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Drug-free and context-dependent locomotor hyperactivity in DBA/2 J mice previously treated with repeated cocaine: Relationship with behavioral sensitization and role of noradrenergic receptors

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ABSTRACT

Drug-associated contexts and discrete cues can trigger motivational states responsible for drug-seeking behavior and relapse. In preclinical research, drug-free conditioned hyperactivity has been used to investigate the expression of memories associated with psychostimulant drug effects. Addictive drugs can produce long-lasting sensitization to their psychomotor actions, a phenomenon known as behavioral sensitization. The neuroplasticity underlying behavioral sensitization appears to be involved in pathological drug pursuit and abuse. In the present study we evaluated drug-free, context-dependent hyperactivity in DBA/2 J mice previously treated with cocaine and ~~we~~ explored whether this conditioned effect was related to behavioral sensitization. Given the role of noradrenergic (NA) neurotransmission in memory retrieval, consolidation and reconsolidation processes, we also investigated whether conditioned hyperactivity in a drug-free state was mediated by NA receptors. Animals underwent a sensitization protocol with six cocaine injections (0, 5, 10 or 20 mg/kg) paired to a particular floor cue. Three days after this sensitization phase, all animals were exposed to the same familiar floor environment without drug treatment. A second test with an unfamiliar floor was conducted 24 h later. Conditioned hyperactivity was also explored after one or three cocaine pairings and was evaluated for its duration (with repeated familiar vs. unfamiliar floor tests). In a series of pharmacological experiments, we evaluated the effects propranolol (a non-selective antagonist of β1- and β2-receptors) and prazosin (α1-receptor antagonist) on conditioned hyperactivity. Cocaine treatment produced both robust sensitization and drug-free conditioned hyperactivity, an effect that lasted up to 17 days (with cocaine 20 mg/kg). A significant correlation between the magnitude of cocaine sensitization and the level of conditioned hyperactivity was found. Propranolol, but not prazosin, blocked context-dependent hyperlocomotion in a drug-free state. Our data, together with a vast body of literature, indicate that the NA system plays a key role in the retrieval and behavioral expression of drug-associated memories.

1. Introduction

Drugs of abuse are powerful natural or artificial compounds that alter brain structure and function through their pharmacological actions on several neurotransmitter systems (Hyman et al., 2006; Nestler, 2001). In vulnerable individuals, the occasional use of addictive drugs can lead to consumption patterns that escalate from sporadic and voluntary to recurrent and unmanageable (Everitt and Robbins, 2016; Piazza and Deroche-Gamonet, 2013; Volkow and Morales, 2015). This transition is often thought to be partly due to maladaptive plasticity of appetitive learning and memory processes crucial for survival (Everitt, 2014; Hyman, 2005; Torregrossa et al., 2011). Drug-induced molecular and cellular plasticity may result in tolerance and/or sensitization (Hyman et al., 2006; Koob and Le Moal, 2008; Stewart and Badiani, 1993). Whereas tolerance refers to a decrease in the effectiveness of a given drug, sensitization involves a progressive increase in its effect (Kalivas and Stewart, 1991; Nestler and Aghajanian, 1997; Stewart and Badiani, 1993). Influential theories of addiction have identified tolerance and sensitization as key neural processes underlying different aspects of the addictive phenotype such as negative emotionality,

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vulnerability to stress and pathological motivation towards drug consumption (Ahmed et al., 2002; Berridge and Robinson, 2016; Koob and Le Moal, 2005; Piazza and Deroche-Gamonet, 2013; Robinson and Berridge, 2000, 2008; Wise and Koob, 2014). Sensitization-related neuroplasticity has been particularly relevant to explain pathological drug-seeking and drug-taking behaviors with psychostimulants (Berridge and Robinson, 2016; Piazza and Deroche-Gamonet, 2013; Robinson and Berridge, 2008; Robinson, 1993; Vanderschuren and Pierce, 2010).

At a behavioral level, drug-induced sensitization generally refers to the progressive and long-lasting increase in the stimulating effects observed after repeated drug administration (Robinson, 1993; Robinson and Berridge, 2008; Steketee and Kalivas, 2011). Behavioral sensitization can occur in response to various drugs of abuse, but it is best characterized for psychostimulants like cocaine and amphetamines (Berridge and Robinson, 2016; Hyman et al., 2006). Many aspects of drug-induced sensitization are thought to be a result of a hyper-responsiveness of dopaminergic mesocorticolimbic neurons to repeated drug-administration (Robinson, 1993; Robinson and Berridge, 2008). Coupled with the loss of medial prefrontal inhibitory control of striatal systems, dopaminergic sensitization is thought to be a key contributor in the shift of manageable to unmanageable drug consumption (Everitt, 2014; Everitt and Robbins, 2005). Drug-induced sensitization has also been observed in humans and correlates with an enhancement in drug seeking and drug taking behaviors, but also with craving after abstinence (Narendran and Martinez, 2008; Steketee and Kalivas, 2011). In pre-clinical research psychomotor sensitization has been considered an indirect measure of mesolimbic DA sensitization, among other neuroplastic changes (Adinoff et al., 2007; Berridge and Robinson, 2016; Koob and Volkow, 2010).

Although sensitization is considered a non-associative type of learning, a strong corpus of data has shown that neuroadaptations underlying drug-induced behavioral sensitization can be modulated by environmental cues (Anagnostaras and Robinson, 1996; Brabant et al., 2011; Robinson, 1993; Tirelli and Heidbreder, 1999). After prolonged drug exposure, environmental stimuli become associated with the unconditioned effects of the drug through a Pavlovian learning process (Everitt and Robbins, 2016; Milton and Everitt, 2012). Contextual stimuli, by acquiring motivational significance, can trigger compulsive drug-seeking behavior and relapse in humans (Childress et al., 1999; Garavan et al., 2000; Grant et al., 1996). Human imaging studies using positron emission tomography and functional magnetic resonance imaging have shown that presentation of drug-related cues induces changes in the mesocorticolimbic system that correlate with craving measures (Goudriaan et al., 2013; Kilts et al., 2004; Li et al., 2005; Volkow et al., 2006; Wong et al., 2006). Animal models have proved key to study drug-induced context-dependent behavioral effects and underlying mechanisms (Cunningham et al., 2006; Li et al., 2015; Wong et al., 2006). Drug-induced conditioned place preference (CPP) and cue-mediated reinstatement of extinguished drug-seeking have been extensively used to investigate drug-associated conditioning (Bardo and Bevins, 2000; Cunningham et al., 2006; Sanchis-Segura and Spanagel, 2006; Tzschentke, 2007). In the case of psychomotor sensitization, context-dependent effects have been investigated in two different ways. Animals that show locomotor sensitization in a given context show a weaker or null expression of drug-induced sensitization when tested in an unfamiliar or unpaired context (Anagnostaras and Robinson, 1996; Badiani and Robinson, 2004; Mattson et al., 2007; Vezina and Leyton, 2009; Wang and Hsiao, 2003). Additionally, previously sensitized animals can show context-dependent hyperactivity in a drug-free state (Post et al., 1992; Wei and Li, 2014; White and Rauhut, 2014). Given the partial overlap and connection between activational and motivational systems (Everitt, 2014; Salamone and Correa, 2012), conditioned hyperactivity tests might allow researchers to investigate memory retrieval and activation of systems that participate in cue-mediated drug-seeking and relapse.

The noradrenergic (NA) system is involved in multiple cognitive processes including arousal, attention, learning, as well as retrieval, consolidation and reconsolidation of memories (Aston-Jones and Cohen, 2005; España et al., 2016; Fitzgerald et al., 2016; Harley, 1987; McGaugh, 2000; Mueller and Cahill, 2010; Sara, 2009). These actions are primarily mediated via alpha- and beta-adrenoceptors (α-, β-AR) (Ferry et al., 1999; Hein, 2006; Schmidt and Weinshenker, 2014). Laboratory animal research shows that β1/β2-AR and α1-AR are involved in a number of conditioned effects induced by drugs of abuse such as cocaine, morphine and amphetamine (Bernardi et al., 2006, 2009; Fricks-Gleason and Marshall, 2008; Latagliata et al., 2017; Otis and Mueller, 2011). The NA system mediates the retrieval of emotional memories by triggering cellular processes that promote new protein synthesis and synaptic plasticity (Sara, 2009). Current evidence indicates that new protein synthesis is required for the reconsolidation of drug-retrieved memories, a phenomenon predominantly investigated using CPP. The non-selective β-AR antagonist propranolol (administered post-CPP test) can prevent reconsolidation and promote extinction learning of cocaine- and morphine-induced CPP in rodents (Bernardi et al., 2006, 2009; Huang et al., 2018). However, α-AR system is also implicated as acute administration of the α-AR antagonist prazosin, administered following memory retrieval, is effective at reducing expression of cocaine-induced CPP (Bernardi et al., 2009; Bernardi and Lattal, 2012). To date, only two studies have examined the role of the NA system in conditioned hyperactivity in drug-sensitized animals. In one of these studies animals were administered morphine during seven consecutive days in a given context. Two days later, animals received a drug-free retrieval session (during which they showed context-dependent hyperactivity) followed by the administration of propranolol. A subsequent test performed 24 h later showed that conditioned hyperactivity was absent in animals that received propranolol after memory reactivation but not in those receiving vehicle (Wei and Li, 2014). These data are relevant because they suggest that propranolol prevented reconsolidation of drug-triggered memories, thus facilitating extinction. Similar effects were found with methamphetamine sensitization and conditioned hyperactivity in mice treated with prazosin (White and Rauhut, 2014). The direct effects of propranolol and prazosin on memory retrieval-induced behavior (i.e. conditioned hyperactivity) in sensitized animals, however, remain to be investigated. In the present study we explored drug-free, context-dependent hyperactivity in DBA/2 J mice previously treated with cocaine. We explored whether this conditioned effect was related to the presence and magnitude of behavioral sensitization and whether it required activation of β- or α1-AR for its expression.

2. Materials and methods

2.1. Subjects

A total of 311 male DBA/2 J mice were used in the present studies (JAX® mice were purchased through Charles River Laboratories España S.A., Barcelona, Spain). Animals (6 weeks old upon arrival) were housed in groups of four per cage and acclimated to the colony room for 2 weeks before experiments started. The colony was maintained in a humidity- (~50%) and temperature-controlled (21 ± 2 °C) environment under a 12-h light/dark cycle (lights on at 8:00 am) with standard laboratory rodent chow (Panlab S.L., Spain) and tap water available ad libitum. Behavioral testing took place between 10:00 am and 2:00 pm. All experimental procedures complied with the European Community Council Directive (86/609/ECC) for the use of laboratory animal subjects.

2.2. Drugs

Cocaine hydrochloride was dissolved at the concentration of 0.5, 1 or 2 mg/ml and administered intraperitoneally (IP) at doses of 5, 10 or

20 mg/kg. Propranolol was dissolved in saline at 1 mg/ml and injected IP at 10 mg/kg. Prazosin was dissolved in saline at 0.05 mg/ml and injected IP at 0.5 mg/kg. All drugs were obtained from Sigma-Aldrich Química S.L. (Spain) and prepared fresh daily in 0.9% saline.

2.3. Apparatus

Four activity monitor chambers (30 cm long × 15 cm wide × 20 cm high) contained in individual sound-attenuated and light-controlled enclosures (Cibertec S.A., Spain) were used in the present studies. The activity boxes were equipped with infrared emitter/detector pairs placed along the length of the box, 2.2 cm above the floor and 5 cm apart. These detectors provided activity counts (beam breaks expressed as cm per min) during all locomotor activity sessions. Flooring cues (interchangeable grid and hole floors) were used as conditioning stimuli. The hole floor consisted of perforated stainless steel sheet metal (16 gauge) containing 6.4 mm round holes on 9.5 mm staggered centers. For the grid hole floor, 2.3 mm stainless steel rods were mounted 6.4 mm apart on acrylic rails. These two floor types were chosen based on previous studies showing drug-induced conditioning and no differences in the spontaneous preference (Cunningham et al., 2006, 2003; Font et al., 2006). Locomotor activity data were generated by MAP1.5 software (Cibertec S.A., Spain) run in Windows XP.

2.4. Experimental procedures

Each experiment consisted of two experimental phases, starting with a cocaine-induced locomotor stimulation and/or sensitization phase followed by subsequent behavioral drug-free tests (see Fig. 1).

2.4.1. Experiment 1: cocaine-induced behavioral sensitization and drug-free hyperactivity evaluated in a context previously associated with cocaine

Mice (N = 95) were randomly assigned to four different groups; saline (n = 23), cocaine 5 mg/kg (n = 24), cocaine 10 mg/kg (n = 24) and cocaine 20 mg/kg (n = 24). The sensitization phase consisted of a total of 6 sessions conducted in 12 days; animals were trained every other day with a two-day break between the first and the last three sessions (days 1, 3, 5, 8, 10 and 12). Mice were weighed and habituated to the experimental room 30 min before experiments began. Prior to experiments, mice were injected with cocaine (0, 5, 10 or 20 mg/kg, IP) and immediately placed into the test apparatus, where floors were either grid or hole (orders counterbalanced across groups). Floor-type remained constant for each animal throughout sensitization experiments. Horizontal locomotor activity (in cm) was registered for a total of 15 min. On days 2, 4, 6, 7, 9 and 11, animals remained in the colony room and were left undisturbed. Sensitization protocol and cocaine doses used in the present study were adapted from previously published literature (Lessov and Phillips, 2003; Michel and Tirelli, 2002; Pastor et al., 2010).

A second phase evaluated drug-free behavior in all animals and included a total of 6 tests (T1-T6; 15-min test session). On T1, T3, and T5 mice were tested for horizontal activity on the same floor previously used during the sensitization phase. On T2, T4, and T6 animals were exposed to a new, unfamiliar floor (animals that received grid during the sensitization phase, now received hole and vice versa). Three days after the last sensitization session, mice received T1 (day 15; familiar cue) followed for T2 24 h later (day 16; unfamiliar cue). The following tests were separated by a 6-day break; T3 and T4 were conducted on days 22 and 23, and T5-T6 on days 29 and 30, respectively. Pilot studies showed that the order of stimulus presentation on T1 and T2 did not influence the expression of conditioned hyperactivity. No treatments or tests were conducted between drug-free testing sessions. All drug-free tests were conducted after habituating animals to the experimental room for 30 min before testing. Mice did not receive a saline IP injection before testing; our pilot studies showed no differences in locomotor activity for both T1 and T2 in animals tested immediately after a saline injection compared to those simply placed in the apparatus without an injection (data not shown). The purpose of T1, T3 and T5 was to investigate whether the mere exposure to a context previously associated with cocaine could induce locomotor hyperactivity per se in a drug-free state. To determine whether this effect was indeed context-dependent, we tested animals in the presence of a novel, unpaired floor (T2, T4 and T6). Given that we were interested in studying the duration of this drug-free effect, we included an additional T7-T8 (familiar cue, day 36; unfamiliar cue day 37) with the dose that showed the strongest and more lasting conditioning (20 mg/kg). This T7–8 experiment was done with a separate set of 24 animals (saline or cocaine) that showed comparable sensitization levels and conditioned stimulation on T1, T3 and T5. Since the effect was lost on T7 no further tests were conducted. Through all experiments floor types were counterbalanced (familiar and unfamiliar; hole vs. grid and vice versa) in all groups.

2.4.2. Experiment 2: effect of one or three cocaine administrations on the expression of drug-free conditioned hyperactivity

On Experiment 1 we found that a 6-session protocol with cocaine produced drug-free, context-dependent locomotor hyperactivity. Experiment 2 was designed to evaluate whether a shorter protocol of 1 or 3 sessions of cocaine exposure could also produce drug-free conditioned hyperactivity. Mice (n = 48) were randomly assigned to either a 1- or a 3-session treatment group (cocaine 0 or 20 mg/kg). Procedures were similar to those followed on Experiment 1, except that only 1 or 3 cocaine pairings (days 1, 3 and 5) were administered. Drug free tests (T1 and T2) were performed 72 h after the last cocaine-induced behavioral test. As described on Experiment 1, T1 and T2 evaluated drug-free behavioral activity under familiar- or unfamiliar-floor conditions (i.e., paired vs. new), respectively.

2.4.3. Experiment 3: effect of the β-AR antagonist propranolol on drug-free conditioned hyperactivity in animals previously treated with cocaine

This experiment evaluated whether the non-selective blockade of β-1AR and β-2AR could prevent the drug-free and context-dependent enhanced locomotion that we found in animals previously treated with

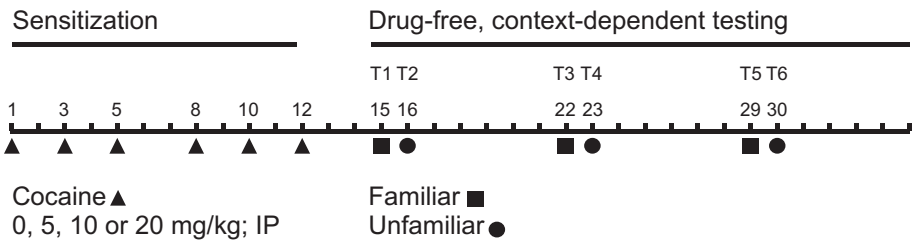

Fig. 1. General experimental design and timeline. All experiments included two phases: a psychomotor sensitization phase with cocaine followed by a drug-free evaluation of locomotor activity in familiar (floor previously associated with cocaine; T1, T3 and T5) and unfamiliar (novel floor; T2, T4 and T6) contexts. Experiment 1 evaluated the effect of three different doses of cocaine on sensitization and the duration of drug-free, conditioned hyperactivity; T1-T6. The rest of the experiments included T1 and T2 only. All experiments, except Experiment 2, used a sensitization protocol with 6 cocaine injections. Experiment 2 explored the effect of 1 vs. 3 cocaine injections on conditioned locomotor activity with drug-free testing (T1 and T2) starting 3 days after the last cocaine administration (mirroring the same time window between completion of cocaine administration and the beginning of drug-free testing used for all experiments). In Experiments 3 and 4, propranolol and prazosin were administered 30 min before T1 and T2. The horizontal line with tick marks represents days. Animals were left undisturbed (no pharmacological treatment or behavioral manipulation) on days not numbered on the timeline.

cocaine. Mice (n = 48) were randomly assigned to a saline or cocaine (20 mg/kg) treatment group. After a 6-session sensitization protocol (as described in Experiment 1) each group was divided into two pretreatment subgroups, saline or propranolol. Three days after the last cocaine challenge (T1; day 15), animals received propranolol (0 or 10 mg/kg), 30 min before being placed in the activity monitor, where locomotor behavior was measured for 15 min under the same floor conditions used during the sensitization phase. T2 (day 16) mirrored the procedure followed for T1 except that an unfamiliar floor was used. The dose of propranolol administered in this study and injection times were based on previous work by Font and Cunningham (2012).

2.4.4. Experiment 4: effect of the α1-AR antagonist prazosin on drug-free conditioned hyperactivity in animals previously treated with cocaine

Experiment 4 was carried out to determine whether a different pharmacological compound selective for α1-AR noradrenergic receptors could produce similar behavioral effects. Experimental protocol was otherwise identical to the previous experiments described. Mice (n = 48) were assigned to a cocaine treatment group (0 or 20 mg/kg), and then dividend into saline or prazosin pretreatment subgroups. Prazosin (0.5 mg/kg) was administered 30 min before T1 and T2 testing. A further test with prazosin 2 mg/kg (only on T1) was conducted with a separate set of 48 animals. Prazosin doses and injection times were based on previously published studies (Bernardi et al., 2009; Gazarini et al., 2013).

2.5. Statistical analyses

Analyses of variance (one-, or two- way ANOVA, with repeated measures when required) were used to analyze behavioral activity data during the sensitization and drug-free testing phases. Cocaine doses (0, 5, 10 or 20 mg/kg) were treated as a between group factor, whereas days and drug-free challenge day was treated as a within-group factor. Significant main effects and interactions were further analyzed using Tukey HSD tests. *t*-tests for unrelated samples were also performed when comparing saline vs. cocaine 20 mg/kg groups. Pearson's correlation analyses were used to explore the relationship between conditioned hyperactivity and the magnitude of cocaine-induced locomotor sensitization, as defined by the following formula: Sensitization index (SNZ_{idx}) = locomotion on Day 12 - locomotion on Day 1. Statistical significance was established at an alpha level below 0.05 for all analyses. Data were analyzed using STATISTICA 6.1 software (StatSoft, Inc., Tulsa, OK) and Prism 7 (GraphPad Software Inc.).

3. Results

3.1. Cocaine-induced behavioral sensitization

Preliminary analyses were conducted to examine potential differences in motor performance linked to floor cues. We found no effect of floor type (hole vs. grid) on horizontal activity, and this absence of effect was found across treatments; no effect of floor was found in both saline and cocaine-stimulated animals. These results confirm previous studies reporting the same lack of effect on spontaneous preference for the hole and grid floors (Cunningham et al., 2003). Floor type was therefore not included as a factor in the analyses. For the sensitization phase (Experiment 1), a two-way ANOVA with repeated measures (day x cocaine dose) showed significant main effects of day [$F(5,455) = 20.27$, $p < 0.01$] and cocaine dose [$F(3,91) = 98.15$, $p < 0.01$]. Also, an interaction effect was found between the two factors [$F(15455) = 4.12$, $p < 0.01$]. As it can be seen in Fig. 2, cocaine produced a clear acute activating effect; Tukey HSD post-hoc analysis revealed significant increases in locomotor activity on day 1 for all cocaine doses ($p < 0.01$), with the highest cocaine dose (20 mg/kg) producing a significantly higher ($p < 0.01$) stimulant response than the lowest (5 mg/kg). Subsequent post-hoc multiple comparisons

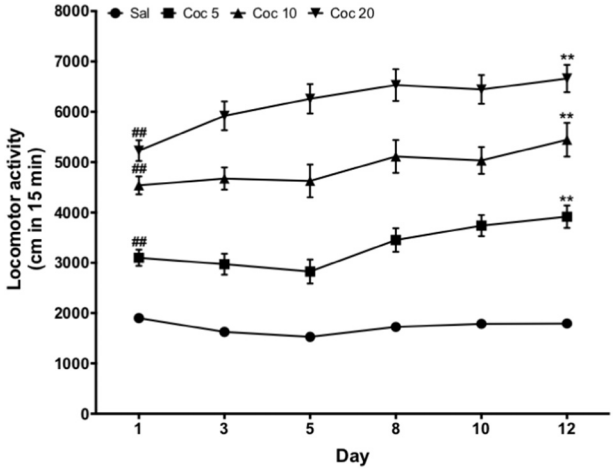

Fig. 2. Acquisition of locomotor sensitization. Locomotor activity of saline and cocaine 5, 10, and 20 mg/kg groups on days 1, 3, 5, 8, 10, and 12. Locomotor activity is expressed as mean ± S.E.M. (S.E.M. in the saline group is not displayed as it is smaller than the size of the symbol). ## Significant at $p < 0.01$ with respect to saline on day 1; ** significant at $p < 0.01$with respect to the same group on day 1. N = 23–24 per group.

revealed a cocaine-induced sensitized response as an increase in locomotor activity across days for all cocaine groups was found; $p < 0.01$, comparing day 12 to day 1.

3.2. Expression and persistence of drug-free, context-dependent hyperactivity

Fig. 3 shows behavior from drug-free tests conducted with Experiment 1 animals. Panel A shows T1, T3 and T5 (paired, familiar floor), and panel B shows T2, T4 and T6 (unfamiliar floor) data respectively. A two-way ANOVA with repeated measures (cocaine dose x test session) on T1, T3 and T5 revealed an effect of cocaine dose [$F(3,91) = 8.5$, $p < 0.01$] and a significant interaction between the two factors [$F(3,182) = 2.3$, $p < 0.05$]. No main effect of test session was found. Post-hoc comparisons showed significant hyperactivity (compared to the saline group) in animals previously treated with cocaine 5 mg/kg ($p < 0.05$, only on T1), 10 mg/kg ($p < 0.01$ on T1 and $p < 0.05$ on T5), and 20 mg/kg ($p < 0.01$ on T1 and T5 and $p < 0.05$ on T3). No main factor effect or interaction effect between factors were found with T2, T4, and T6 (unfamiliar floor) data. With respect to T7-T8 data (data not graphed), a *t*-test comparing saline vs. cocaine 20 mg/kg groups revealed no differences for both tests, indicating that T5 was the last session where a drug-free conditioned effect was found.

3.3. Associations between the magnitude of cocaine-induced locomotor sensitization to cocaine and its expression of conditioned hyperactivity

With all animals tested with cocaine 20 mg/kg using a 6-exposure protocol (including animals in Experiments 1, 3 and 4; n = 72) we performed correlation analyses between SNZ_{idx} and conditioned hyperactivity scores on T1 and T2. A significant and positive relationship between the magnitude of sensitization and drug-free conditioned hyperactivity (familiar floor; T1) was found ($r = 0.51$, $p < 0.01$; Fig. 4A). Analysis between Snz_{idx} and T2 data (Fig. 4B; unfamiliar floor) showed no relationship between variables ($r = 0.12$, $p = 0.32$). This pool of data with 72 animals was also used to assign mice to the following two categories: those with the 25% highest SNZ_{idx} (mean D12-D1 difference in cm in 15 min, S.E.M. = 3390 ± 304) and those with the 25% lowest SNZ_{idx} (−826 ± 239). Fig. 4C shows locomotor activity on T1 and T2 of these two groups (Low vs. High). A two way ANOVA with repeated measures revealed a significant effect of group [$F(1,17) = 18.6$,

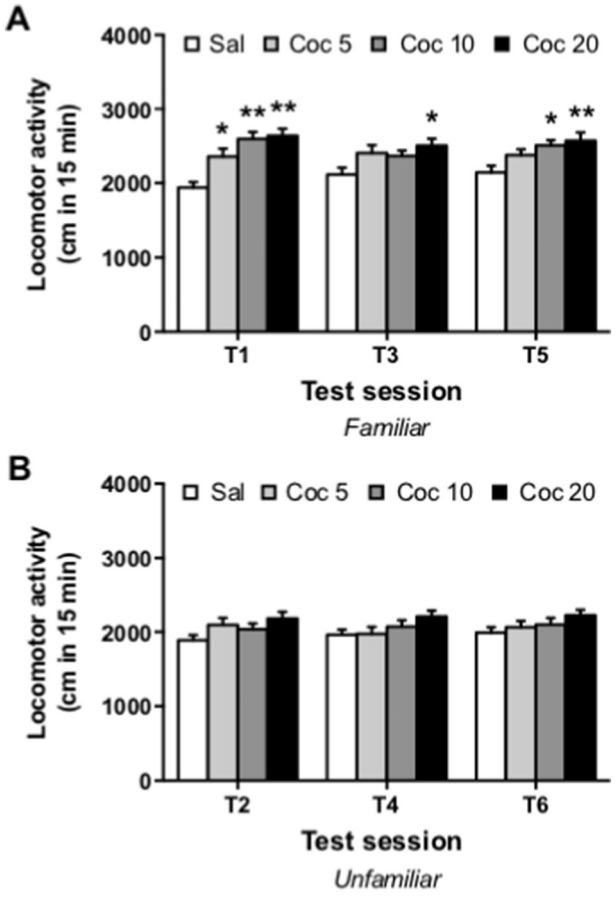

Fig. 3. Expression of conditioned hyperactivity in drug-free conditions. Locomotor activity of saline and cocaine 5, 10, and 20 mg/kg groups tested in a drug-free condition on a familiar (A; T1, T3, T5) or unfamiliar (B; T2, T4, T6) context. Locomotor activity is expressed as mean ± S.E.M. * and ** significant at $p < 0.05$ and $p < 0.01$ with respect to the saline group. N = 23–24 per group.

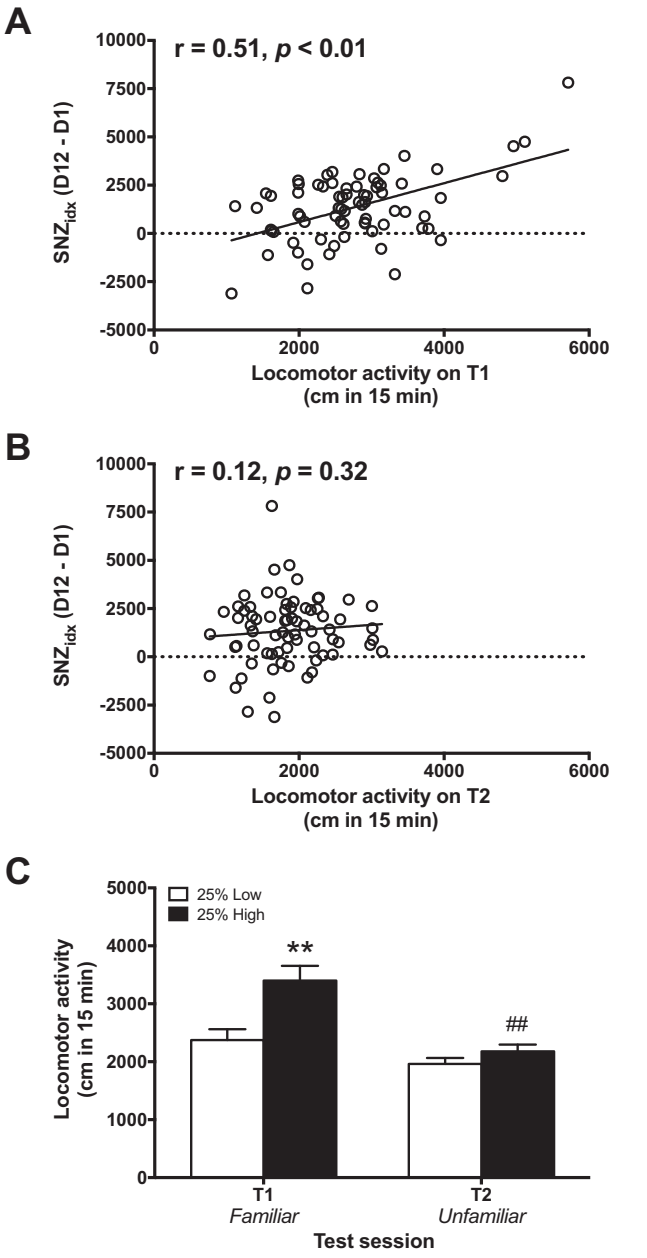

Fig. 4. Relationship between conditioned hyperactivity and sensitization index. A; correlation between locomotor activity on T1 and sensitization index (SNZ_{idx}) of all animals (n = 72) treated with cocaine 20 mg/kg. B; correlation between locomotor activity on T2 and SNZ_{idx} (same animals shown on panel A). C; locomotor activity (T1 and T2) of the 25% highest and lowest scores according to the SNZ_{idx} (n = 18). Locomotor activity is expressed as mean ± S.E.M. ** significant at $p < 0.01$ between T1 Low and High groups. ## $p < 0.01$ between T1 and T2 (High groups).*Effect of one* vs. *three cocaine administrations on drug-free conditioned hyperactivity.*

$p < 0.01$], testing day [F (1,17) = 15.3, $p < 0.01$] and an interaction between both factors [F (1,17) = 5.3, $p < 0.05$]. Pairwise comparisons showed that Low vs. High SNZ_{idx} animals were different on T1 ($p < 0.01$) but not on T2. Locomotor scores of High SNZ_{idx} animals were also found to be different between T1 and T2 ($p < 0.01$; familiar vs. unfamiliar floor). This effect was not found in Low SNZ_{idx} animals. With animals sensitized to cocaine 5 mg/kg (n = 24) no relationship between SNZ_{idx} and T1 was found ($r = 0.19$, $p = 0.381$), however a positive relationship between these two variables was found with animals treated with 10 mg/kg ($R = 0.52$, $p = 0.01$, n = 24). SNZ_{idx} mean (and S.E.M.) values for the 5, 10 and 20 mg/kg dose groups were 817.3 ± 263, 906.6 ± 317 and 1346.3 ± 192 cm in 15 min, respectively. Although it was clear that the highest dose produced the highest SNZ_{idx}, no statistical analysis comparing doses for their respective SNZ_{idx} was conducted given the different total n of subjects per group (n = 24 for 5 and 10 mg/kg, n = 72 for 20 mg/kg).

Table 1 shows cocaine-induced and drug-free (T1 and T2) locomotor activity in animals administered with 1 or 3 cocaine (20 mg/kg) challenges. Animals treated with a single cocaine exposure showed locomotor stimulation [t (22) = 9.00, $p < 0.01$; saline vs. cocaine]. A single cue-paired administration of cocaine, however, failed to induce context-induced hyperactivity in a subsequent drug-free challenge (T1). No differences between groups were seen in T2. A 3-day injection schedule induced locomotor sensitization; two-way ANOVA with repeated measures (cocaine dose x day) showed effects of cocaine dose [F (1,22) = 341.36, $p < 0.01$], day [F (2,44) = 6.5, $p < 0.01$] and a significant interaction between factors [F (2,44) = 14, $p < 0.01$]. Tukey HSD post-hoc analysis showed a cocaine-induced stimulated response on day 1 ($p < 0.01$ cocaine vs. saline) which increased on days 3 and 5 ($p < 0.01$ with respect to the first cocaine session). This indicates that animals were already sensitized on the second cocaine challenge. After this 3-session cocaine treatment, a statistically significant conditioned hyperactivity response was shown on T1 [t (22) = 6.2, $p < 0.01$] but not on T2.

Table 1
One vs. three cocaine-administration protocols: cocaine-induced and drug-free locomotor activity (mean cm in 15 min ± S.E.M.).

	Day 1			*T1*	*T2*
Saline	2083 ± 121	- - - - - - -	- - - - - - -	2001 ± 164	1978 ± 60
Cocaine	5883 ± 404**	- - - - - - -	- - - - - - -	2183 ± 96	1977 ± 79
	Day 1	*Day 3*	*Day 5*	*T1*	*T2*
Saline	1677 ± 95	1557 ± 120	1405 ± 92	1498 ± 97	1314 ± 74
Cocaine	6061 ± 265**	7135 ± 382**,##	7754 ± 231**,##	2396 ± 127**	1657 ± 190

Dashed lines indicate no treatment. T1 and T2 were conducted 3 and 4 days, respectively, after the last cocaine administration.
** Significant ($p < 0.01$) with respect to the respective same-day saline group.
Significant ($p < 0.01$) with respect to the first cocaine session.

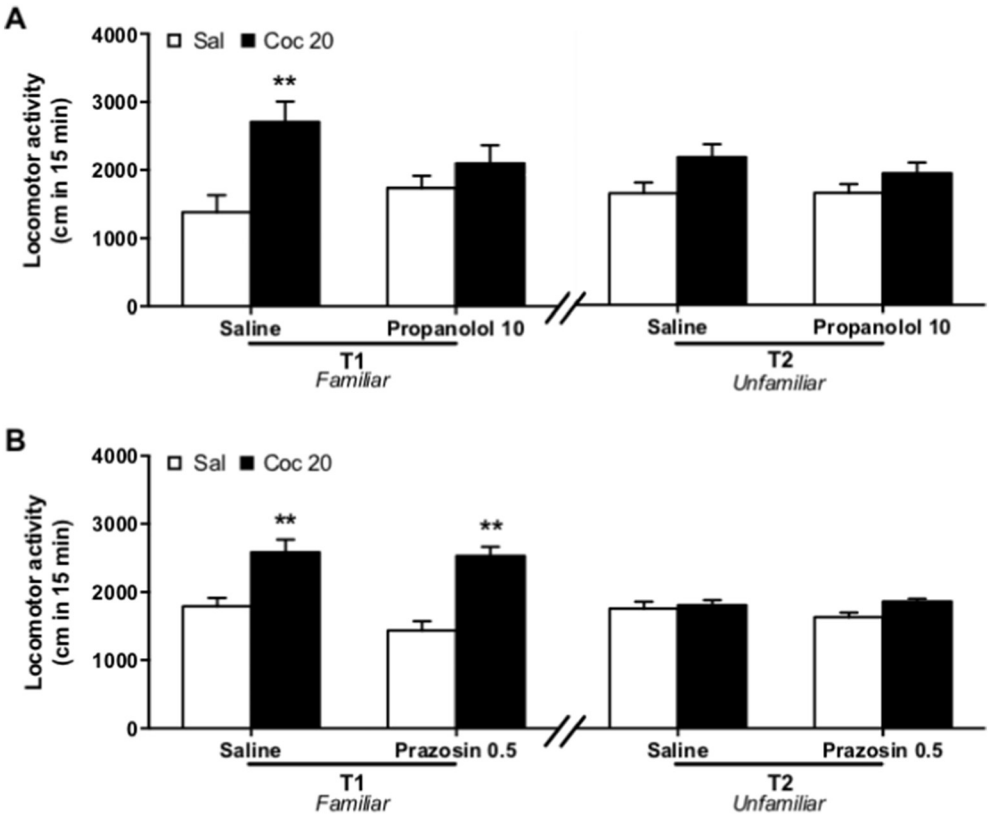

Fig. 5. Effects of noradrenergic receptor antagonism on the expression of conditioned hyperactivity. Locomotor activity of saline- and cocaine-treated mice (during the acquisition phase), pretreated with saline, propranolol (A; 10 mg/kg) or prazosin (B; 0.5 mg/kg) 30 min before a drug-free challenge on T1 (familiar floor) and T2 (unfamiliar floor). Locomotor activity is expressed as mean ± S.E.M. ** Significant at $p < 0.01$ with respect the saline group. N = 12 per group.

3.4. Propranolol, but not prazosin, inhibits the expression of drug-free conditioned hyperactivity in animals previously treated with cocaine

Significant cocaine-induced sensitization (6-sessions protocol; data not shown) was found in animals used for the propranolol and prazosin experiments. In experiment 3 (propranolol; Fig. 5A) a two-way ANOVA (cocaine dose x propranolol dose) showed a significant effect of cocaine [$F(1,44) = 5.9$, $p < 0.01$] and a significant interaction between cocaine and propranolol dose on T1 [$F(1,44) = 4.1$, $p < 0.05$]. Pairwise post-hoc comparisons revealed that conditioned hyperactivity on T1 was found in animals pretreated with saline ($p < 0.01$) but not with propranolol. No main effects or interaction between factors were found on T2. In experiment 4 (Fig. 5B) we found a significant effect of cocaine treatment [$F(1,44) = 40$, $p < 0.01$], but no effects of prazosin and a cocaine dose × prazosin dose interaction were found on T1. Conditioned hyperactivity was found in sensitized animals pretreated with saline and prazosin ($p < 0.01$) (Fig. 5B). No differences between groups were found on T2. A higher dose of prazosin (2 mg/kg) was tested in an additional experiment (only on T1; data not graphed). T1 data (mean ± S.E.M. cm in 15 min) were: 1695 ± 127 for saline-saline, 2414 ± 210 for cocaine-saline, 728 ± 192 for saline-prazosin and 1768 ± 298 for cocaine-prazosin. This dose of prazosin produced a general decrease in locomotor activity. A two-way ANOVA (cocaine dose x propranolol dose) confirmed a main effect of cocaine dose [$F(1,44) = 17$, $p < 0.01$] and prazosin dose [$F(1,44) = 14$, $p < 0.01$], but no interaction between factors was found.

4. Discussion

Converging clinical and pre-clinical literature indicates that cues and contexts associated with addictive drugs can precipitate craving (Bonson, 2002; Childress et al., 1999; Crombag and Shaham, 2002; Garavan et al., 2000; Grant et al., 1996; Meil and See, 1996; Shaham et al., 2003). In human addicts, drug-associated cues can induce relapse even after long periods of abstinence, suggesting that drug-induced neuroadaptations of learning and memory systems are long-lasting and resistant to extinction (Kalivas and Volkow, 2005; Sinha and Li, 2007). Animal models focusing on drug-related conditioning represent key efforts in elucidating the neurobiology that mediates such pathological memories. In the present study we show that the mere exposure to a

context previously associated with the stimulant actions of cocaine can elicit behavioral activation in a drug-free state. This context-dependent effect appeared to be related to the presence and magnitude of cocaine-induced psychomotor sensitization and was mediated by β-, but not α1-AR.

Our experimental findings suggest that repeated cocaine treatment can promote behavioral sensitization in DBA/2 J mice at three different doses (5, 10 and 20 mg/kg). The higher dose also resulted in a sensitized psychomotor response visible after a shortened three-session period. These data are in agreement with a vast number of studies showing that drugs like psychostimulants, ethyl alcohol, and opioids can produce behavioral sensitization in rodents under a variety of conditions (Itzhak and Martin, 1999; Pastor et al., 2008; Vanderschuren et al., 1997). The current studies found that cocaine-sensitized animals showed a drug-free context-dependent locomotor hyperactivity. Mice receiving repeated pairings of cocaine associated with a distinctive cue displayed conditioned hyperactivity when later tested on the same context, without the drug. This drug-free phenomenon was previously described in rats treated with psychomotor-sensitizing doses of cocaine (Post et al., 1992). Some authors have suggested that rodents trained under the effects of cocaine are not only hyperactive, but also more distractive and less attentive to contextual stimuli, which in turn makes the drug-paired environment relatively novel to animals that experience it without the effects of the drug (Carey et al., 2008; Carey and Damianopoulos, 2006). It could be thus argued that drug-free hyperactivity, like that found here, would reflect increased arousal levels due to novelty rather than a floor-specific activation associated with cocaine. In our case, however, this conditioned effect was not found when the same cocaine-treated animals were tested in an unfamiliar context; the same testing environment, with a new floor. Novelty, therefore, was not sufficient to induce conditioned hyperactivity and suggests that cocaine-induced sensitization did not produce augmented locomotion per se. It is important to highlight that the same cocaine-treated animals that showed no hyperactivity in an unfamiliar floor (T2, T4) recovered the conditioned response when presented with the cocaine-paired cue in later tests (T3, T5). This pattern was particularly clear for the dose of 20 mg/kg and was found for a total of three retrieval sessions after the last cocaine administration; the last context-dependent hyperactivity episode was found 17 days after termination of cocaine exposure. The lowest sensitizing cocaine dose (5 mg/kg) also produced a conditioned response on T1, but this response only lasted for one session, and was already absent on T3. Previous data using Wistar rats have shown that drug-free conditioned hyperlocomotion can last for up to 42 days after a 6-day 30 mg/kg cocaine treatment (Johnson et al., 2012).

We observed that a single challenge with the higher dose of cocaine (20 mg/kg) induced a robust psychomotor response but did not produce context-dependent hyperactivity in a drug-free state. Because our T1 test was performed 3 days after the cocaine challenge, we conducted pilot studies (data not shown) to explore whether shortening the interval between cocaine exposure and T1 could affect this response. Our results suggest that this was not the case. This dose of cocaine of 20 mg/kg induced higher acute stimulation levels than those found on the last sensitization session (D12) with the lowest cocaine dose (5 mg/kg). Therefore, an across-sessions increase in cocaine-induced activation (even if it was achieved with three drug exposures), rather than absolute locomotor stimulation levels, seemed to be linked to the cue-dependent response. We explored the relationship between cocaine-induced sensitization and context-induced hyperactivation in a drug-free state. Using all animals treated with cocaine (20 mg/k), we found a significant correlation between the magnitude of sensitization and the level of drug-free conditioned hyperactivity. A significant relationship between these two variables was also found for animals treated with cocaine 10, but not with 5 mg/kg. Interestingly, our sensitization index (D12-D1) did not predict locomotor activity on T2. Direct D1 scores (acute drug-induced activity) also failed to predict T1 performance. In addition to these findings, a quartile study with the 25% highest- and lowest-scoring subjects according to the sensitization index showed clear T1-specific and respective high and low locomotor activity. It is interesting to mention that the 25% lowest-scoring subjects showed a negative D12-D1 value (indicating that this group of animals did not develop sensitization) and presented T1 data that were similar to saline-treated animals. Additional factors not evaluated in our study, such as baseline locomotion, can also influence sensitization and/or conditioned effects of drugs. Bernardi and Spanagel (2014), for example, found that C57BL/6 N mice with high basal locomotor activity scores were particularly sensitive to the psychomotor-sensitizing effects of nicotine. Future experiments will need investigate whether basal locomotion levels are associated with drug-free conditioned hyperlocomotion in cocaine-sensitized animals.

Previous behavioral data have shown a lack of relationship between sensitization and conditioned hyperactivity (Brabant et al., 2011; Hotsenpiller and Wolf, 2002; Michel and Tirelli, 2002; Tirelli et al., 2003). According to these authors, the mechanisms underlying drug-induced behavioral sensitization and conditioned responses may be dissociable. Procedural differences such as duration of conditioning and test sessions, route of administration, and mice strain may explain discrepancies between our results and previous findings. Our procedure involved the presentation of environmental cues during the first 15 min after drug injection whereas other studies that failed to demonstrate a relationship between sensitization and conditioned responses involved longer presentations; the conditioning and testing phases in the cocaine studies of Michel and Tirelli (2002) and Tirelli et al. (2003) involved 40-min and 60-min sessions, respectively. Also, the conditioning and testing sessions in Brabant et al. (2011) lasted for 120 min (with amphetamine). By using a much shorter testing window we might have induced stronger associations between cues and the ascending part of the stimulant action of the drug. On the other hand, studies comparing different strains of mice, including DBA/2 J and C57/BL6J have shown differences in the psychomotor stimulant effects of cocaine between these two mice strains, being DBA/2 J more active than C57/BL6J mice (Orsini et al., 2005; Thomsen and Caine, 2011). Other studies found that DBA/2 J mice are more sensitive to the positive incentive properties of cocaine than C57/BL6J (Fish et al., 2010; Van Der Veen et al., 2007). Therefore, differences in sensitivity to the activational and motivational properties of drugs of abuse might also explain differences among studies.

Altogether, our data suggest a relationship between the presence and magnitude of sensitization and the expression of conditioned hyperactivity. It seems clear that some of the psychostimulant properties induced by cocaine were partially transferred to the context where animals were later tested in the absence of the drug. A causal relationship between sensitization and conditioning, however, cannot be supported. Sensitization-related plasticity might be directly linked to the development and persistency of conditioned hyperactivity, yet it might also occur in parallel to those adaptations that underlie the conditioned effect. Further research, perhaps focusing on molecular markers, will need to be conducted to clarify whether there is a direct or indirect link between both phenomena. It has been suggested that certain types of drug-induced neuroplasticity are not merely dependent of past drug history, but are mediated by conditioning processes (Badiani and Robinson, 2004). Li et al. (2003) showed differential neuroplastic changes following mere repeated cocaine administration (for example in the home cage) in contrast to repeated injections of cocaine given in a distinctive environment, in which behavioral sensitization was observed. These authors found increases in spine density of nucleus accumbens *core* in animals showing contextual sensitization whereas, structural changes in nucleus accumbens *shell* were related to repeated treatments that did not necessarily induce sensitization. Contextual cues, therefore, appear to be important not only for drug-associated effects per se, but also for their ability to modulate drug-induced neuroplasticity.

In our pharmacological studies we found that a blockade of β-AR

with propranolol prevented drug-free conditioned hyperactivity in mice previously sensitized to cocaine. NA neurotransmission plays a key role in learning and memory processes, and the involvement of AR in the conditioned effects of abused drugs have been thoroughly investigated in rodents (Bernardi et al., 2009; Bernardi and Lattal, 2010, 2012; Cummins Jacklin et al., 2015; McGaugh, 2002; Otis and Mueller, 2017, 2011; Sara, 2009). Otis and Mueller (2011) demonstrated that propranolol, administered before testing, prevented the expression of cocaine CPP. Additionally, when administered post-retrieval (immediately after a CPP test), it inhibited subsequent (tested 24 h later) cocaine CPP (Bernardi et al., 2009; Fricks-Gleason and Marshall, 2008). Wei and Li (2014) evaluated the effects of propranolol on drug-free conditioned hyperactivity in morphine-sensitized animals; they administered propranolol immediately after a conditioned hyperactivity test and found that this conditioned effect was absent when tested 24 h later (animals treated with saline remained hyperactive). One important difference between our protocol and that used by Wei and Li (2014) is that we tested animals under the effects of propranolol. It is noteworthy to mention that we did not find any nonspecific effects of propranolol on T1 or T2, demonstrating that β-AR antagonism only decreased locomotor activity when tested in the presence of cocaine-associated cues. Together, these data suggest that β-AR are involved in the expression and reconsolidation of memories that underlie cue-triggered behavioral drug effects.

Both α1- and β-AR have been seen to modulate memory formation and retrieval and mediate synaptic plasticity (Hein, 2006). In our experiments, however, the selective α1-AR antagonist prazosin did not prevent conditioned hyperactivity. Drug-free conditioned hyperlocomotion was only reduced by prazosin when administered at doses (2 mg/kg) that produced general locomotor-depressant effects. Previous data indicate that prazosin, administered immediately after a CPP test, reduced cocaine CPP in a subsequent test, only with a high dose (1 mg/kg), but not with a low dose (0.3 mg/kg) (Bernardi et al., 2009; Bernardi and Lattal, 2012). In agreement with this, White and Rauhut (2014) found that, from a range of doses of 0.5, 1 and 2 mg/kg, only the highest dose of prazosin (given 30 min prior to each methamphetamine sensitization session) was able to attenuate conditioned hyperactivity in a drug-free test conducted 48 h following the last conditioning session. The authors also reported that this dose produced nonspecific impairments in locomotion in control animals. Both, α1- and β-AR seem to be heterogeneously expressed within brain regions involved in memory consolidation, reconsolidation and retrieval (Nicholas et al., 1996). Interestingly, α1-AR appear to be highly expressed in brainstem and spinal motor nuclei (Pieribone et al., 1994), which might be related to prazosin's nonspecific effects. Further research, nevertheless, needs to be conducted to explore the nature of the nonspecific effects of prazosin that affect locomotion.

Drug-free, context-dependent hyperactivity in cocaine-treated animals might require participation of systems involved in arousal, attention, memory retrieval and behavior activation. NA neurons in the locus coeruleus respond to salient stimuli (Sara, 2009); presentation of conditioned cues previously paired to unconditioned stimuli increases firing of NA neurons in the locus coeruleus, and local stimulation of this structure facilitates memory retrieval (Bouret and Sara, 2004; Devauges and Sara, 1991; Sara and Devauges, 1988; Sara and Segal, 1991). As previously suggested, both α1- and β-AR mediate cue-dependent arousal and memory retrieval (Hein, 2006; Puumala et al., 1997; Sara, 2009), which might in turn activate conditioned behavior. The difference between propranolol and prazosin found in the present and other studies could be related to prazosin's more direct role in motor output systems. This would explain why both compounds are effective in reconsolidation studies, where these compounds are generally administered after, and not before behavior testing. In direct pharmacological tests (when animals are under the effect of the drug) that evaluate behavior, prazosin seems to be, compared to propranolol, a more limited tool. This hypothesis, however, should not disregard other factors such as conditioning strength. It could be possible that, under weaker conditioning procedures, prazosin could be able to reduce conditioned hyperlocomotion at doses that do not produce nonspecific motor effects. Future studies exploring different conditioning regimes as well as the neuroanatomy of propranolol and prazosin actions on the conditioned effects of cocaine (and other drugs of abuse) will be critical to disentangle the differential role of adrenergic receptors.

5. Conclusions

Drug-associated stimuli can acquire motivational properties that contribute to craving and relapse. Here we presented preclinical data revealing that animals previously sensitized to the psychomotor actions of cocaine show context-dependent hyperactivity in a drug-free state. The magnitude of cocaine-induced behavioral sensitization was associated with the level of context-specific drug-free hyperactivity. Our data also showed that a strong acute response to cocaine was not sufficient to produce, and did not predict, conditioned hyperlocomotion. Sensitization-related neuroplasticity, therefore, seems to be associated with the behavioral expression of drug-induced conditioned memories. Pharmacological manipulations that disrupt long-term drug-associated memories may serve as potential therapeutic tools to reduce environment-precipitated craving and abuse in abstinent addicts. Our data, together with a growing body of literature, suggest that β-AR antagonists could prevent the expression and reconsolidation of context-dependent memories associated with drugs of abuse. By mitigating the potency of such pathological memories, reduced environment-mediated vulnerability and longer abstinent periods could be achieved.

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Conflicts of interest

The authors declare no conflict of interest.

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