing these memories has not proven efficacious in reducing relapse/reinstatement of both humans or rodents (Everitt, 2014, Pitchers et al., 2017). In fact, we found that exposure of mice to social/emotional stress, such as defeat in a social interaction, was highly effective in reinstating cocaine-CPP, whereas an agonistic encounter with nonaggressive mouse (control) did not induce the reinstatement of CPP.

Importantly, the present study showed that an acute administration of 24 mg/kg of the selective DAD3R antagonist SB-277011-A completely prevented the social defeat-induced CPP reactivation in following CPP extinction. To our knowledge, these results provide the first illustration (examining the therapeutic potential of DAD3R antagonists as prevention of cocaine relapse induced by social stress) that DAD3R stimulation might play an essential role in social stress-induced reactivation of a previously extinguished cocaine CPP expression. It has been shown that social defeat encounters increase DA release in the mesolimbic pathway (Berton et al., 2006; (Tidey and Miczek, 1996). Our findings are consistent with these reports showing that stress increases the activity of mesolimbic DA neurons. Thus, by antagonizing DA signaling at postsynaptic DAD3R, SB-277011-A would inhibit the effects DA release.

One of the most consistently reported effects of DAD3R antagonists is their ability to block drug-seeking triggered by drug-paired environmental stimuli in different animal models of reinstatement (Ashby et al., 2015, Gilbert et al. , 2005, Heidbreder et al. , 2007, Vorel et al. , 2002). The DAD3R is highly expressed in brain neurons innervated by mesolimbic dopaminergic reward pathway, such as NAc and postulated to be critically involved in reward,

emotions, motivation, and diseases such as schizophrenia and addiction (Diaz et al. , 2000, Stanwood et al. , 2000). It has been postulated that stress can induce cocaine reinstatement by reactivating the motivational value of the drug-conditioned cues (Goddard and Leri, 2006). Accordingly, DAD3R-selective antagonists have been shown to decrease motivation for drugs of abuse and drug-seeking behavioral (Higley et al. , 2011, Sokoloff and Le Foll, 2017, Xi et al. , 2005). Although the level at which the DAD3R intervenes in social stress-induced cocaine conditioned effect is not well known, the current study might provide evidence that activation of DAD3R is involved in the associations between cues and the subjective effects of the drug and/or in the motivation to obtain the conditional cues, since the selective DAD3R antagonist SB-277011-A attenuated that effect.

In our study, the number of total entries and entries to the cocaine-paired compartment were elevated during social defeat-induced CPP reactivation, which indicate that social stress induced locomotor activity during reactivation of CPP that resembles the response to cocaine. This may indicate that hyperactivity is driven by the drug-paired contextual stimuli, which has been thought to be associated with incentive motivational properties (Le Foll et al. , 2002), although the motivational aspects could not be tested in the procedure used in the present work, as the animals did not perform any operant task. Our findings also showed that defeated animals treated with SB-277011-A showed decreased of both total entries and cocaine entries, and no differences were seen between the number of entries to the cocaine chamber and to the saline chamber, which is consistent with a selective DAD3R occupancy by the antagonist.

Inhibition by DAD3R blockade of cocaine-CPP also would involve disruption of memory. However, this is unlikely since it has been reported that SB-277011-A does not alter learning or memory in rodents, but enhances social memory (Nakajima et al. , 2013, Pich and Collo, 2015). In human, improved cognition, including memory, social recognition, attention and executive function was found in schizophrenic patients receiving selective DAD3R antagonists (Gross et al. , 2013). Together, present data and these previous results may in part explain how DAD3R antagonism attenuates social stress-induced reinstatement of cocaine-CPP.

Here, we used the social stress versus two kind or physiological stressors (restraint and tail pinch) to compare the effects of the DAD3R antagonism on both kind of stress. According to previous findings using physiological stressors, such as foot-shock in rats (Xi et al. , 2004), our results showed that SB-277011-A also decreased reinstatement of CPP elicited by acute restraint and tail pinch, although a higher dose was needed.

The present experiments with SB-277011-A raise the issue of whether or not the presently observed robust decreases in physiological stress-induced CPP reactivation might be attributable to DAD2R antagonism rather than the DAD3R-selective antagonism. This is unlike, as SB-277011-A (up to 90 mg/kg, PO) and other selective DAD3R antagonists have no effect on spontaneous or stimulant-induced locomotion and failed to produce catalepsia when given at doses in excess of two times the higher dose used in the present experiment, in contrast to D2 antagonists (Vorel et al., 2002). In fact, SB-277011-A has 80- to 100-fold selectivity over other DA receptor and high affinity for human (pKi 7.95) and rat

(pKi 7.97) DAD3R (Heidbreder, 2005). Additionally, SB-277011-A reverses the extracellular DA levels in the NAc (DAD3R-rich) produced by the DAD3R agonist quinerolane, but this agonist effects on extracellular DA in the dorsal striatum (DAD3R-poor; DAD2R-rich) is not reversed by higher doses of SB-277011-A than the highest dose used in the present study (Reavill et al. , 2000).

Our study also shows that, by contrast to what was observed during the reactivation of CPP induced by social stress, mice exposed to physiological stressors did not displayed hyperlocomotion, as seen by the number of entries to the different chambers. This would imply that they spent more time in the cocaine-paired chamber without increasing the number of crossings between chambers and might indicate that the drug-paired contextual stimuli acquire stronger emotional value after reactivation of CPP by restraint or tail pinch than by social stress. It is also possible that these differences likely reflect the highly context-dependent nature of mice stress response, as has been suggested (Farrell et al. , 2018). It could also be hypothesized that physiological stress augmented the rewarding memory of cocaine, which motivated animals to remain in the conditioned chamber for longer periods, in agreement with previous studies (Shinohara et al. , 2018). However, it can not be ruled out a possible nonspecific SB-277011-A-induced decreased of locomotor activity at the highest doses used in the present study, and further studies are necessary to address this issue. Taken together, we can conclude that the attenuating effect of SB-277011-A on physiological stress-induced CPP reinstatement cannot be easily interpreted on the basis of the present data. On the other hand, the difference in the apparent potency of SB-277011A in reducing social stress- versus physiological stressors-induced reinstatement would be related to the anatomical mechanisms by which

these stresses influence the mesolimbic dopamine system.

SB-277011-A did not reduce cocaine-primed reinstatement

Cocaine addiction is well characterized by a persistent susceptibility to drug relapse. Drug re-exposure has been identified as one of the most important determinants of relapse in humans and in experimental animals. As stated above, the mesolimbic DA system is critically involved in cocaine- or cue-induced drug-seeking behavior (Kalivas and McFarland, 2003, Volkow et al., 2017). In addition, growing evidence suggests that brain DAD3Rs (particularly in the NAc) are up-regulated after prolonged withdrawal from chronic cocaine self-administration in both experimental animals and humans (Conrad et al. , 2010, Neisewander et al. , 2004). Accordingly, blockade of DAD3Rs might well be explained to antagonize cocaine- or cue-induced cocaine-seeking behavior. It is consistent with the report of (Xi et al. , 2013) in which microinjections of SB-277011-A into the NAc or amygdala significantly inhibited contextual cue-induced cocaine seeking under the same experimental conditions.

We here show that a priming injection of cocaine reactivated the extinguished CPP response, according to previous reports (Ashby et al., 2015). The present study also showed that administration of 24 or 48 mg/kg of SB-277011-A did not attenuate cocaine reactivation of the CPP response following CPP extinction. It has been reported that the DAD3R antagonist NGB 2904 significantly inhibited reinstatement elicited by 2 mg/kg, but not 10 mg/kg of cocaine (Xi et al. , 2006). Similarly, SB-277011-A inhibit cocaine-induced reinforcement except at high cocaine doses (Xi et al., 2005). It is well known that cocaine, by blocking DAT induces increase in extracellular DA in the NAc (Ritz et

al. , 1987). Thus, it is possible that the dose of cocaine used in the present study may produce an important increase in DA levels and that SB-277011-A antagonism actions may depend on endogenous DA levels, which in turn may explain why the same doses of SB-277011-A that produce inhibition of social defeat- and physiological stressors-triggered reinstatement of drug seeking behavior had no effect on 12.5 mg/kg cocaine prime. Overall, these data suggest a fundamental difference in the potential therapeutic efficacy of DAD3R antagonist agents is depending on how much cocaine is systemically present. Since previous findings have shown that SB-277011-A attenuated the rewarding properties of cocaine as measured by CPP (Vorel et al., 2002), another possible explanation is that cocaine-induced CPP is more vulnerable to DAD3R antagonism than cocaine-induced reinstatement.

The fact that the DAD3R antagonism did not blockade the drug prime-provoked reinstatement of CPP but did prevent its reestablishment when it was provoked by social and physiological stressors supports the existence of already known neurobiological differences between drug- and stress-induced relapse (McFarland et al. , 2004).

Dissociable role of glucocorticoids in attenuation of stress- and prime-induced reinstatement through the blockade of DAD3R

The HPA axis through glucocorticoids release and subsequently brain glucocorticoids concentration, plays a key role in mediating the reinforcement effects of drugs of abuse and stress-induced relapse (Koob et al. , 2014, Volkow et al. , 2011). These effects of glucocorticoids are hypothesized to be attributable to activation of DA neurons in the VTA and increased DA release in the NAc

(Barrot et al. , 2001) and support that glucocorticoids may be involved on the vulnerability to relapse after abstinence, particularly during stressful events (George and Koob, 2010). We addressed here the possible involvement of glucocorticoids in the inhibition of social stress-induced reinstatement of cocaine-seeking behavior that was seen in animals pretreated with the DAD3R antagonist SB-277011-A. Our results showed that there was an increase in corticosterone release during reinstatement induced by social defeat, restraint, tail pinch and a cocaine prime. In addition, we found that higher levels of glucocorticoids were correlated with higher scores during both psychosocial and physiological stress-induced reinstatement, but not in cocaine prime-induced reinstatement of CPP. Furthermore, corticosterone response in defeated animals as well as in mice that suffered a physiological stress was correlated significantly to cocaine entries. Therefore, the HPA axis-related glucocorticoids release could at least partially mediate stress-induced cocaine-seeking behavior, as has been proposed (Volkow et al., 2011).

In opposition, the results of our study point out that glucocorticoids are not critical for the cocaine prime-induced reinstatement of CPP, given that we did not find significant correlation between plasma corticosterone concentrations and the reinst-CPP score. In agreement, it has been reported that, while adrenalectomy did not block the reestablishment of cocaine self-administration induced by a cocaine prime in rats, this reestablishment was attenuated by the i.c.v. administration of a selective CRF1 receptor antagonist (Erb et al. , 1998), which suggests that CRF but not glucocorticoids would be involved in the cocaine prime-induced reinstatement. Additionally, it also has been shown that corticosterone synthesis inhibitors also failed to block cocaine-primed

reinstatement of drug seeking in rats (Mantsch and Goeders, 1999). Altogether, these data support a dissociable role for glucocorticoids in the reinstatement of CPP and suggest that subjective and behavioral aspects of cocaine-CPP reinstatement are possibly not associated with glucocorticoids.

In social defeated animals pretreated with SB-277011-A there was a parallelism between levels of corticosterone and behavior. Thus, the lower dose of SB-277011-A did not prevent either reinstatement or corticosterone release, whereas mice receiving SB-277011-A at the dose of 24 mg/kg, which inhibited social defeat-induced reinstatement, showed levels of corticosterone as in basal conditions. These data would suggest that DAD3R activation could at least partially mediate the social defeat-induced reinstatement through HPA-related corticosterone release. Such findings are consistent with reports that social defeat triggered the release of DA in the NAc, whereas this effect was diminished in mice deprived of glucocorticoid receptor (Barik et al. , 2013). Thus, by antagonizing DAD3R receptor, SB-277011-A may inhibit the effects of stress-induced release of glucocorticoids. Together, these findings suggest that the DAD3R may be a crucial intersection point between social stress and drug reinstatement.

Levels of plasma corticosterone differed neither in animals undergone acute social encounter with unfamiliar mice (undefeated mice, controls), which did not show reinstatement, and in defeated mice. These findings suggest that social defeat-induced reinstatement was not only dependent on HPA axis response and could led to the idea that undefeated and defeated individuals process motivationally salient information in different ways and by different neural

systems and, thus, may be or not sensitive to different triggers of reinstatement/relapse.

On the other hand, present results showed that in animals pretreated with SB-277011-A at doses of 24 and 48 mg/kg before suffering physiological stress there were a dose-dependent decrease of corticosterone plasma levels. However, only the higher dose of SB-277011-A did prevent reinstatement. This could mean that the dopaminoceptive neurons in the mesolimbic system would respond more sensitively to the corticosterone released by physiological stress, which would result in a strong activation of DA neurons and consequently in an amplified release of DA in the NAc, and could explain that a high dose of SB-277011-A is necessary to antagonize stress-induced reinstatement.

Our study also showed that pretreatment with SB-277011-A did reverse the enhanced corticosterone plasma levels induced by a cocaine prime but did not block the reestablishment of the CPP. These data seem to validate the hypothesis of a dissociable role for glucocorticoids in the reinstatement of cocaine seeking behavior, which would depend on the type of stimulus that triggers the reinstatement.

Summarizing, cocaine relapse is a complex phenomenon, engaging a variety of brain circuits and receptors that vary due to experimental details of animal studies. Our study could indicate that DAD3R modulates the reactivation of the incentive value and/or the rewarding memory of cocaine-associated cues induced by social and physiological stress, which seems to be associated to a glucocorticoid-dependent mechanism. Here we provide an overview of studies

pointing out DAD3R as a target for pharmacological therapy to treat drug addiction.

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Contributors

MVM and BRDC designed the study. MLL and BRDC wrote the protocol. RGB, BRDC, FC, GM, JMH and CN conducted the experiments. CN and MVM performed the statistical analysis and wrote the manuscript. All the authors read and approved the final version of the manuscript

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Figure legends

Fig. 1. Schematic representation of the experimental procedure. After 5 habituation and handling days, on day 0 animals were placed in the central corridor and allowed to explore the apparatus freely for 15 min. For each mouse, one chamber was randomly chosen to be paired with cocaine and the other chamber with saline. During days 1-4, animals were treated with cocaine and saline (conditioning sessions). The CPP test was conducted on day 5, exactly as in the preconditioning phase. The extinction sessions (twice a week for 7-8 weeks) were conducted as in the preconditioning phase. Once achieved the criterion of extinction, a second session was performed 48 h later in order to confirm extinction. One day after the second extinction test, different groups of mice received A) vehicle or SB-277011-A (24 or 48 mg/kg i.p.) 30 min before saline or a cocaine prime dose; B) vehicle or SB-277011-A (12 or 24 mg/kg i.p.) 30 min before an agonistic encounter or a social defeat stress; C) vehicle or SB-277011-A (24 or 48 mg/kg i.p.) 30 min before a tail pinch; and D) vehicle or SB-277011-A (24 or 48 mg/g i.p.) 30 min before restraint). Fifteen min after saline or cocaine prime and after the social or physiological stressful stimuli beginning, mice were allowed to explore the apparatus freely (reinstatement test) and were sacrificed 15 min later, immediately after the reinstatement.

Fig. 2. DAD3R blockade (24 or 48 mg/kg) did not attenuate the reinstatement of the conditioned place preference (CPP) paradigm induced by a priming dose of cocaine. (A, B, C, D) Time spent in the cocaine-paired chamber during the pre-conditioning (pre-C), post-conditioning (post-C), post-extinction (post-ext) and reinstatement (reinst) by male mice pretreated with vehicle plus saline (A),

vehicle plus cocaine prime (B) and SB-277011-A (24 or 48 mg/Kg i.p.) plus cocaine prime (C, D, respectively). *p < 0,05; **p < 0,01; ***p < 0,001 vs pre-C; $^{++}$p < 0,01 vs post-C; $^{\#}$p < 0,05 vs post-ext. Difference between the number of total entries to both the dots and stripes chambers (E), to the cocaine-paired chamber (F) and to the saline-conditioned chamber (G) during the reinst-test minus the number of entries during the pre-C test. Each bar corresponds to mean ± standard error of the mean. *p < 0,05 vs veh + prime saline; $^{+}$p < 0,05 vs SB 24 + priming cocaine. (H) Correlation between the reinstatement CPP-score and the difference in the number of entries to the saline-conditioned chamber during the reinst-test minus the number of entries during the pre-C test. The difference in the number of entries to the saline-paired chamber was negatively correlated with the reinst CPP-score.

Fig. 3. DAD3R blockade (24 mg/Kg) antagonized the reinstatement of CPP induced by social stress. Times spent in the cocaine-paired chamber by non-defeated (controls; A) and defeated (B, C, D) mice pretreated with vehicle (A, B), or SB-277011-A (12 or 24 mg/kg i.p.; C, D, respectively). **p < 0,01; ***p < 0,001 vs pre-C; $^{++}$p < 0,01; $^{+++}$p < 0,001 vs post-C; $^{\#}$p < 0,05 vs post-ext. Difference between the number of total entries to both the dots and stripes chambers (E), to the cocaine-paired chamber (F) and to the saline-conditioned chamber (G) during the reinst-test minus the number of entries during the pre-C test. Each bar corresponds to mean ± standard error of the mean. $^{+++}$p < 0,001 vs veh + not-defeat; ***p < 0,001 vs veh + social defeat. Correlations between the reinst CPP-score and the difference in the number of total entries (H), entries to the cocaine- (I) or saline-paired (J) chamber during the reinst-test minus those in the pre-C

test in socially-defeated mice. The difference in the number of total entries and of entries to the cocaine- or saline-paired chamber were positively correlated with the reinst CPP-score in a significant way.

Fig. 4. DAD3R blockade (48 mg/Kg) antagonized the reinstatement of the CPP elicited by restraint. (A, B, C) Time (in seconds) spent by mice in the cocaine-paired chamber during the pre-conditioning (pre-C), post-conditioning (post-C), post-extinction (post-ext) and reinstatement (reinst) test after 15 min of restraint when pretreated with vehicle (A) or SB-277011-A (24 mg/kg or 48 mg/kg i.p.) (B, C, respectively). ***p < 0,001 vs pre-C; $^{+++}$p < 0,001 vs post-C; $^{\#}$p < 0,05; $^{\#\#}$p < 0,01 vs post-ext. Difference between the number of total entries to both the dots and stripes chambers (D), to the cocaine-paired chamber (E) and to the saline-conditioned chamber (F) during the reinst-test of restrained animals minus those during the pre-C test. Each bar corresponds to mean ± standard error of the mean. *p < 0,05; **p < 0,01; ***p < 0,001 vs veh + restraint; $^{+}$p < 0,05 vs SB 24 + restraint. Correlation between the reinst CPP-score and the difference in the number of total entries (G), entries to the cocaine- (H) or saline-paired (I) chamber during the reinst-test in restrained animals minus those in the pre-C test. The difference in the number of total entries and in the entries to the cocaine- or saline-paired chamber were positively correlated with the reinst CPP-score in a significant way.

Fig. 5. DAD3R blockade (48 mg/Kg) antagonized the reinstatement of the CPP provoked by a tail pinch stimulus. (A, B, C) Time (in seconds) spent by mice in the cocaine-paired chamber during the pre-conditioning (pre-C), post-

conditioning (post-C), post-extinction (post-ext) and reinstatement (reinst) test after 15 min of tail pinch when pretreated with vehicle (A) or SB-277011-A (24 mg/kg or 48 mg/kg i.p.) (B, C, respectively). ***p < 0,001 vs pre-C; $^{+}$p<0,05, $^{++}$p < 0,01 vs post-C; $^{\#\#\#}$p < 0,001 vs post-ext. Difference between the number of total entries to both the dots and stripes chambers (D), to the cocaine-paired chamber (E) and to the saline-conditioned chamber (F) during the reinst-test of animals subjected to tail pinch minus those during the pre-C test. Each bar corresponds to mean ± standard error of the mean. *p < 0,05 vs veh + tail pinch. Correlation between the reinst CPP-score and the difference in the number of total entries (G), entries to the cocaine- (H) or saline-paired (I) chamber during the reinst-test minus those in the pre-C test in animals suffering a tail pinch stimulus.

Fig. 6. DAD3R blockade (24 mg/kg) antagonized the increase in plasma corticosterone concentration induced by a cocaine priming or by social or physiological stress. (A, F, K, P) Plasma corticosterone levels after the reinst-test of animals that received saline or a cocaine priming (A), mice that were subjected to an agonistic encounter (F), mice that were restrained (K) and animals subjected to tail pinch (P) 30 min after an injection of vehicle or SB-277011-A (12, 24, or 48 mg/kg i.p.). Each bar corresponds to mean ± standard error of the mean. (A) **p<0,01, ***p<0,001 vs basal; $^{+++}$p<0,001 vs veh + saline; $^{\#\#\#}$p<0,001 vs veh + cocaine prime. (F) ***p<0,001 vs basal; $^{+++}$p<0,001 vs veh + not-defeat; $^{\#\#\#}$p<0,001 vs veh + social defeat; $^{\$\$\$}$p<0,001 vs SB 12 + social defeat. (K) **p<0,01, ***p<0,001 vs basal; $^{+++}$p<0,001 vs veh + restraint. (P) ***p<0,001 vs basal; $^{+++}$p<0,001 vs veh + tail pinch. (B,G,L,Q) Correlation between the plasma levels of corticosterone after reinst-test of cocaine-primed mice (B), socially

defeated animals (G), restrained animals (L) and mice subjected to tail pinch (Q). Plasma corticosterone levels correlated positive and significantly with the reinstatement CPP-score of socially and physiologically stressed mice but not with the reinstatement CPP-score of cocaine-primed animals (Pearson correlation). (C-E) Correlation between the corticosterone plasma concentration after the reinstatement test and the difference in the number of total entries (C), entries to the cocaine- (D) or saline-paired (E) chamber during the reinst-test minus those in the pre-C test in cocaine-primed animals. Plasma corticosterone levels correlated positive and significantly with the difference in the number of total entries. (H-J) Correlation between the plasma corticosterone concentration after the reinstatement test and the difference in the number of total entries (H), entries to the cocaine- (I) or saline-paired (J) chamber during the reinst-test minus those in the pre-C test in socially defeated animals. Plasma corticosterone levels correlated positive and significantly with the difference in the number of total entries, and in the entries to the cocaine- and saline-paired chambers. (M-O) Correlation between the plasma corticosterone concentration after the reinstatement test and the difference in the number of total entries (M), entries to the cocaine- (N) or saline-paired (O) chamber during the reinst-test minus those in the pre-C test in restrained animals. Plasma corticosterone levels correlated positive and significantly with the difference in the number of total entries, and in the entries to the cocaine- and saline-paired compartments. (R-T) Correlation between the corticosterone plasma concentration after the reinstatement test and the difference in the number of total entries (R), entries to the cocaine- (S) or saline-paired (T) chamber during the reinst-test minus those in the pre-C test in socially-defeated animals. Plasma corticosterone levels correlated positive and

significantly with the difference in the number of total entries, and in the entries to the cocaine- and saline-paired chambers.