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Blockade of D3 receptor prevents changes in DAT and D3R expression in the mesolimbic dopaminergic circuit produced by social stress- and cocaine prime-induced reinstatement of cocaine-CPP

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ABSTRACT

Background: Cocaine may cause persistent changes in the brain, which are more apparent in DA transporter (DAT) and DA receptor availability within the nucleus accumbens (NAc). On the other hand, the DA D3 receptor (D3R) has emerged as a promising pharmacotherapeutic target for substance use disorders.

Aims: To assess the impact of selective D3R antagonism on DAT and D3R after reinstatement of cocaine preference (CPP) induced by an acute session of social defeat stress and a cocaine prime in mice after a period of abstinence.

Methods: Male mice were conditioned with 25 mg/kg of cocaine for 4 days. After 60 days of extinction training mice were pretreated with the selective D3R antagonist SB-277011A before the re-exposure to a priming dose of cocaine or to a single SDS session. CPP scores were determined and levels of DAT, D3R, phospho Akt (pAkt) and phospho mTOR (pmTOR) were assessed in the NAc shell.

Results: An increase in DAT and D3R expression was seen in the NAc after both a cocaine prime- and SDS-induced reinstatement of CPP. Pretreatment with SB-277011A blocked elevated DAT and D3R expression as well as SDS-induced reinstatement. By contrast, the blockade of D3R did not modified the cocaine prime-induced CPP. Changes in DAT and D3R expression does not seem to occur via the canonic pathway involving Akt/mTOR.

Conclusions: Our results suggest that the selective D3R antagonist ability to inhibit DAT and D3R up-regulation could represent a possible mechanism for its behavioral effects in cocaine-memories reinstatement induced by social stress.

INTRODUCTION

A primary feature of drug addiction, including cocaine addiction, is the high tendency to relapse during abstinence (Mantsch et al., 2016; O'Brien, 2005) for which there are not effective treatments. Despite lots preclinical and clinical studies onto drug abuse, relapse rate has not changed over the last decades (Sinha, 2011; Kalivas and O'Brien, 2008). In abstinent animal and human drug users, drug relapse can be triggered by acute exposure to the drug (drug prime in animals), drug-associated stimuli and contexts and stressful events (for review, see (Dong et al., 2017).

Drug-induced alterations in mesolimbic dopaminergic circuit mediate the acquisition of some types of rewarding memories, such as what is called *conditioned reinforcement*, that occurs when a former neutral stimulus reinforces or hardens conducts by its association with a primary reinforcer and develops into a reinforcer per se. This effect is mediated by an increase in phasic dopamine (DA) levels (Koob and Volkow, 2016; Volkow and Morales, 2015; Di Chiara, 2002; Baik, 2013). DA binds to DA receptors to trigger many molecular and behavioral changes (Neve et al., 2004). Among the five subtypes of DA receptors that have been identified (Beaulieu and Gainetdinov, 2011), the D3 receptor (D3R) subtype has several characteristics that have led to researches into its potential target for the treatment of DA-related diseases, supported by the fact of its restricted pattern of distribution, with the greatest abundance in the limbic system (Heidbreder and Newman, 2010; Sokoloff and Le Foll, 2017), including the nucleus accumbens (NAc, a component of the ventral striatum), a key component of the reward system. Addictive drugs, including cocaine, mediate their reinforcement properties by targeting the

mesocorticolimbic DA system (Radwanska et al., 2011). Hence, the presence of D3R in the NAc suggests a potential role of this receptor in reinforcement processes. Accordingly, the D3R has gained attention as a clinical target for treatment of addiction-related behavior (Ashok et al., 2017; Sokoloff and Le Foll, 2017; Ashby et al., 2015a; Maramai et al., 2016). Thus, blockade of D3R has been shown to inhibit psychostimulant and opiates self-administration (Xi et al., 2005; You et al., 2019), conditioned-place preference (CPP) to drugs of abuse (Xi et al., 2006), and reinstatement of drug-seeking behavior and CPP triggered by stress (Guerrero-Bautista et al., 2019; Xi et al., 2004).

A converging body of evidence from laboratory and human studies is pointing to a key role for stress in drug abuse (Koob and Schulkin, 2018; Sinha et al., 2011; Mantsch et al., 2016). Using the extinction-reinstatement model, data from this and other laboratories have shown that reinstatement of cocaine-induced CPP was assessed in mice after a short period of social defeat stress (SDS), as well as after different types of physiological stress (Guerrero-Bautista et al., 2019; Ribeiro Do Couto et al., 2009). Furthermore, stressful life events can promote relapse in addicts drive drug seeking by impinging upon the mesolimbic DA system, although underlying mechanisms are poorly understood (Cabib and Puglisi-Allegra, 2012; McCutcheon et al., 2012). Early studies have shown that stressors strongly activate the dopaminergic projection from VTA to NAc and that the subregion NAc shell is especially important for contextual control over relapse (Gibson et al., 2019).

The availability of DA in the brain is modulated by the DA transporter (DAT), a plasma membrane protein that translocates the released neurotransmitter from the extracellular space into the presynaptic neuron and

thus maintaining DA homeostasis. On the other hand, in addition to serving as classical postsynaptic receptor, D3R also functions as presynaptic receptor for controlling DA release and synthesis (Diaz et al., 2000; Chen et al., 2009). Thus, within the NAc it functions both as a presynaptic inhibitory autoreceptor on DA terminals arising from the ventral tegmental area (VTA) and as postsynaptic heteroreceptor on GABAergic medium spiny neurons (MSN) (Beaulieu and Gainetdinov, 2011; Liu et al., 2009).

There are several regions within the mesolimbic DA circuits that are directly affected by cocaine. Cocaine enhances DA neurotransmission in the brain, specifically in regions such as the NAc, by increasing extracellular DA levels by binding to and blocking the function of DAT in presynaptic terminals of DA neurons, leading a prolonged intensity of DA signaling (dela Pena et al., 2015). Chronic use of cocaine and other psychostimulant leads to long-term impact on DAT levels and activity, indicating that these compounds induce not only pharmacological but also physiological changes that could lead to transporter adaptation during addiction (Schmitt and Reith, 2010).

Since extinction therapy for addiction to different drugs has not been always a successful rehabilitation strategy (Milton, 2013; Conklin and Tiffany, 2002), it is important to dilucidate how drug-associated memories reinstate the rewarding memory of cocaine as well as the mechanism by which D3R antagonism reduces addiction-relevant behaviors in animal models. Therefore, characterizing how the D3R antagonism modulates dopaminergic striatal circuitry and associated behaviors is likely be essential to determine how stressful life events cause relapse to drug use. Using the CPP paradigm, the objective of the present study was to determine the impact of selective D3R antagonism on those

factors that control DA neurotransmission in the NAc shell (where D3R expression is high and is critical for stress-induced reinstatement of drug seeking; (Sokoloff et al., 2006; Mantsch et al., 2016), such as DAT and D3R, after reinstatement of CPP induced by i) an acute session of SDS; we have adopted the social defeat paradigm that profoundly alters the motivation for social interaction in rodent (Berton et al., 2006; Avgustinovich and Kovalenko, 2005) and ii) a cocaine prime in mice after a period of abstinence. To achieve a specific D3R blockade, we used the highly-selective D3R antagonist SB-277011A, which exhibit 100-fold selectivity over the D2R (Thanos et al., 2005). We first measured changes in DAT and D3R expression following CPP reinstatement, induced by SDS and by cocaine prime in mice pretreated with the D3R antagonist and in their respective controls. D3R not only activates $G_{i/o}$ proteins to inhibit cyclic adenosine monophosphate (cAMP) production, but also regulates other intracellular pathways, including extracellular mitogen-activated protein kinase (MEK)-signal-regulated kinases (ERK), phosphatidyl inositol 3-kinase (PI3K)-protein kinase B (Akt) and mecanistic target of rapamicine (mTOR) intracellular signaling (Collo et al., 2012). In addition, neurobiological findings indicate that drugs of abuse can lead to significant structural plasticity in rodent brain and that this is dependent on the availability of functional dopamine receptors, whose activation increased phosphorylation in the Akt/mTORC pathway (Le Foll et al., 2014). Because of the importance of Akt-mTOR signaling pathway in modulation of mood-related behavior and long-term memory formation (Radwanska et al., 2011; Le Foll et al., 2014), the current study has also tested the impact of SDS- and cocaine prime-induced CPP reinstatement on activated (phosphorylated) Akt

and mTOR protein levels in the NAc shell, as well the possible expression of Akt and D3R in GABAergic neurons.

METHODS

Subjects

Adult male C57BL/6 mice were purchased from Charles River Laboratories (France). The animals arrived at the laboratory at 42 days of age (23 ± 0.19 g when they arrived and 23 ± 0.26 g when the experiment begun) and were housed in groups of four in in polypropylene cages (25 L × 25 W × 14,5 H cm) for 10 days (n=139 at the beginning of the study) or for 1 month (n=8; used as nonaggressive opponents in the test of social interaction). Animals 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). Mice were maintained on arrival in a room with controlled temperature (22 ± 2°C) and humidity (50 ± 10%), with free access to water and food. 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.

Drugs

Cocaine hydrochloride (Alcaliber, Madrid, Spain) was dissolved in sterile saline (NaCl 0,9%). SB-277011-A (N-[trans-4-[cyano-3,4-dihydro-2(1H)-isoquinolinyl)ethyl]cyclohexyl]-4-quinolinecarboxamide dihydrochloride; Tocris,

St. Louis, MO, USA; selective D3R antagonist) was dissolved in desionized distilled water (vehicle). Injections were administered intraperitoneally (i.p.) in a volume of 0.01 ml/g body weight.

Conditioned place preference (CPP)

Acquisition of cocaine-induced conditioned place preference. The CPP chambers (Panlab, Barcelona, Spain) consisted in two equally sized compartments (20L×18W×25H cm) with distinct visual and tactile cues (one room had a black smooth floor and walls with black dots, and the other had a grey rough floor and walls with equally spaced grey stripes), which were interconnected by a rectangular corridor (20Lx7Wx25H cm) with transparent walls. A weight transducer technology and PPCWIN software allowed detection and analysis of animal position throughout the test and the number of entries in each compartment. The acquisition of CPP consisted of three distinct phases: a preconditioning phase, a conditioning phase, and a testing phase (Fig. 1A,B). During the preconditioning phase (Pre-test), mice freely explored the two compartments for 900 second and the time spent in each compartment during the exploratory period was recorded. Animals showing strong unconditioned aversion (<33% of session time) or preference (>67%) for any compartment were excluded from subsequent procedures (n=3). In the second phase (conditioning), guillotine doors blocked access from both chambers to the central corridor. Eight groups of animals were conditioned with cocaine. On days 2 and 4, 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 4h, 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 (Rodriguez-Arias et al., 2017; Guerrero-Bautista et al., 2019). On days 3 and 5, 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 (25 mg/kg i.p.) immediately before confinement in the drug-paired compartment for 30 min. One animal was discarded in this phase. The third phase or post-conditioning (Post-test) was conducted on day 6, exactly as in the preconditioning phase (free entry to each chamber for 900 s). The CPP scores were calculated by subtracting time spent in the cocaine-paired compartment during the post-test from that spent during the Pre-test. A positive score indicated a place preference; a negative score indicates a place aversion.

Extinction of cocaine-conditioned place preference. Animal acquired cocaine-CPP, as described previously (Guerrero-Bautista et al., 2019), followed by extinction training. From day 7 and during seven-eight weeks, depending on the group (which is approximately equivalent to 4 years in human (Quinn, 2005) animals underwent extinction sessions two days a week, which were conducted exactly as in the pre-test and the post-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 in the drug-associated chamber during the extinction (ext)-test with regard to that in the Pre C-test for each group. Thus, all the animal in each group underwent the same number of extinction sessions, independently of their individual scores. Once achieved the criterion (Pre C-test=ext), a new session was performed to confirm extinction. Two days after extinction was

ratified, mice were subjected to a psychological stressful stimulus or were administered with a cocaine priming (12.5 mg/kg i.p.) and then tested to evaluate the reinstatement in the cocaine-induced CPP. The dose of cocaine prime, half of that used for conditioning, was chosen as it has been previously shown it elicits CPP reactivation in male mice that have undergone extinction of cocaine-induced CPP (Rodriguez-Arias et al., 2009; Guerrero-Bautista et al., 2019; Maldonado et al., 2006). The reinstatement (reinst) test was similar to the pre-C, post-C and ext-tests, that is, free entry to each chamber for 900 s. All procedures were performed in the colony room, a non-contingent place to the previous conditioning injections.

Social defeat

The effects of SDS in a social interaction (which is considered a type of social stress; (Ribeiro Do Couto et al., 2009; Miczek et al., 2011), 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), which had previous fighting experience and had been previously screened for an elevated level of aggression. This encounter lasted 15 min and took place in a neutral transparent plastic cage (23L×13,5W×13H 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 (Rodríguez-Arias et al. 1998). All defeated mice experienced similar levels of aggression because attack behavior from the opponent initiated immediately after seeing the experimental mouse (latency<30 s). With the aim of demonstrating the lack of effects of the procedure itself, an

additional group underwent an agonistic encounter with another animal that was previously grouped. These animals elicit attack but never initiate it, and no aggressive behaviors were observed.

Experimental groups

Experiment 1. Effect of D3R antagonism on the reinstatement of cocaine-induced CPP evoked by a cocaine prime. In order to provoke the reinstatement in 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 (Fig. 1A). Control animals were administered with a saline injection instead of cocaine to confirm that the restoration of the CPP was due to the drug. The possible involvement of D3R in the reinstatement in the cocaine-induced CPP evoked by a cocaine priming was studied by means of the administration of a single dose of a D3R antagonist, SB-277011-A (24 or 48 mg/kg) 30 min before the cocaine priming. Control mice received a vehicle injection instead of the D3R antagonist. The conditioning and priming doses of cocaine were based on previously published studies in mice (Guerrero-Bautista et al., 2019; Rodriguez-Arias et al., 2009).

Experiment 2. Effect of D3R antagonism on the reinstatement of the cocaine-induced CPP evoked by social stress. In a separate cohort of animals, the reinstatement test was performed immediately after social defeat. To evaluate the implication of D3R 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. Control mice were injected with vehicle instead of the antagonist (Fig. 1B).

Doses of SB-277011-A in experiments 1-2 were selected based on previous studies from our laboratory and from other authors, in which similar doses were evaluated in mice and rats in the CPP paradigm or drug self-administration (Xi et al., 2004; Gilbert et al., 2005; Xi et al., 2005; Ashby et al., 2015b; Guerrero-Bautista et al., 2019).

Tissue collection and Western blot analysis for D3R, DAT, Akt and mTOR

Fifteen min after the reinstatement test, mice were killed by cervical dislocation, and the brains were removed and stored at -80ºC until use for Western blot. Brains were sliced on a cryostat and kept at −20°C until each region of interest comes into the cutting plane. For NAc (medial shell) study, three consecutive 500-µm coronal slides were made corresponding to approximately +1.70 to +0.98 mm from bregma, according to the atlas of Franklin & Paxinos (2008). Bilateral 1-mm^2 punches of the NAc shell were collected into Eppendorf tubes, according to the method of (Leng et al., 2004). Four bilateral NAc punches were placed in Eppendorf tubes containing 50 µl homogenization buffer containing phosphate buffered saline (PBS), 10% sodium dodecyl sulfate (SDS), protease inhibitors and an inhibitor of phosphatases cocktail set. The tubes were immediately frozen in dry ice and stored at -80°C until assaying. Samples were sonicated, vortexed and sonicated again before centrifugation at 10.000 rpm for 10 min at 4°C. Protein content was determined using the BCA method. Samples containing equal quantities of total proteins (10–20 µg, depending on the protein of interest) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes

(EMD Millipore Corporation, USA). Western blot analysis was performed using the following primary antibodies: mouse monoclonal anti-D3R (1:100; sc-136170, Santa Cruz Biotechnology Inc.); rat monoclonal anti-DAT (1:2000; MAB369, EMD Millipore Corporation); rabbit anti-pAkt (1:750; #4060, Cell Signaling Technology); and rabbit anti-pmTOR (1:500; #2983, Cell Signaling Technology). After three washings with TBST (Tris buffered saline tween), the membranes were incubated 1 h at room temperature, with the peroxidase-labeled secondary anti-mouse and anti-rabbit antibodies (#31460; #31430; 1:10000, Thermo Fisher Scientific, Waltham, MA, USA). After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent Western blot detection system (ECLPlus, Thermo Fisher Scientific) and visualized by a LAS 500 imager (GE Healthcare). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control for all the experiments. The integrated optical density of the bands was normalized to the background values. Relative variations between bands of the experimental and the control samples were calculated in the same image. Antibodies were stripped from the blots by incubation with stripping buffer (glycine 25 mM and SDS 1%, pH 2) for 1 h at 37 ºC. The blots were subsequently reblocked and probed with rabbit monoclonal anti-GAPDH (#2118; 1:5000, Cell Signaling Technology Inc.) as our loading control for D3R, DAT, pAkt and pmTOR. The ratios of D3R/GAPDH, DAT/GAPDH, pAkt/GAPDH and pmTOR/GAPDH were plotted and analyzed. Protein levels were corrected for individual levels.

Immunofluorescence

Immediately following SD or cocaine priming reinstatement, another set of mice were deeply anesthetized with pentobarbital and then transcardially perfused with saline followed by a cold fixative solution containing 4% paraformaldehyde in 0.1 M borate buffer, pH 9.5. Brains were removed and kept in the same fixative solution containing sucrose (30%) for 3 h and then placed in phosphate buffered saline (PBS) containing 30% sucrose overnight. Twenty-five micrometer rostro-caudally coronal sections containing the NAc were obtained using a freezing microtome (Leica, Nussloch, Germany), collected in cryoprotectant and stored at -20ºC until processing. The atlas of (Frankin and Paxinos, 2008) was used to identify different brain regions. Brain sections were rinsed in PBS, and an antigen retrieval procedure was applied by treating sections with citrate buffer (10 mM citric acid in 0.05 Tween-20, pH 6.0) at 90°C for 20 minutes before the blocking procedure. Non-specific Fc binding sites were blocked with 7% BSA/0.3% Triton-X-100 in PBS for 2 h (at RT), and the sections were incubated for 60 h (4ºC, constant shaking) with the following primary antibodies: rabbit anti-pAkt (1:200, #4060, Cell Signaling, Danvers, Massachusetts, USA); mouse anti-D3R (1:150, sc-136170; Santa Cruz, Dallas, Texas, USA); and chicken anti-glutamate decarboxylase (GAD; 1:750, NBP1-02161 Novus Biologicals, Centennials, Colorado, USA). Alexa Fluor 488 donkey anti-rabbit IgG (1:1000; A-21206, Invitrogen, Eugene, OR, USA), Alexa Fluor 555 donkey anti-mouse IgG (1:1000; A-31570, Invitrogen), and Alexa Fluor 647 goat anti-chicken IgG (1:1000; A-21449, Invitrogen) labelled secondary antibodies were applied for 4 h. After washing, sections were incubated in 4, 6-diamino-2-phenylindole (DAPI, 1:25,000) for 1 min and mounted in ProLong® Gold antifade reagent (Invitrogen).

Confocal analysis

The brain sections were examined using a Leica TCS SP8 (Leica, Illinois, USA) confocal microscope and LAS X Software (Leica Microsystems). Images from the NAc shell were captured from low magnification to high magnification (10X to 40X objective). Confocal images were obtained using 405-nm excitation for DAPI, 488-nm excitation for Alexa Fluor 488, 555-nm excitation for Alexa Fluor 555 and 647-nm excitation for Alexa Fluor 647. Emitted light was detected in the range of 405-490 nm for DAPI, 510-550 nm for Alexa Fluor 488, 555-640 nm for Alexa Fluor 555 and 647-775 nm for Alexa Fluor 647. Every channel was captured separately to avoid spectral cross-talking. The confocal microscope settings were established and maintained by Leica and local technicians for optimal resolution.

Statistical analysis

Data were analyzed using GraphPad Prism 7.0 (GraphPad Software; CA, USA), and p-values < 0.05 were considered statistically significant. All descriptive data were presented as means and standard error of mean (SEM). Results of behavioral tests (Score) and Western blot were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. The analysis of the difference between the times spent by mice in the cocaine-paired compartment during the Pre-C and Post-C tests was performed using paired Student's *t*-test.

Results

Effects of the selective D3R antagonist SB-277011A on the reinstatement of cocaine-CPP induced by a cocaine prime and by social stress

Mice underwent cocaine-CPP for 4 days and were tested for preference on day 5. A significant preference for the cocaine-paired compartment was found for all the series of animals during the posttest compared with that during the pretest (Fig. 2A-D, veh+sal (n = 14): Pre-C T, 290.4±12.66 s, Post-C T, 372.3±13.76 s; t_{13}=7.398, p<0.0001; veh+coc prime (n = 20): Pre-C T, 291.0±13.36 s, Post-C T, 416±20.24 s; t_{19}=7.627, p<0.0001; SB24+coc prime (n = 11): Pre-C T, 264.4±13.49 s, Post-C T, 394.8±18.48 s; t_{10}=7.154, p<0.0001; SB48+coc prime (n = 7): Pre-C T, 291.9±10.94 s, Post-C T, 397.1±9.90 s, t_6=10.10, p<0.0001). One-way ANOVA with repeated measured revealed significant differences between treatments for all the series of animals (Fig. 2A: F(1.9,25) = 21; p < 0.0001); Fig. 2B: F(1.4,27) = 13; p = 0.0004, n = 20; Fig. 2C, F(1.35,32) = 31, p < 0.0001,; Fig.2D, F(1.2,16) = 65, p < 0.0001,). Next, mice were given a 60 days abstinence period before subsequent re-exposure to a single priming dose of cocaine. Consistent with previous work (Guerrero-Bautista et al., 2019), our results showed that the preference could be significantly extinguished and that cocaine-CPP could be reinstated by a priming dose of cocaine (Fig. 2B) but not by an injection of saline, as shown the CPP scores in Fig. 2A). Since CPP expression occurred on a cocaine-free test day, the behavior is dependent upon the retrieval of the association between the environment and the affective state produced by the drug (White and Carr, 1985). Therefore, behavior in the place

preference depended upon both the affective and the memory improving properties of the reinforcers under examination (White and Carr, 1985). To study whether D3R is implicated in the reinstatement of cocaine-CPP promoted by a cocaine prime, two different doses of SB-277011-A were administered (24 and 48 mg/kg, i.p.) 30 min before the priming injection. Our results revealed that the blockade of D3R with any of the two doses of the antagonist did not inhibit the reinstatement of cocaine-induced CPP evoked by a drug prime, given the higher CPP scores in the cocaine-conditioned chamber during the post-reinst test than during the post-ext tests (Fig.2C,D).

We next explored the behavioral responsiveness to an acute episode of social stress and the effects of the selective blockade of D3R on social stress-induced reinstatement of cocaine-CPP (Fig. 3). A significant preference for the cocaine-paired compartment was found for all the series of animals during the posttest compared with that during the pretest (Fig. 3A-D, veh+non-defeated (n = 16): Pre-C T, 273.0±8.48 s, Post-C T, 377.3±14.63 s; t_{15}=8.579, p<0.0001; veh+social defeat (n = 23): Pre-C T, 263.9.±10.06 s, Post-C T, 426.4±18.81 s; t_{22}=7.863, p<0.0001; SB12+social defeat (n = 14): Pre-C T, 276.4±14.51 s, Post-C T, 360.4±13.46 s; t_{13}=8.709, p<0.0001; SB24+social defeat (n = 30): Pre-C T, 255.0±14.51 s, Post-C T, 361.2±22.78 s; t_{29}=7.684, p<0.0001). One-way ANOVA showed significant differences between treatments for all the series of animals ($F(1.3,20 = 32$, $p < 0.0001$ for veh + not defeat,; $F(1.7,37) = 34$, $p < 0.001$ for social defeat,; $F(1.1,15) = 9$, $p = 0.0076$ for SB 12 + social defeat, n = 14; $F(1.9,36) = 25$, $p < 0.0001$ for SB 24 + social defeat). Post hoc test showed that cocaine-CPP could be extinguished (Fig. 3A,B) and reinstated by a single episode of social stress (Fig. 2B) but not by a social interaction without defeat

(Fig. 3A). Administration of 12 mg/kg of SB-277011A 30 min before the social defeat episode did not modifies the reinstatement of the cocaine-CPP (Fig. 3C), given that the CPP score in the cocaine-paired compartment was similar to that obtained during the post-C test and significantly higher than that obtained during post-ext test. By contrast, a higher dose of the D3R antagonist (24 mg/kg i.p.) did block the reinstatement of cocaine-CPP by social stress (Fig. 3D). Thus, post hoc test showed that mice injected with this dose of the antagonist before the antagonistic encounter did not augment the CPP score during the post-reinst test with regard to the post-ext test. Additionally, this CPP score was lower than that in the post-C test (Fig. 3D).

Changes in DAT and D3R protein expression in the NAc following reinstatement of cocaine-CPP induced by a cocaine prime and by social stress

We first studied the changes in DAT and D3R protein expression in the NAc after the reinstatement of place preference induced by a cocaine prime in mice that had extinguished the CPP, given that this nucleus is enriched in both DAT and D3R, which are known to play a critical role in DA homeostasis. In addition, D3R is postulated to be critically involved in mood, emotion, and diseases, included addiction (Sokoloff and Le Foll, 2017). One-way ANOVA for DAT and D3R expression after cocaine prime-induced reinstatement showed significant differences between treatments ($F(2,19) = 26.08$, $p<0.0001$ for DAT; $F(2,22) = 5.958$, $p = 0.0086$ for D3R). Post hoc test showed that there was a significant increase of DAT expression after cocaine prime-induced CPP reinstatement (Fig. 4B; n = 8), compared with that observed in the group receiving saline instead

cocaine prime (n = 8). This increase was significantly prevented by administration of SB-277011A (24 mg/kg i.p.) 30 min before reinstatement of cocaine-induced CPP evoked by a drug prime (Fig. 4B; n = 6). Similar results were obtained when the expression of D3R was studied (Fig. 4C), with a significant increase of the protein expression (n = 9) compared with its control (n = 8) that was antagonized by SB-277011A (24 mg/kg i.p.; n = 8). Since the NAc mainly contains GABA output neurons (medium spiny neurons; MSN), we also performed an immunofluorescence study to investigate if D3R expression after memory retrieval occurred in GAD-positive neurons in the NAc shell. As depicted in Fig. 4D, we found that D3R-positive neurons were also GAD-positive, although some cells that expressed GAD did not exhibit D3R.

We next investigated DAT and D3R protein expression in the NAc after the reinstatement of cocaine place preference induced by an episode of social stress (Fig. 5). One-way ANOVA with repeated measures revealed significant differences between treatments for all the series of animals ($F(3,28) = 6.474$, $p = 0.0018$ for DAT; $F(F(3,25) = 6.048$, $p = 0.0030$ for D3R). Post hoc test indicated that there was a significant increase in DAT expression in the NAc after social defeat-induced reinstatement (n = 6), when compared with non-defeated mice (n = 9). The administration of SB-277011A (12 and 24 mg/kg i.p., n = 8, n = 9, respectively) 30 min before the reinstatement of cocaine-CPP induced by social defeat antagonized the increased DAT expression in a dose-dependent manner (Fig. 5A). Similar results were obtained when D3R was quantified (Fig. 5B). A significant increase in D3R expression was found in mice suffering CPP reinstatement induced by social stress (n = 6), when compared with the non-

defeated group (n = 9). In addition, administration of SB-277011A (12 and 24 mg/kg i.p.; n = 7, n = 7, respectively) 30 min before the episode of social defeat did block elevated D3R expression in a dose-dependent manner (Fig. 5B). To extend our results, we performed a phenotypic study to characterize the neuronal populations involved in D3R expression. We discover that D3R-positive cells were GABAergic neurons (Fig. 5C).

Regulation of Akt and mTOR expression in the NAc following reinstatement of CPP induced by a priming dose of cocaine and by social stress

Given the proposed role of Akt-mTOR signaling cascade in the striatum in dopaminergic transmission and behaviors (Beaulieu et al., 2005), we next measured the impact of cocaine priming on the levels of phosphorylated (activated) Akt (pAkt) and mTOR (pmTOR) in the NAc shell (Fig. 6A). One-way ANOVA revealed no significant differences in pAkt expression between treatments after the reinstatement of cocaine place preference induced by cocaine prime ($F(2,16) = 2.445$, $p = 0.1184$). Statistical significance was not found in the levels of pAkt in the NAc shell after cocaine prime-induced reinstatement of the cocaine-CPP (n = 6), when compared with the control group (n = 7). On the other hand, administration of 24 mg/kg of SB-277011A prior the cocaine prime injection (n = 6) did not induce significant alterations in pAkt levels. In a like manner, one-way ANOVA did not show significant differences between groups in pmTOR levels in the NAc after the reinstatement of CPP evoked by a priming dose of cocaine ($F(2,16) = 1.371$; $p = 0.2822$). We did not find significant modifications in pmTOR expression after the reinstatement of cocaine CPP (n=6)

regarding control group (n=7). SB-277011A injection prior social defeat session (24 mg/kg; n=6) did not alter significantly pmTOR levels.

We next performed a characterization of the neural populations involved in pAkt expression. As shown in Fig. 6B, the majority of pAkt-positive neurons are GABAergic. In addition, D3R was found in pAkt-positive neurons (Fig. 6C), which adds evidence to the notion that D3R may regulate Akt signaling following exposure to cocaine prime. Supporting this hipothesis, when we studied the colocalization of pAkt, D3R and GAD in the neurons of the NAc shell after relapse in cocaine-CPP induced by a priming drug injection, we observed that the majority of pAkt-positive cells that co-expressed D3R were GABA-ergic (Fig. 8).

When we evaluated the effects of social defeat on pAkt and pmTOR expression in the NAc, one-way ANOVA revealed not significant differences between treatments. As depicted in Fig. 7A, pAkt levels after social defeat (n = 6) were similar to those obtained in non-defeated mice (n = 7). When SB-277011A (12 mg/kg, n = 6; 24 mg/kg, n = 6) was administered prior the social defeat episode, there was a small and not significant decrease in pAkt expression either. Likewise, one-way ANOVA did not reveal significant changes in pmTOR levels in the NAc after the reinstatement of cocaine CPP provoked by social defeat ($F(3,21) = 2.000$; $p = 0.1513$). Reinstatement of CPP induced by social stress (n=5) did not significantly alter pmTOR/GAPDH ratio in NAc when compared with non-defeated animals (n=6). No significant modifications were observed either after the administration of 12 mg/kg (n=5) or 24 mg/kg (n=5) of SB-277011-A.

Then, we performed immunofluorescence study to characterize the possible co-localization of pAkt and GAD in the NAc shell following social defeat.

The results revealed that pAkt was localized in GAD-positive neurons (Fig. 7B). As seen in Fig. 7C, D3R was colocalized with pAkt in the NAc shell, which adds evidence to the notion that D3R may regulate Akt signaling. Next, we studied the colocalization of pAkt, D3R and GAD in the neurons of the NAc shell after reinstatement of cocaine-induced CPP evoked by acute social stress. Our data revealed that most pAkt-expressing neurons that were D3R-positive were also GAD-positive (Fig. 8), which support that D3R might control Akt signaling in GABA-ergic neurons of the NAc following relapse in cocaine-CPP induced by social stress.

Discussion

The behavioral results of this study corroborated that the D3R may have a great implication on susceptibility to relapse to cocaine-induced CPP provoked by social stress after a long extinction training (60 days, approximately) in mice. Specifically, stress reinstated extinguished cocaine-induced CPP, and administration of a selective D3R antagonist prior to a single social defeat episode significantly attenuated social defeat-induced reinstatement, which corroborate previous results showing that both social and physiological stress-induced cocaine-CPP reinstatement were prevented by blocking D3R (Guerrero-Bautista et al., 2019). These findings suggested a mechanism wherein the D3R is activated by dopaminergic inputs during social defeat, leading to the subsequent enhance of the rewarding memory of cocaine (cocaine-CPP). Previous results have shown that social defeat encounters increase DA release in the mesolimbic pathway (Berton et al., 2006). Our findings are consistent with the hypothesis that stress would increase the mesolimbic DA neurons activity. Thus, antagonizing DA signaling at the postsynaptic D3R, the selective antagonist SB-277011A would inhibit the effects of DA release. This effect was not evident when reinstatement was induced by a priming dose of cocaine, which would suggests that D3R activation is not necessary for reinstatement of cocaine-associated memories, and support previous findings indicating the existence of neurobiological differences between stress- and drug-induced relapse (McFarland et al., 2004). In fact, we previously also reported that the increased levels of plasma corticosterone that were seen during psychosocial stress-induced CPP reinstatement were correlated with higher scores, but not in the

case of cocaine prime-induced reinstatement of CPP (Guerrero-Bautista et al., 2019).

The mesolimbic DA system and in particular DA in the NAc shell is largely responsible for the reward and reward-based learning (Wise, 1996; Di Chiara, 2002; Wise, 2008), which reinforce behaviors essential for survival. Drugs of abuse modify this system to sustain compulsive drug seeking and consumption. DA neurotransmission is regulated by DAT in pre-synaptic terminals of DA neurons, specifically in the regions such as the NAc, which is involved in execution of motivated behavior based in previous experience (Day and Carelli, 2007). In addition to DAT, DA release and synthesis are modulated by inhibitory autoreceptors D2R and D3R located on DA nerve terminals, thus regulating synaptic DA levels (Diaz et al., 2000). Other studies also propose that D3 autoreceptor also regulates DA neurotransmission through its interaction with DAT (McGinnis et al., 2016). Recent reports have suggested a long lasting impact of cocaine on DA system function that provided a putative mechanism for relapse, which can occur in cocaine addicts after years of abstinence (Siciliano et al., 2016). However, the regulation of DA signaling by aversive stimuli or a cocaine prime that cause CPP reinstatement is not well characterized yet. We addressed here the effects of D3R antagonism on changes in DAT and D3R levels induced by a cocaine priming- and social stress-induced reinstatement of extinguished cocaine-induced CPP. Stressors negatively impact emotional state and drive drug seeking and promote relapse in addicts by inducing craving (Sinha et al., 2000; Wemm and Sinha, 2019). We found that during reinstatement to cocaine-induced CPP produced by both social stress and cocaine prime there was an elevation of DAT and D3R levels in the NAc shell of mice. Other studies also

evidenced that cue-induced incubation of cocaine craving coincided with an increase of D3R expression in the NAc after prolonged withdrawal from cocaine, suggesting a possible role for increased D3R signaling in incubation of cocaine craving (Conrad et al., 2010), which is in accordance with present data. It is well stablished that cocaine binds to DAT, which is a plasma membrane protein that reuptake the released DA back into the presynaptic DA nerve terminal, thus inducing an increase of extracellular DA levels and related subsequent signaling during drug abuse (Kristensen et al., 2011). On the other hand, and in addition to its role as classical postsynaptic receptor, the D3R is expressed in dopaminergic terminals, serving as presynaptic autoreceptor that decreases DA synthesis and release. Taking together, our findings would indicate that the increase in DAT expression in the NAc in mice after a drug priming and an episode of social stress may be a neuronal compensatory mechanism to decrease synaptic DA levels and its action at postsynaptic level. Since the present study was centered in the NAc shell, changes in DAT and D3R in the terminals arising from the VTA innervating the NAc (DAT and presynaptic D3R) and D3R in the MSN in the NAc would reflect their involvement in reinstatement of cocaine-induced CPP at the NAc shell level. However, it cannot be rule out the possible involvement of DAT and/or D3R at VTA as well as in dendrites on that reinstatement. In fact, it has been shown that since midbrain DA neurons also release DA from their dendrites, the DAT inhibition induced by cocaine causes not only an increase of DA in the NAc and other limbic areas innervated by the VTA, but also the VTA itself (Beckstead et al., 2004; Lüscher and Malenka, 2011).

The up-regulation of D3R shown in the present study would serve as parallel feedback mechanism aimed at reducing extracellular DA levels when

high DA levels activate D3R. In accordance with these findings, chronic use of cocaine leads to long-term impact on DAT regulation, and membrane trafficking and activities by mechanisms that are not well defined (Urban et al 2012), indicating that psychostimulants induce physiological changes that could lead to transporter and presynaptic receptors D2 and D3 adaptation during psychostimulant addiction (Schmitt and Reith, 2010). Nonetheless, the acute effect of cocaine on DAT expression are less consistent, since some research findings have shown that cocaine produces a rapid increase in DAT levels whereas others have found no effects in DAT expression (Daws et al., 2002; Gorentla and Vaughan, 2005).

Accumulating evidence suggests limited efficacy of the conditioned stimulus memory retrieval extinction procedure in modifying pathological memories (Mortensen and Amara, 2003). Recently, the D3R has gained attention with respect to the meso-accumbens VTA-NAc DA system, drug-induced reward, motivation and relapse and the behavioral manifestation of many drugs of abuse. This has led to the suggestion that the D3R may be a useful therapeutic target for anti-addiction medications (for review, see (Sokoloff and Le Foll, 2017). Our results showed that the pretreatment with the selective D3R antagonist SB-277011A antagonized in a dose-dependent manner the increase in DAT and D3R expression in the NAc shell induced by social stress, which paralleled the attenuated social defeat-induced reinstatement that was seen in animals receiving the D3R blocker. Overall, these data suggest that the potential therapeutic efficacy of D3R antagonist agents in attenuating stress-induced relapse of cocaine-CPP memories would involve down-regulation of DAT and D3R in the NAc.

Within the NAc, D3R functions both as a presynaptic autoreceptor on dopaminergic terminals arising from the VTA and as postsynaptic receptor on MSNs, and thus positioned to modulate presynaptic DA release and postsynaptic DA receptor-mediated changes in neuronal activity, respectively (Beaulieu and Gainetdinov, 2011). Although from our results we cannot distinguish if the decrease in D3R induced by the D3R antagonist affects pre- and/or postsynaptic D3R, the D3R antagonist-induced downregulation of DAT and D3R availability would lead to an increase in extracellular DA levels in the NAc, which was concomitant with the ability of the antagonist in reducing the social stress-induced reinstatement of cocaine CPP. This is somewhat surprising, as NAc DA is essential for reward-related learning and to drug seeking. Recently, it has been shown that stress-induced drug seeking can occur in a DA terminal environment of low DA tone (Twining et al., 2015). In addition, significant evidence from both animal and human studies suggest that a hypoactivity of reward function may occur following protracted withdrawal from drugs of abuse (Koob and Schulkin, 2019), and it has been speculated that blocking D3R may increase extra-neuronal DA (Sokoloff et al., 2006), known to be low in addicted individuals (Volkow et al., 2009; Martinez et al., 2007). In fact, previous clinical trials have investigated the effect of medication that increase striatal DA transmission as treatment of cocaine dependence, although inconsistent results have been obtained (Urban and Martinez, 2012). Taken together, these findings and present results may suggest that the decrease of DAT and D3R that was seen in animals pretreated with the D3R antagonist would increase synaptic DA levels in the NAc then attenuating social defeat-induced reinstatement. So, the selective D3R antagonist ability to inhibit DAT and D3R up-regulation could represent a possible

mechanism for its behavioral effects in cocaine reinstatement induced by social defeat.

We previously reported that D3R subtype is critical for the stress-induced cocaine CPP but not for cocaine priming-induced CPP (Guerrero-Bautista et al., 2019), which has been corroborated in the present study. Present findings showed that although the D3R antagonist prevented the synaptic plasticity induced by both of these manipulations (an increase of DAT and D3R levels), cocaine-CPP was reinstated by a single injection of cocaine, which would suggest that SB-277011A did not disrupt cocaine-associated memory when it was reactivated by the drug. This is difficult to interpret as similar plasticity in the NAc was induced by both cocaine prime and an episode of social defeat. The mechanisms by which D3R antagonism alters addiction-relevant behaviors in animal models is currently unknown (Payer et al., 2014). From present results, we propose that an additional and different plasticity induction involving other neurotransmitter systems might be required for cocaine prime-induced CPP reinstatement, which would not involve the D3R. The complexity of NAc circuitry generated by its multiple different inputs from the prefrontal cortex, the hyppocampus and the basolateral amygdala provides a major change for understanding the neural circuit modifications that underlie reinstatement of drug-seeking behavior after a withdrawal period.

The mechanisms underlying neuronal D3R signaling are poorly characterized. Thus, Akt-mTOR has been proposed as a signaling cascade in the striatum in dopaminergic transmission and behaviors (Beaulieu et al., 2005). As mentioned previously, it is well characterized that D3R activates $G_{i/o}$ proteins to inhibit cAMP production, but D3R also regulates other intracellular pathways,

including the ERK and Akt-mTOR cascades (Collo et al., 2012). Since the Akt signaling pathway has been recently implicated in psychostimulant-induced behavioral and cellular effects, in the present study we measured the impact of social stress- and cocaine prime induced cocaine-CPP reinstatement on the levels of phosphorylated Akt-mTOR In the NAc shell. The data presented herein showed that pAkt and mTOR were expressed in MSN, according to previous studies (Schwartz et al., 1998). Although statistical significance in pAkt and pmTOR in the NAc was not found when cocaine-associated memory was reactivated by a cocaine prime, small trends toward increased in those phosphorylated proteins was detected in the NAc shell. These effects tended to be decreased when animals were pretreated with the selective D3R antagonist. Likewise, no significant modifications in pAkt and pmTOR were seen either after social defeat-induced cocaine reinstatement or in animals pretreated with the D3R antagonist prior to social stress. Therefore, it would be tempting to speculate that Akt-mTOR signaling in the NAc is not regulated by the reactivation of cocaine-associated memory. Further experiments are needed to determine the signaling cascade that are linked to cocaine prime- and social stress-induced CPP reinstatement. Hence, other studies and recent unpublished results from our laboratory indicate that mTOR pathway in hippocampus and amygdala would participate in the reactivation of cocaine-related memory (Shi et al., 2014).

In summary, the results presented here indicated that DAT and D3R in the NAc are engaged by reactivation of cocaine reward memories. Blockade of D3R prevented the increase in DAT and D3R levels produced by social stress, which was accompanied by attenuated social defeat-induced reinstatement that was seen in animals receiving the D3R blocker. Overall, these data suggest that the

potential therapeutic efficacy of D3R antagonist agents in attenuating stress-induced relapse of cocaine-CPP memories may involve down-regulation of DAT and D3R in the NAc.

Declaration of conflicting interests

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FIGURE LEGENDS

Figure 1. Timeline of the behavioral experimental procedure. A: reinstatement induced by cocaine prime. B: reinstatement induced by a social defeat session. 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 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. Fifteen min after saline or cocaine prime and after the social stressful stimuli beginning, mice were allowed to explore the apparatus freely (reinstatement test) and were sacrificed 15 min later, immediately after the reinstatement.

Figure 2. Effect of selective D3R blockade with SB-277011-A (24 or 48 mg/kg i.p.) on the reinstatement of conditioned place preference (CPP) induced by a priming dose of cocaine. Mean preference score in the cocaine-paired chamber during the post-conditioning (post-C), post-extinction (post-ext) and reinstatement (Post-reinst) in 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). Preference scores indicate the time spent in the cocaine-paired compartment (in seconds) during the post-extinction (Post-ext) test or during the post-reinstatement (Post-reinst) test minus that spent during the post-conditioning test (Post-C). ***$p < 0,001$ vs post-C; $^{+++}p < 0,001$ vs post-ext. Each bar corresponds to mean ± standard error of the mean.

Figure 3. Effects of selective D3R blockade with SB-277011-A (12 or 24 mg/kg i.p.) on the reinstatement of conditioned place preference (CPP) induced 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). Preference scores indicate the time spent in the cocaine-paired compartment (in seconds) during the post-extinction (Post-ext) test or during the post-reinstatement (Post-reinst) test minus that spent during the post-conditioning test (Post-C). As shown in D, D3R blockade (24 mg/kg) antagonized the reinstatement induced by social stress. ***$p < 0,001$ vs post-C; $^{+}p < 0,05$, $^{+++}p < 0,001$ vs post-ext.

Figure 4. Semiquantitative analysis and representative immunoblot of dopamine transporter (DAT; B) and dopamine D3 receptor (D3R; C) protein expression in the NAc shell. Mice were conditioned to cocaine and after 60 days of abstinence received an injection of saline (veh+saline; controls), a priming dose of cocaine (12,5 mg/kg; veh+coc prime) or an injection of the D3R antagonist SB-277011-A (24 mg/kg) prior a priming dose of cocaine (SB 24+coc prime). A preference test was conducted giving the animals free access to both compartments of the CPP apparatus for 15 min. The analyzed region within the NAc rostral and NAc shell caudal is schematically illustrated in A (modificated from Franklin and Paxinos,

2008). *p < 0,05, **p < 0,01 vs veh+sal; $^{+}$p < 0,05, $^{+++}$p < 0,001 vs veh+priming coc. D: Representative confocal images showing NAc shell coronal sections immunostained for D3R (green), GAD (red; GABAergic neurons) and DAPI (nuclear stain, blue) from controls (VEH-SAL) and from cocaine prime-induced reinstatement (VEH-COC) mice. Z-axis: z = -6,4 µm (veh + sal); z = -8,9 µm (veh + coc priming); z = 90,2 µm (veh + ND); z = 93,9 µm (veh + SD); z =97,5 µm (SB + SD). *Scale bars*, 50 µm.

Figure 5. Semiquantitative analysis and representative immunoblot of dopamine transporter (DAT; A) and dopamine D3 receptor (D3R; B) protein expression in the NAc shell. Mice were conditioned to cocaine and after 60 days of abstinence underwent a non-aggressive social encounter (veh+non-defeated; controls), an antagonistic encounter (veh+social defeat) or an injection of the D3R antagonist SB-277011-A (12 or 24 mg/kg) prior to the antagonistic encounter (SB 12 and SB 24+social defeated, respectively). A preference test was conducted giving the animals free access to both compartments of the CPP apparatus for 15 min. **p < 0,01 vs veh+not-defeated; $^{+}$p < 0,05, $^{++}$p < 0,01 vs veh+social defeat. C: Representative confocal images showing NAc shell coronal sections immunostained for D3R (green), GAD (red; GABAergic neurons) and DAPI (nuclear stain, blue) from controls (veh-ND), from social defeat-induced reinstatement (veh-SD) and from mice receiving an injection of SB-277011-A (24 mg/kg) prior to the antagonistic encounter (SB-SD). Z-axis: z = -6,4 µm (veh + sal); z = -8,9 µm (veh + coc priming); z = 90,2 µm (veh + ND); z = 93,9 µm (veh + SD); z =97,5 µm (SB + SD). *Scale bars*, 50 µm.

Figure 6. Semiquantitative analysis and representative immunoblot of phosphorylated (activated) Akt (pAkt) and mTOR (pmTOR) protein expression in the NAc shell (A). Mice were conditioned to cocaine and after 60 days of abstinence received an injection of saline (veh+saline; controls), a priming dose of cocaine (12,5 mg/kg; veh+coc prime) or an injection of the D3R antagonist SB-277011-A (24 mg/kg) prior a priming dose of cocaine (SB 24+coc prime). A preference test was conducted giving the animals free access to both compartments of the CPP apparatus for 15 min. No significant changes in pAkt levels were observed between groups. B, C: Representative confocal images showing NAc shell coronal sections immunostained for pAkt (green; B, C), GAD (B, red; GABAergic neurons), D3R (C, red) and DAPI (B, C; nuclear stain, blue) from controls (VEH-SAL) and from cocaine prime-induced reinstatement (VEH-COC). Z-axis: z = -6,4 µm (veh + sal); z = -8,9 µm (veh + coc priming); z = 90,2 µm (veh + ND); z = 93,9 µm (veh + SD); z =97,5 µm (SB + SD). *Scale bars*, 50 µm.

Figure 7. Semiquantitative analysis and representative immunoblot of phosphorylated (activated) Akt (pAkt) and mTOR (pmTOR) proteins expression in the NAc shell (A). Mice were conditioned to cocaine and after 60 days of abstinence underwent a non-aggressive social encounter (veh+not-defeat; controls), an antagonistic encounter (veh+SD) or an injection of the D3R antagonist SB-277011-A (12 or 24 mg/kg) prior to the antagonistic encounter. A preference test was conducted giving the animals free access to both compartments of the CPP apparatus for 15 min. No significant changes in pAkt levels were observed between groups. B, C: Representative confocal images showing NAc shell coronal sections immunostained for pAkt (green; B, C), GAD

(B, red; GABAergic neurons), D3R (C, red) and DAPI (B, C; nuclear stain, blue) from controls (veh+ND), from social defeat-induced reinstatement (VEH-SD) mice and from mice receiving an injection of SB-277011-A (24 mg/kg) prior to the antagonistic encounter (SB24-SD). Z-axis: z = -6,4 µm (veh + sal); z = -8,9 µm (veh + coc priming); z = 90,2 µm (veh + ND); z = 93,9 µm (veh + SD); z =97,5 µm (SB + SD). *Scale bars*, 50 µm.

Figure 8. Representative confocal images showing NAc shell coronal sections immunostained for pAkt (green), D3R (red) and GAD (yellow; GABAergic neurons), from cocaine prime-induced reinstatement (VEH-COC) and its control (VEH-SAL), and from social defeat-induced reinstatement (VEH-SD) mice, their mice controls (VEH-ND), and from mice receiving an injection of SB-277011-A (24 mg/kg) prior to the antagonistic encounter (SB24-SD). Z-axis: z = -6,4 µm (veh + sal); z = -8,9 µm (veh + coc priming); z = 90,2 µm (veh + ND); z = 93,9 µm (veh + SD); z =97,5 µm (SB + SD). *Scale bars*, 50 µm.