Effects of different CPP cue configuration and extinction procedures on acquisition, extinction and reinstatement of cocaine-associated memories

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ABSTRACT

Addiction is a brain disease characterized by compulsive drug-seeking and taking in spite of aversive consequences, along with craving and relapse even after long periods of abstinence. Drugs of abuse induce long-lasting changes in brain function and structure, through neuroplastic mechanisms on the learning and memory system that lead to compulsive drug-taking behavior and eventually to addiction. After several drug-context associations, context re-exposure can activate those associative-memories eliciting physiological responses and drug-seeking directed behaviors. Thus, drugs of abuse induce an aberrant enhancement and consolidation of drug-related memories and its activation by context exposure promotes compulsive drug-directed behaviors. Among all the neural systems involved in the formation and consolidation of drug-related memories, the noradrenergic system seems to be a critical component. The NE responds to emotional arousal, novel stimuli or stimuli that represent a sensory challenge and require attention. Previous data suggests that the agonism of the NE system improves appetitive and aversive emotional learning. Specifically, the evidence suggests the implication of the β_2-receptors in conditioned memories induced by abused drugs. The aim of these experiments is twofold; first to compare different CPP configurations and procedures on acquisition, extinction and reinstatement of cocaine-associated memories. Second, to explore the effects of the β_2-AR agonist CLE, on extinction and reinstatement of cocaine-induced CPP using the most effective procedure. For the first aim, we used two different CPP apparatus configurations (one- (A1) or two- (A2) compartment). Also, we used two different extinction procedures (force- (FE) and choice- (CE) extinction). For the second goal, we used a wide range of CLE doses following a A2 apparatus configuration and a CE procedure.

Keywords: *Conditioned place preference, CPP configuration, extinction procedure, reinstatement, noradrenergic system, clenbuterol*

INTRODUCTION

Addiction is a brain disease characterized by compulsive drug-seeking and taking in spite of aversive consequences, along with craving and relapse even after long periods of abstinence. Drugs of abuse induce long-lasting changes in brain function and structure, through neuroplastic mechanisms, leading to compulsive drug-taking behavior and eventually to addiction (Duka, Crombag & Stephens, 2011; Everitt & Robbins, 2005; Lüscher & Malenka, 2011). These permanent and semi-permanent changes have been found in the neural systems responsible for learning and memory, motivation and emotion. Thus, abused drugs usurp the natural circuits evolutionarily selected to learn associations between the consequences of survival stimuli and the contexts in which they occur, creating pathological associative-memories. After several drug-context associations, context re-exposure can activate those associative-memories eliciting physiological responses and drug-seeking directed behaviors. Thus, drugs of abuse induce an aberrant enhancement and consolidation of drug-related memories and its activation by context exposure promotes compulsive drug-directed behaviors (Torregrossa & Taylor, 2013; Hitchcock & Lattal, 2018; Vanderschurenm & Everitt, 2005).

A great deal of evidence demonstrates that development and persistence of drug-seeking behavior, depends on the strength of drug-associated cues to motivate behavior. Thus, in preclinical models, a behavioral strategy that has received attention is to reduce the ability of conditioned cues to promote relapse, after periods of abstinence. Presentation of cues or contexts previously paired with the drug (conditioned stimuli; CS+) without the expected reward induces the formation of a new memory in which the CS+ does not predict the presence of the drug any more. Extinction memory is an inhibitory and context-dependent memory that competes with the original memory, that now it is less likely to guide drug-seeking behavior (Shaham et al., 2003). Both, original and extinction memories co-exist so, the recovery of the conditioned response is likely to occur under certain circumstances following extinction training (Torregrossa & Taylor, 2013; Duka, Crombag & Stephens, 2011; Bender & Torregrossa, 2020; Berke & Hyman, 2000). For all these reasons, it is interesting to study treatments directed to reduce the emotional and motivational impact of drug-related cues on drug-seeking behavior. The goal, therefore, will be to find a combination of behavioral and pharmacological therapies directed to increase the strength of extinction memory and to prevent any kind of reinstatement.

Among all the neural systems involved in the formation and consolidation of drug-related memories, the noradrenergic system (NE) seems to be a critical component. The NE responds to emotional arousal, novel stimuli or stimuli that represent a sensory challenge and require attention. Each of these conditions could be present during a learning task and, consequently, would explain why noradrenaline (NA) is involved in learning and memory. In fact, we know that activation of the NE is necessary to induce LTP, at least in the hippocampus, amygdala and in the medial prefrontal cortex (mPFC) (Sara, 2009). NA is released mainly by neurons found in the locus coeruleus (LC), a pontine nucleus which is the main source of NA synthesis in the whole brain. LC sends projections to several brain regions such as cerebellum, brainstem or diencephalon and specifically to frontal and all sensory areas, thalamic and hypothalamic nuclei, olfactory bulb and limbic regions such as hippocampus, amygdala or septum (Sara,

2009; Berridge & Waterhouse, 2003). LC activation through the NE mediates attention, decision making, memory formation, retrieval and consolidation by promoting synaptic changes. Upon release, NA binds to different types and subtypes of receptors that are widely distributed in many brain regions, expressed in different cell types and show different pharmacological profiles. To date, they are classified into three distinct subclasses, β1-2-3, α1-, and α2-AR. α_1- and β-ARs are postsynaptic while α_2-receptor could be both pre and postsynaptic. Each receptor type is related to different responses. Specifically, β-receptors are metabotropic and coupled to Gs-proteins, which promotes the activation of the cycling adenosine monophosphate (cAMP), facilitating thus, the plastic changes necessary to LTP. Hence, synaptic plasticity induced through β-receptors activation seems to be necessary in memory formation and long-term memory consolidation (Sara, 2009; Tully & Bolshakov, 2010; Mueller & Cahill, 2010).

Extensive evidence using animal models indicates that stimulating or blocking NE produces opposite effects in memory consolidation. It has been showed that a systemic or intra-amygdala post-training injection of clenbuterol (CLE), a β2-AR agonist, enhances memory retention of an inhibitory avoidance task in rats (Introini-Collison, Miyazaki, & McGaugh, 1991; Introini-Collison, Dalmaz & McGaugh, 1996) and mice (Introini-Collison & Baratti 1992; Introini-Collison, Castellano & McGaugh, 1994). In addition to aversive memories, the NE also modulates the acquisition of appetitive memories (Mueller & Cahill, 2010). Pre-training injections with epinephrine enhance memory retention and performance in a Y-maze in which water-deprived rats and mice received water on the test day as a reward (Sternberg et al., 1985). Similarly, rats trained in 8-arm maze with food-reward improved memory retention after being treated with atomoxetine, a NE reuptake inhibitor (Tzavara et al., 2006). Extinction memory can also be modulated by changes in noradrenergic activity. For example, it has been demonstrated that NE injected in basolateral amygdale (BLA) following extinction training decreased the level of expression of conditioned response in rats trained in conditioned fear task. Rats spent less time in freezing demonstrating an enhancement of extinction of aversive memories (Berlau & McGaugh, 2006). NE also modulates extinction memory of operant or conditioned appetitive learning. It has been shown that microinjections of CLE in the infralimbic cortex (IL) after extinction training enhanced consolidation of extinction memory in rats. Rats treated with CLE showed a lesser number of cocaine self-administration lever presses, suggesting an improvement in extinction learning (LaLumiere, Niehoff & Kalivas, 2010; Schmidt & Weinshenker, 2014). Chai, Wang, Yasheng & Zhao (2016) found that mice treated with clenbuterol spent less time finding the platform compared with control subjects in water maze task, it means their spatial memory was improve after being treated with clenbuterol. Injections of atomoxetine before (Economidou, Dalley & Everitt, 2011; Janak, Bowers & Corbit, 2012) or after training (Broos et al., 2015) enhanced extinction memory decreasing the number of lever presses and preventing cocaine-seeking behavior in a self-administration procedure in rats. Also, rats injected with atomoxetine before extinction training decreased time spent on cocaine-associated compartment in conditioned place preference (CPP) paradigm. This result demonstrates an enhancement of extinction learning associated with a decrease in cocaine-seeking behavior (Brenhouse, Dumais & Andersen, 2010).

To our knowledge, no study has been conducted on the effects of β_2-AR agonists in appetitive pavlovian conditioned memories. CPP paradigm is a Pavlovian conditioning model widely used

to study drug-induced conditioned effects in the laboratory. CPP allow us to modulate extinction of pavlovian associations and to explore the consequences on the reinstatement of drug-seeking responses. CPP procedures have been used among laboratories. The aim of these experiments is twofold; first to compare different CPP configurations and procedures on acquisition, extinction and reinstatement of cocaine-associated memories. Second, to explore the effects of the β_2-AR agonist CLE, on extinction and reinstatement of cocaine-induced CPP using the most effective procedure. For the first aim, we used two different CPP apparatus configurations (one- (A1) or two- (A2) compartment). Also, we used two different extinction procedures (force- (FE) and choice- (CE) extinction). For the second goal, we used a wide range of CLE doses following a A2 apparatus configuration and a CE procedure.

METHODS

Subjects

C57BL/6J adult male mice (N=124) purchased from Charles River Laboratories España S.A. (Barcelona, Spain) were 6 weeks old at the arrival (25-35g). Animals were housed in groups of three per cage with *ad libitum* access to food and water and under 12/12h light and dark cycle (light on at 8:00 AM). Room temperature was 21±1ºC and humidity levels were 50±5%. Behavioral procedure starts, at least, one week after the acclimation (8 weeks old). Daily manipulations start 2 hours after lights were on. All procedures were conducted in accordance with the provided by the European Community Council Directive (2010/63/EU), Spanish directive BOE 34/11370/2013 and local directive DOGV 26/2010.

Drugs

Cocaine hydrochloride (Sigma-Aldrich, Spain) was dissolved in 0.9% saline solution at a concentration of 1 mg/ml. It was administered at a dose of 20 mg/kg before conditioning and 10 mg/kg before the reinstatement sessions. Clenbuterol hydrochloride (Sigma-Aldrich, Spain) was dissolved in 0.9% saline solution and administrated at the doses of 0, 0.1 and 0.5 mg/kg. 0.9% saline was the vehicle solution. All treatments were administered intraperitoneally (i.p.) and adjusted to a volume of 0.01 ml/g of weight. Cocaine and saline were administered immediately before each conditioning trial and reinstatement sessions. Clenbuterol and saline were administered immediately after each extinction tests.

Place Conditioning Apparatus

In this investigation we used six activity monitor chambers (30 cm long × 15 cm wide × 20 cm high) with a light-controlled, sound-attenuating and ventilation system (Cibertec S.A., Spain). The conditioning boxes were provided with photocells running the length of the boxes (22 cm above the floor and 5 cm apart). These detectors were connected to a computer and measured the time that animal spends in each side and the motor activity (expressed as beam breaks per min).

Tactile cues (interchangeable grid or hole floors) were used as the Conditioned Stimulus (CS). The interchangeable floor was placed under each box. The grid floor was 2.3 mm stainless

steel rods were mounted 6.4 mm apart on acrylic rails. The hole floor was mounted on acrylic base and stainless steel (16 gauge) perforated with 6.4 mm holes on 9.5 mm staggered centers. These stimuli have been selected based on previous studies demonstrating similar levels of preference (Hitchcock, Cunningham & Lattal, 2014; Hitchcock & Lattal, 2018; Cunningham, Gremel & Groblewski, 2006; Cunningham, Ferree & Howard, 2003).

General protocol

The protocol used in this investigation consisted of the following experimental phases: 1) habituation 2) pre-test (PT), 3) acquisition (A), 4) preference test (T1), 5) extinction (E) 6) reinstatement (R). Habituation consisted of acclimatizing the animals to the conditioning room for 1 hour. For this, animals were left undisturbed left in their homecages. Following this, every animal was handled during 2 minutes, and then animals were left undisturbed for 1 additional hour in the conditioning room (homecages). This phase lasted 2 days. 24 hours later we started the PT phase. It consisted of a preference test in which animals were exposed to both stimuli. That is, half of the chamber had a grid floor and the other half had a hole floor. Animals were placed in the middle of the chamber and allowed to explore the whole apparatus for 5 minutes. We recorded the time spent in each side of the chamber and set the innate preference for each compartment. Animals spent around 50% time in each compartment demonstrating the absence of initial bias for any of the stimuli. Thus, all treatments were randomly assigned. Acquisition and extinction phases were different for each experiment, and then they will be described later. Following tests; T1, E and R tests, were carried out as PT. In order to consider that animals were developing cocaine-induced CPP, the preference criterion established was that a given group of animals should spent more or equal than 60% of time on the cocaine-associated compartment (CS+). In PT and T1 animals didn't received any treatment. In E tests depending on the experiment animals received no treatment, saline or CLE immediately after the test. R phase was identical for all experiments. It consisted of three tests: first (R1) was 24 hours after last extinction test, second (R2) was 72 hours after last extinction test and third (R3) was 30 days after last extinction test. In all reinstatement tests animals were randomly divided in two groups and received saline or cocaine-priming immediately before.

All treatments and procedures were counter balanced for stimulus-drug assignment. Left-right position of the floors and the order of the injections; (saline or cocaine) were also counterbalanced. Half of the animals were assigned to the GRID+ group; animals received cocaine on the GRID floor (G+) and saline on the HOLE floor (H-). The other half of animals was assigned to the GRID- group; animals received saline on the GRID floor (G-) and cocaine on the HOLE floor (H+). We used two different CPP configurations for the acquisition phase: *one-compartment* (A1) and *two-compartment* (A2). In the *one-compartment* configuration the animals were exposed to the whole chamber in each conditioning session. In the *two-compartment* configuration, the chamber was divided in half with a transparent barrier, so each day animals were conditioned to half of the chamber. In this particular set up, animals were confined to one of the compartments, but they could see the other side. The configuration in which animals were trained was randomly assigned. Half of animals were trained following *one-compartment* configuration and the other half were trained following *two-compartment* configuration.

In these experiments we also compare two different extinction procedures: *force-extinction* (FE) and *choice-extinction* (CE). Both procedures consisted of two phases. FE procedure consisted of extinction training and extinction test. During the extinction training animals were daily exposed to the cocaine-associated floor (CS+) without any treatment for 30 minutes. Animals were trained for 1 day and received a test 24 hours later. CE procedure consisted of testing the animals every other day. The days that there was no training, animals were left undisturbed in their homecages. The criterion for extinction was to observe two extinction tests in a row in which behavior was no different from the PT and still different from T1. Half of animals were trained following the FE procedure and the other half following the CE procedure

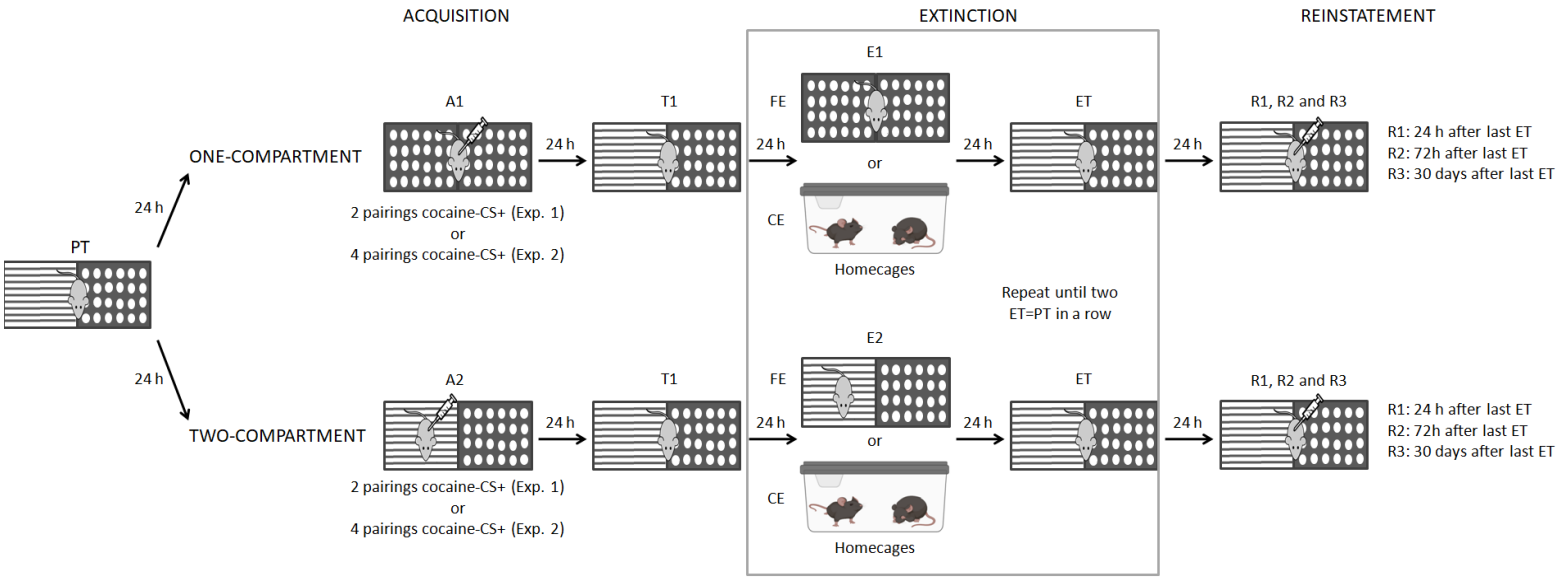

Fig. 1A

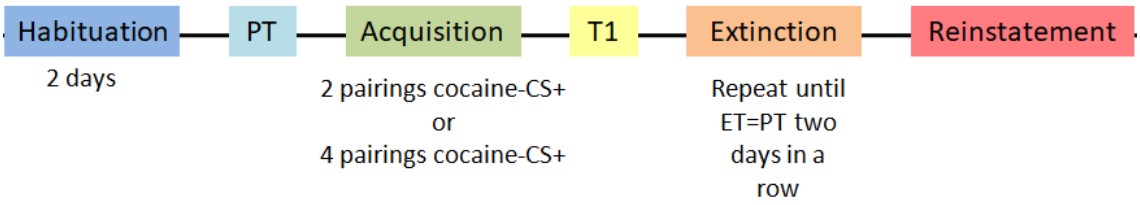

Fig. 1B

Fig. 1A. Schematic representation of the general protocol with a detailed description of all possible combinations studied. **1B.**General timeline. PT, T1 and reinstatement were identical in all experiments. Acquisition and extinction were different in each experiment. Each phase was separated by a 24 hours period.

Experiment 1a. Effects of one- (A1) or two-compartment (A2) apparatus configuration on the acquisition of cocaine-induced CPP

This experiment was designed to compare the effects of different chamber configurations (A1/A2) during acquisition of cocaine-induced CPP. Animals were randomly assigned to A1 (n=28) or A2 (n=30) groups. Habituation, PT and T1 were described in the General protocol. One day after PT, conditioning phase (4 days) started. Acquisition consisted of two pairings of cocaine (CS+) and two pairings of saline (CS-). Each conditioning session lasted 15 minutes and CS+ was presented on alternate days. Treatments were injected immediately before conditioning trials. T1 took place 24 hours after last conditioning day. Time spent in each compartment was recorded for subsequent analysis.

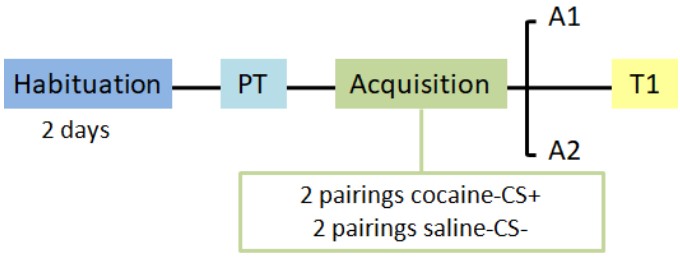

Fig. 2.

Fig. 2. Timeline of experiment 1a.

Experiment 1b. Effects of compartment configuration during acquisition (A1 or A2) on extinction and reinstatement of cocaine-induced CPP

The purpose of this experiment was to evaluate the effects of two different CPP configuration and extinction procedures on extinction and reinstatement of cocaine-induced CPP. This experiment is the second part of Experiment 1a, using the same animals and starting following T1. Therefore, habituation, PT, acquisition and T1 were exactly the same as Experiment 1a. 24 hours after T1, on extinction phase, animals trained in A1 were randomly divided and assigned to FE or CE procedure and animals trained in A2 were randomly assigned to FE or CE. FE and CE procedures were explained in the general protocol. Extinction phase finished when the preference of a given group of animals was not different from PT two days in a row. One day later, reinstatement phase started. This was also explained in the General protocol.

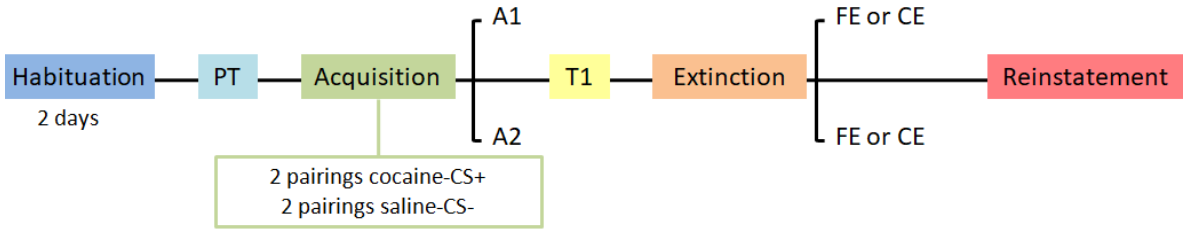

Fig. 3.

Fig. 3. Timeline of experiment 1b. Extinction phase finished when ET=PT two days in a row.

Experiment 2. Effects of Clenbuterol on extinction and reinstatement of cocaine-induced CPP

The purpose of this experiment was to evaluate the effects of CLE, a β_2-AR agonist, on extinction and reinstatement of cocaine-induce CPP. For this experiment, we decided to follow a 4 cocaine pairings procedure, A2 chamber configuration for CPP acquisition and CE procedure for extinction. Because our goal was to obtain a strong CPP procedure in order to evaluate future NE pharmacological manipulations on extinction and reinstatement of cocaine CPP, we chose this combination of factors. Habituation, PT, T1 and reinstatement sessions were the same as in all experiments. Conditioning phase consisted of 8 conditioning days; 4 cocaine pairings (CS+) and 4 saline pairings (CS-). 66 animals were divided in three groups and randomly assigned to different clenbuterol doses (CLE; 0, 0.1, 0.5 mg/kg). 24 animals were injected with 0 mg/kg CLE, 24 animals injected with 0.1 mg/kg CLE and 18 animals injected with 0.5 mg/kg CLE. CLE or saline was given immediately after each extinction test.

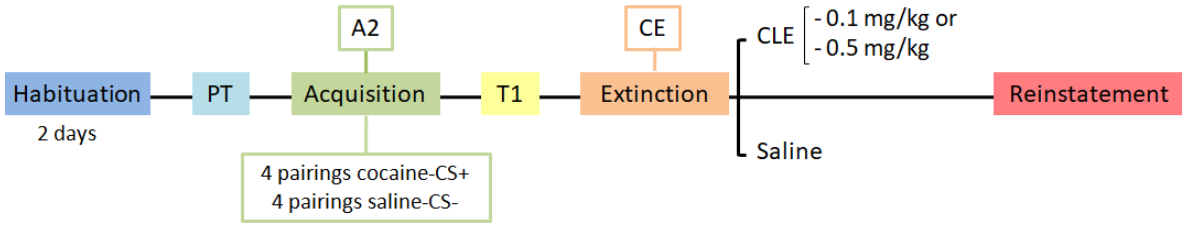

Fig. 4.

Fig. 4. Timeline of experiment 3. Acquisition phase lasted for 8 days. Extinction phase finished when ET=PT two days in a row.

Data analyses

Data were analyzed using GraphPad Prism 7.0 software (© 1997-2017 GraphPad Software, Inc). The main dependent variable was the percentage of time spent on the cocaine-associated floor on test day (acquisition test; T1/extinction tests; ET and reinstatement tests; R). A T-test comparing flooring subgroup assignment at pretest was undertaken across all experiments. The aim of this analysis was to evaluate initial preference or aversion (before conditioning) for one of the two stimuli (hole floor vs. grid floor). Because we found no differences between floors, on T1 and subsequent tests, the percent of time that animals spent on their cocaine-paired floor (G+ and H+ subgroups) was collapsed. The preference criterion was to spent equal or more than 60% time on CS+. Test data were analyzed by means of Student's t test for the initial preference analysis (T1). Also, a Chi-square test was used to analyze number of animals that reach the preference criterion in both; A1 and A2 groups on T1. The criterion for behavioral extinction was to observe two extinction tests in a row in which behavior was no different from the PT and still different from T1. The criterion for extinction using pharmacology was twofold; first, to find differences between T1 and extinction. Second, in order to be extinguished should be below 60%. For reinstatement, the criterium was to obtain differences between the last extinction day and reinstatement. Post hoc analyses were performed using Tukey´s test. In all phases, two-way repeated measures ANOVAs were conducted when required. Alpha-level was set at 0.05 for all analyses.

RESULTS

Experiment 1a. Effects of one- (A1) or two-compartment (A2) apparatus configuration on the acquisition of cocaine-induced CPP

This experiment was designed to compare the effects of two different CPP apparatus configurations (A1 or A2) on the acquisition of cocaine-associated memory. To analyze differences in the magnitude of CPP based on apparatus configuration, we used a two-way repeated measures ANOVA that showed a significant effect of session [F(1, 56) = 159.6, P<0.0001], but neither a significant effect of compartment configuration[F(1, 56)=1.584, P=0.2134] nor interaction between factors [F(1, 56)=2.133, P=0.1498](Fig. 5A). These results suggest that both configurations produce similar magnitude of cocaine-induce expression of CPP. To further analyze our data, we explored the number of animals that fulfill the CPP criterium in both groups using a Chi-square test. The results showed non-significant differences between groups, indicating that the same number of animals were able to show conditioning following A1 or A2 training (X^2= 2.74, *P*= 0.0978) (Fig. 5B).

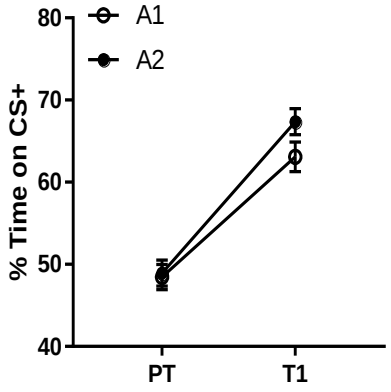

Fig. 5C

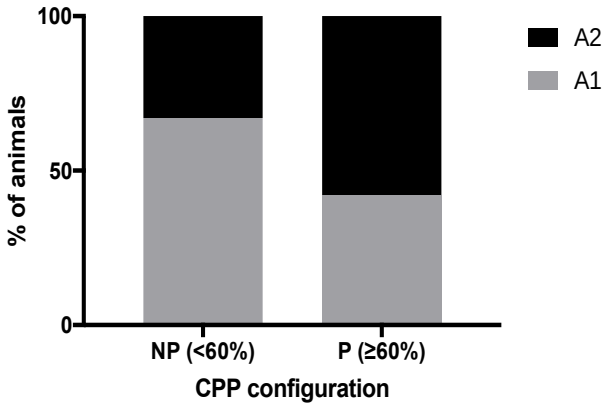

Fig. 5D

Fig. 5A. Mean+SEM (percentage of time) spent on CS+ on the Pretest (PT) and Preference test (T1) for one-*compartment* (A1) and *two-compartment* (A2) groups. White circles represent A1 group and black circles represent A2 group. **5B.**Percentage of animals that reach the preference criterion following A1 (graybar) (n=18) or A2 (blackbar) (n=25) on T1 (P>0.05).

Experiment 1b. Effects of compartment configuration during acquisition (A1 or A2) on extinction and reinstatement of cocaine-induced CPP

Effects of compartment configuration during acquisition (A1 or A2) on extinction

To study the impact of conditioning animals following A1 or A2 apparatus configuration on two different extinction procedures; choice and forced (CE and FE) we used independent two-way repeated measures ANOVAs. A two-way repeated measures ANOVA for CE showed a significant effect of the session factor [F(5, 125)=6.791, P<0.0001] but neither a significant effect of compartment configuration [F(1, 25)=0.3355, P=0.5676] nor an interaction between

factors [F(1, 125)=0.9818, P=0.4317(Fig. 6A). A separate two-way ANOVA for FE displayed a significant effect of the session factor [F(5, 145) = 16.55, P<0.0001], and a non-significant effect of the compartment configuration factor [F(1, 29)=0.3645, P=0.5507] or an interaction [F(5, 145)=0.6929, P=0.6296](Fig. 6B). Because no differences were found among procedures, a follow-up analysis (two-way repeated measures ANOVA) collapsing data from A1 and A2 groups was conducted. The analysis showed a significant effect of the session factor [F(5, 280)=21.21, P<0.0001] but no other significant difference [F(1, 56)=0.3394, P=0.0707]. These data suggests two things. First, that the compartment configuration during acquisition of CPP did not have an impact on the ratio of extinction. Second, that both procedures, CE and FE, produce similar levels of CPP extinction.

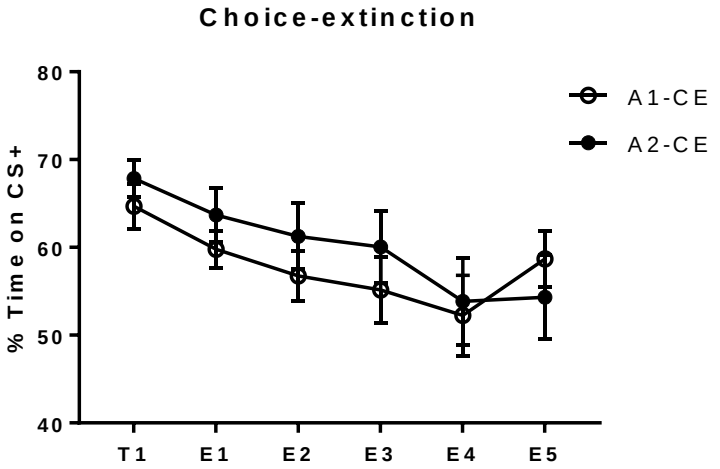

Fig. 6A

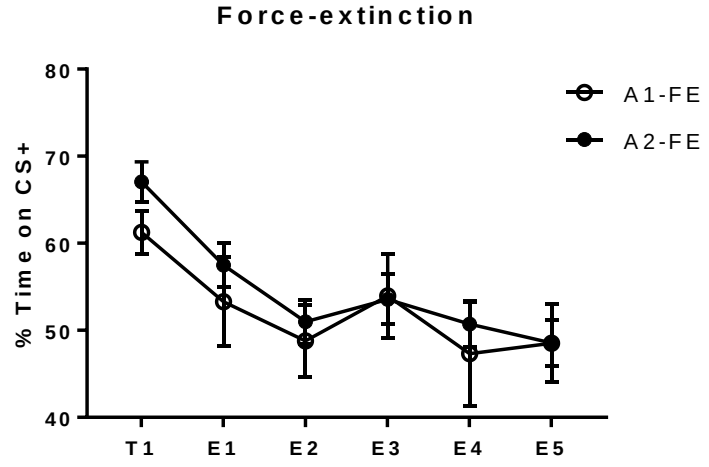

Fig. 6B

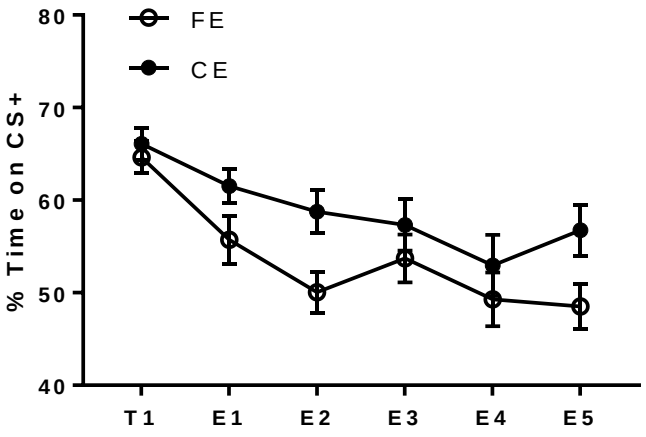

Fig. 6C

Fig. 6A. Mean±SEM percent time spent on the CS+ on T1 and the subsequent extinction tests (E1-E5) following a CE procedure. White circles represent A1 group and black circles represent A2 group. **6B.**Mean ± SEM percent time spent on the CS+ on T1 and subsequent extinction tests following a FE procedure. White circles represent A1 group and black circles represent A2 group. **6C.** Mean ± SEM percent time spent on the CS+ on T1 and subsequent extinction tests following FE or CE procedure. Data from A1 and A2 groups were collapsed for these analyses.

Because we found a marginal significance when analyzing FE and CE together (data collapsed data from A1 and A2 groups), we decide to assess extinction using independent T-test student tests for each extinction trial. The aim of these analyses was to investigate individual differences between CE and FE procedures in the ratio of extinction.

A Student's t test for T1 and E1 following CE showed non-significant differences between them [t(26)=1.857, P=0.0747, R^2=0.1171]. The rest Student's test for CE showed significant differences; for T1 and E2 [t(26)=3.168, P=0.0039, R^2=0.2785], for T1 and E3 [t(26)=3.253, P=0.0032, R^2=0.2893], for T1 and E4 [t(26)=4.096, P=0.0004, R^2=0.3921] and for T1 and E5 [t(26)=3.212, P=0.0035, R^2=0.2841]. Five more independent Student's t tests were used to compare T1 with each extinction tests following FE. Student's t test for all comparisons showed significant differences. For T1 and E1 [t(30)=4.207, P=0.0002, R^2=0.3711], for T1 and E2 [t(30)=8.159, P<0.0001, R^2=0.6894], for T1 and E3 [t(30)=4.539, P<0.0001, R^2=0.4071], for T1 and E4 [t(30)=6.336, P<0.0001, R^2=0.5723], for T1 and E5 [t(30)=7.815, P<0.0001, R^2=0.6706].

Although the analysis of CE displayed non-significant differences between T1 and E1, suggesting that on E1 animals still show CPP, the analysis of FE procedure showed significant differences between T1 and E1, indicating that preference was already extinguished. These results, altogether could suggest that the ratio of extinction would be slower in CE than in FE.

Treatment		T1-E1	T1-E2	T1-E3	T1-E4	T1-E5
CE	T1	66.0803±1.702				
	ET	61.5167±1.832	58.76±2.282	57.3211±2.785	52.9552±3.3	56.7373±2.735
			**	**	***	**
FE	T1	64.6135 ±1.738				
	ET	55.7225±2.565	50.0521±2.235	53.7047±2.577	49.2828±2.905	48.5324±2.403
		***	****	****	****	****

Table 1

Table 1. Effects of training animals on different extinction procedure (CE or FE) comparing percentage time spent on T1 with each extinction test. * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001.

Effects of A1 compartment configuration during acquisition on reinstatement of CPP following a forced or choice extinction procedure

In order to analyze the impact of these configurations on the reinstatement of an extinguished cocaine-CPP we used three separated two-way repeated measures ANOVAs to compare the last extinction test with each reinstatement test in the A1-FE group. A Two-way repeated measures ANOVA for last extinction test and R1 showed no effect of the treatment [F(1, 11)=1.627, P=0.2284], neither the test factor [F(1, 11)=4.825, P=0.0504] nor the interaction between factors [F(1, 11)=0.2152, P=0.6518]. Another Two-way repeated measures ANOVA for the last extinction test and R2 displayed non-significant effect of the treatment [F(1, 11)=1.32, P=0.275] neither the test factor [F(1, 11)=2.025, P=0.1825] nor the interaction between factors [F(1, 11)=0.003787, P=0.952]. One more for the last extinction test and R3 showed non-significant effect of the treatment [F(1, 11)=1.334, P=0.2725], nor the test factor [F(1, 11)=0.1674] neither the interaction between factors [F(1, 11)=0.04127, P=0.8427]. These results suggest that in animals that were initially trained following an A1 apparatus configuration and a FE were not able to reinstate CPP behavior after receiving saline or 10 mg/kg of cocaine in any of the reinstatement tests.

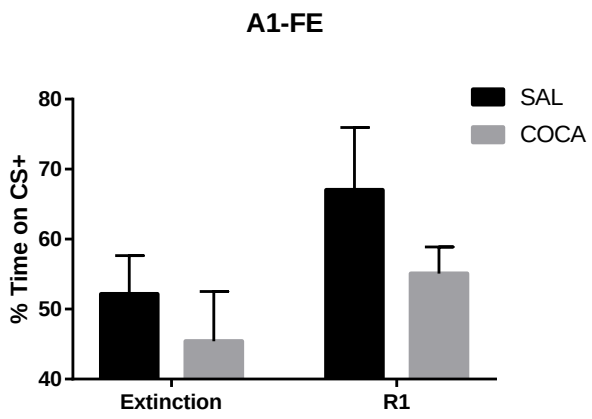

Fig.7A

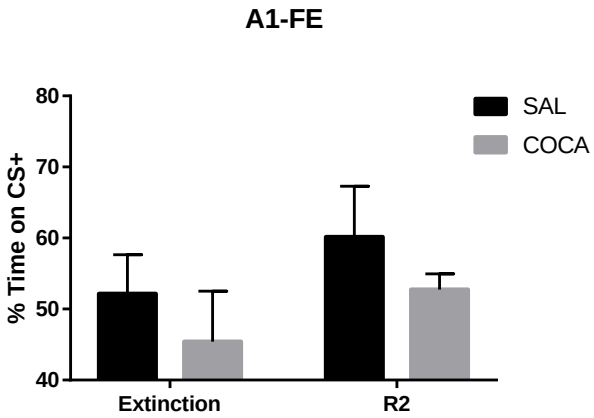

Fig. 7B

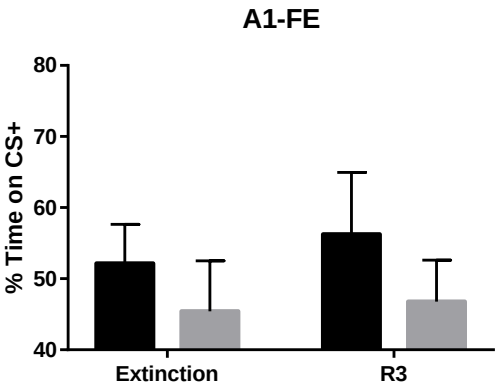

Fig. 7C

Fig. 7A. Mean±SEM percentage time on CS+ on the last extinction test and R1 of animals trained in A1-FE. Black bars refer to animals injected with saline in reinstatement sessions and gray bars to animals injected with cocaine-priming (10 mg/kg) in all reinstatement tests. **7B.**Mean±SEM percentage time on CS+ on last extinction test and R2 of animals trained in A1-FE. Black bars represent animals injected with saline in reinstatement sessions and gray bars to animals injected with cocaine-priming (10 mg/kg) in all reinstatement tests. **7C.**Mean±SEM percentage time on CS+ on last extinction test and R3 of animals trained in A1-FE. Black bars represent animals injected with saline in reinstatement sessions and gray bars to animals injected with cocaine-priming (10 mg/kg) in all reinstatement tests.

To study the effect of A1 configuration and CE procedure on reinstatement CPP three separated Two-way repeated measures ANOVA were used comparing last extinction test with each reinstatement test in animals trained in A1-CE. A Two-way repeated measures ANOVA for the last extinction test and R1 showed a non-significant effect of the treatment [F(1, 13)=0.1922, P=0.6683], neither the test factor [F(1, 13)=0.01101, P=918] nor the interaction between factors [F(1, 13)=0.6104, P=0.4486]. A Two-way repeated measures ANOVA for the last extinction test and R2 displayed no effect of the treatment [F(1, 13)=0.01506, P=0.9042], neither the test factor [F(1, 13)=0.07238, P=0.7921] nor the interaction between factors [F(1, 13)=0.2164, P=0.6495]. One more for the last extinction test and R3 showed non-significant

effect of the treatment [F(1, 13)=0.01292, P=0.9112] neither the test factor [F(1, 13)=1.192, P=0.2947] nor the interaction between factors [F(1, 13)=0.1994, P=0.6625]. These results demonstrated that animals trained under an A1 compartment configuration did not reinstate preference for the cocaine-associated compartment, when injected with saline immediately before the test. This suggests that the presentation of the context where they previously received cocaine is not enough to recover the conditioned response. In conclusion, neither saline, nor cocaine (10 mg/kg) were able to reinstate CPP in animals trained following A1 compartment configuration in any of the extinction procedures; FE or CE.

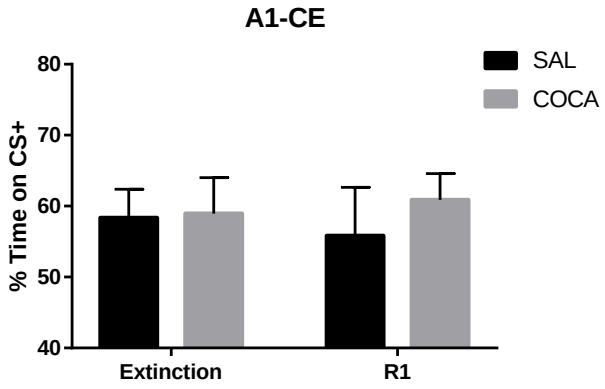

Fig. 8A

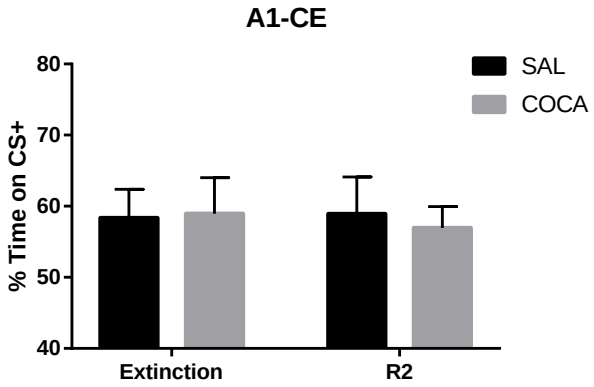

Fig. 8B

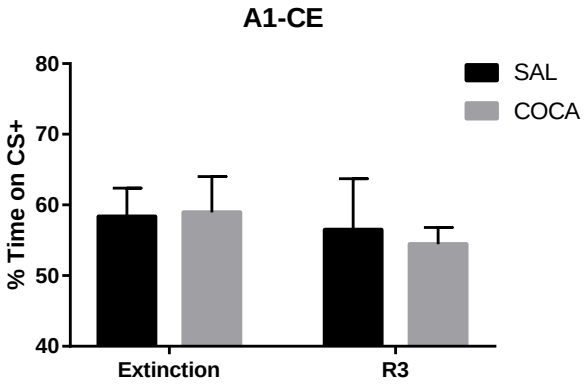

Fig. 8C

Fig. 8A. Data indicated as mean±SEM percentage of time on CS+ on last extinction test and R1 for animals trained in A1-CE. Black bars refer to animals treated with saline and gray bars represent animals injected with cocaine-priming (10 mg/kg) before all reinstatement tests. **8B.**Data are shown mean±SEM percent time on CS+ on last extinction test and R2 for animals trained in A1-CE. Black bars indicate animals treated with saline and gray bars represent animals injected with cocaine-priming (10 mg/kg) before all reinstatement tests. **8C.**Data indicated as mean±SEM percentage of time on CS+ on last extinction test and R3 for animals trained in A1-CE. Black bars refer to animals treated with saline and gray bars represent animals injected with cocaine-priming (10 mg/kg) before all reinstatement tests

Effects of A2 compartment configuration during acquisition on reinstatement of CPP following a forced or choice extinction procedure

Three more independent two-way repeated measures ANOVAs were conducted for the A2-FE group to study the effect of A2 compartment configuration during acquisition on reinstatement of CPP, following FE. A Two-way ANOVA for the last extinction test and R1 displayed a significant effect of the treatment [F(1, 16)=5.107, P=0.0381], also the test factor [F(1, 16)=18.07, P=0.0006] and the interaction between factors [F(1, 16)=10.39, P=0.0053]. Given that we found a significant interaction between factors, post-hoc analyses were performed using Tukey's multiple comparisons test. There were significant differences between last extinction test and R1 on coca group (P=0.0019) and between saline and coca group on R1 (P=0.0023).

Another Two-way repeated measures ANOVA for the last extinction test and R2 showed non-significant effect of the treatment [F(1, 16)=2.061, P=0.1704], a significant effect of the test factor [F(1, 16)=8.561, P=0.0099] and non-significant effect of the interaction between factors [F(1, 16)=3.597, P=0.0761]. Finally, the analysis of the last extinction test and R3 displayed non-significant effect of the treatment [F(1, 16)=2.027, P=0.1737], a significant effect of the test factor [F(1, 16)=14.81, P=0.0014] but a non-significant effect of interaction between factors [F(1, 16)=2.958, P=0.1047]. These results suggested differences between the last day of extinction and the subsequent reinstatement sessions, which indicate a recovery in the extinguished response. However, only on T1 we observed differences between treatments. On R1, 10 mg/kg of cocaine was able to reinstate the extinguished response.

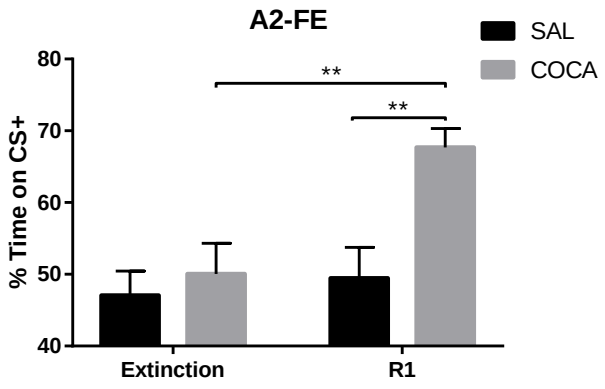

Fig. 9A

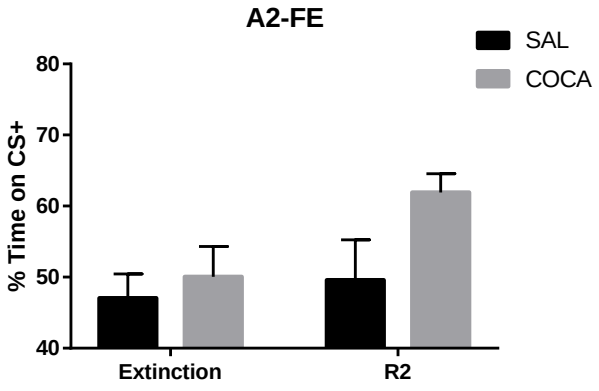

Fig. 9B

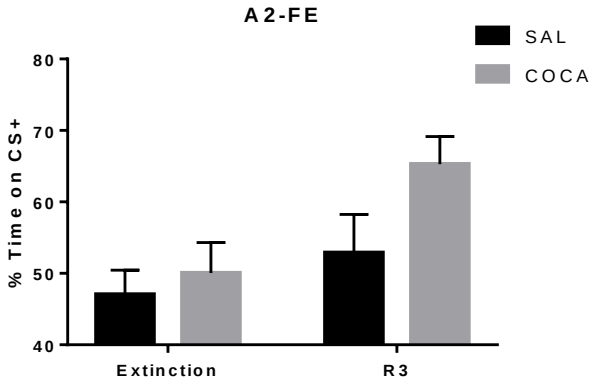

Fig. 9C

Fig. 9A. Mean±SEM percentage time on CS+ on the last extinction test and R1 of animals trained in A2-FE. Black bars represent animals injected with saline in reinstatement sessions and gray bars represent animals injected with cocaine-priming (10 mg/kg) in all reinstatement tests. **9B.**Mean±SEM percentage time on CS+ on last extinction test and R2 of animals trained in A2-FE. Black bars represent animals injected with saline in reinstatement sessions and gray bars to animals injected with cocaine-priming (10 mg/kg) in reinstatement tests. **9C.**Mean±SEM percentage time on CS+ on last extinction test and R3 of animals trained in A2-FE. Black bars represent animals injected with saline in reinstatement sessions and gray bars to animals injected with cocaine-priming (10 mg/kg) in all reinstatement tests

Three independent Two-way repeated measures ANOVA for A2-CE were used to compare the last extinction test with each reinstatement test. A Two-way repeated measures ANOVA for the last extinction test and R1 displayed a non-significant effect of the treatment [F(1, 10)=0.3723, P=0.5553], neither the test factor [F(1, 10)=3.454, P=0.0927] nor the interaction between factors [F(1, 10)=0.1747, P=0.6848]. One more for the last extinction test and R2 showed non-significant effect of the treatment [F(1, 10)=0.4775, P=0.5053] but a significant effect of the test factor [F(1, 10)=8.129, P=0.0172] and a non-significant effect of the interaction between factors [F(1, 10)=0.8073, P=0.39]. A Two-way repeated measures ANOVA for the last extinction test and R3 showed a non-significant effect of the treatment [F(1, 10)=0.07524, P=0.7894], a significant effect of the test factor [F(1, 10)=5.723, P=0.0378] and a non-significant effect of the interaction between factors [F(1, 10)=0.1138, P=0.7428]. The results indicate a significant difference between tests in R2 and R3 and a marginal significance for R1, but no significant differences between cocaine-priming (10 mg/kg) and saline on reinstatement of CPP.

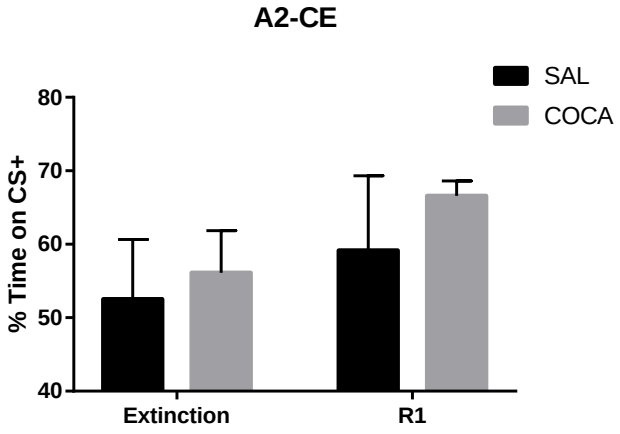

Fig. 10A

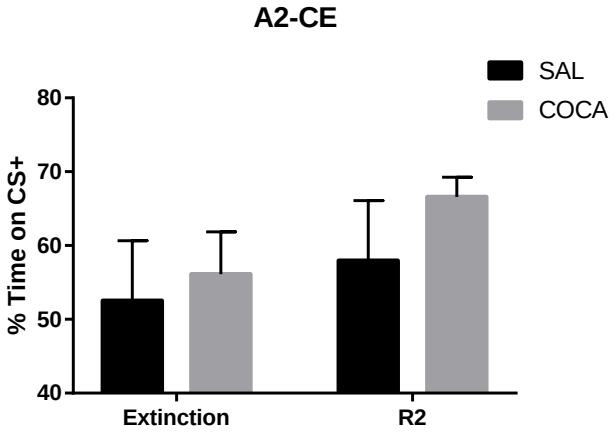

Fig. 10B

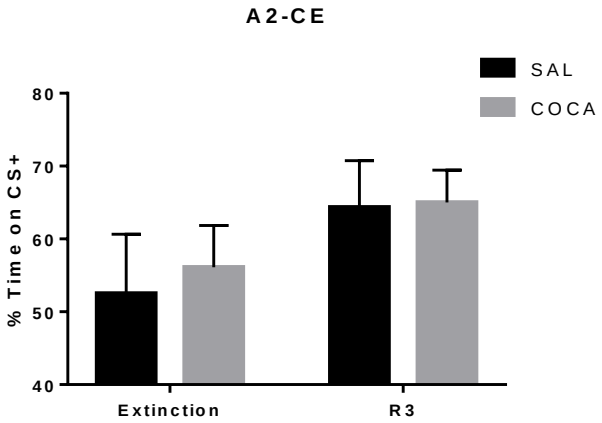

Fig. 10C

Fig. 10A. Mean±SEM percentage time on CS+ on last extinction test and R1 of animals trained in A2-CE. Black bars refer to animals injected with saline and gray bars to animals injected with cocaine-priming (10 mg/kg) in all reinstatement tests. **10B.**Mean±SEM percentage time on CS+ on last extinction test and R2 of animals trained in A2-CE. Black bars represent animals injected with saline in and gray bars to animals injected with cocaine-priming (10 mg/kg) in reinstatement tests. **10C.**Mean±SEM percentage time on CS+ on last extinction test and R3 of animals trained in A2-CE. Black bars represent animals injected with saline in reinstatement sessions and gray bars to animals injected with cocaine-priming (10 mg/kg) in all reinstatement tests.

Experiment 2. Effects of Clenbuterol on extinction and reinstatement of cocaine-induced CPP

For this experiment we follow an A2 compartment configuration during acquisition and a CE procedure. One important difference regarding the studies presented above is that we followed a stronger conditioning procedure than in the studies described above (4 cocaine pairings instead of 2). This was based on additional studies that we performed and that are not presented here. To compare the effect of different doses of CLE on extinction of CPP we conducted a two-way repeated measures ANOVA. Preference scores were calculated by determining the ratio of the time spent in the drug-paired side after conditioning minus the

total time spent in the paired side before conditioning (Fig. 11). The analysis displayed an effect of the session factor [F(5, 315)=28.96, P<0.0001] but no effect of treatment [F(2, 63)=0.4751, P=0.6241] nor interaction between factors [F(10, 315)=0.6328, P=0.7855]. These results suggest a progressive decrease on cocaine-induced CPP regardless of the CLE treatment.

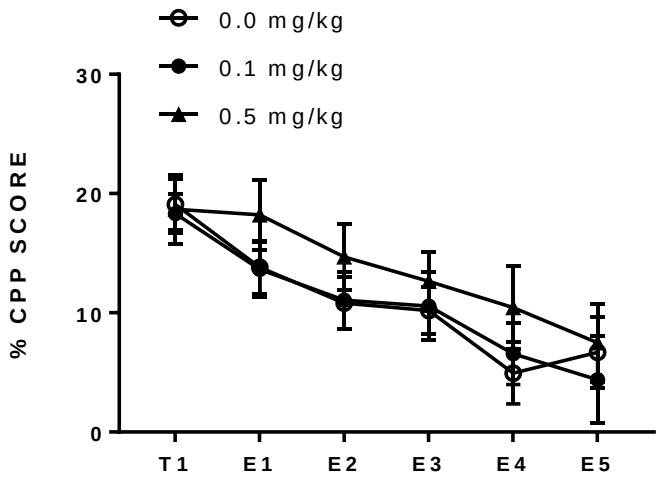

Fig. 11

Fig. 11. Mean±SEM percent time animals spent on CS+ on extinction tests as CPP score. CPP score was calculated by subtracting percentage time that animals spent on CS+ after conditioning minus the percentage time spent on CS+ before conditioning. White circles represent 0.0 mg/kg CLE, black circles 0.1 mg/kg CLE and black triangles 0.5 mg/kg CLE.

To analyze the consequences of CLE treatment during extinction, on the reinstatement of cocaine-CPP (animals treated with cocaine before reinstatement), separated two-way repeated measures ANOVAs were used. For animals treated with cocaine before R1, a two-way repeated measures ANOVA showed a significant effect of the test factor [F(1, 39)=28.07, P<0.0001] but neither a significant effect of CLE dose [F(2, 39)=0.1194, P=0.8877] nor interaction between factors [F(2, 39)=1.374, P=0.265]. For animals treated with cocaine on R2, a two-way repeated measures ANOVA, displayed a significant effect of test [F(1, 39)=10.34, P=0.0026], but a non-significant effect of CLE dose [F(2, 39)=0.2329, P=0.7933] nor interaction between factors [F(2, 39)=1.28, P=0.2895]. These results indicate that cocaine was able to reinstate CPP in all animals. However, treating animals with CLE during extinction did not prevent reinstatement of cocaine-induced CPP.

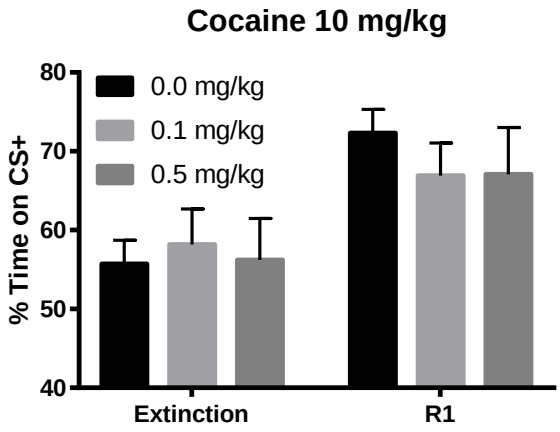

Fig.12A

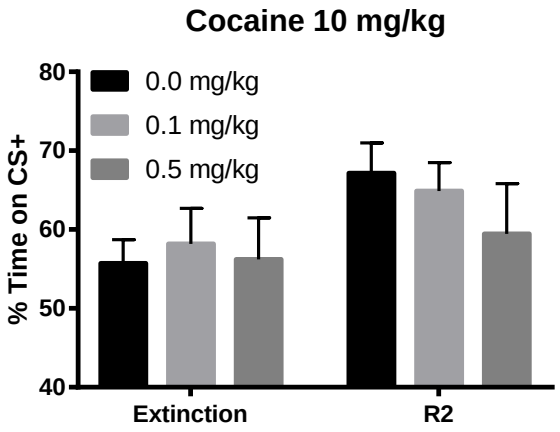

Fig.12B

Fig. 12A. Mean±SEM percentage time on CS+ on last extinction test and R1 (animals treated with 10 mg/kg of cocaine immediately before the reinstatement test). Black bars represent 0.0 mg/kg of CLE, light grey 0.1 mg/kg and dark grey 0.5 mg/kg of CLE. **12B**.Mean ± SEM percentage time on CS+ on last extinction test and R2 on animals treated with cocaine-priming (10 mg/kg) before the reinstatement test. Black bars refer to 0.0 mg/kg CLE, light grey 0.1 mg/kg and dark grey 0.5 mg/kg CLE.

To analyze the consequences of CLE treatment during extinction on the reinstatement of cocaine-CPP (animals treated with saline before reinstatement), separated two-way repeated measures ANOVAs were performed. For animals treated with saline on R1, a two-way repeated measures ANOVA showed a significant effect of the test factor [$F(1, 38)=4.212$, $P=0.0471$], a non-significant effect of the CLE factor [$F(2, 38)=0.5843$, $P=0.5624$], and a non-significant interaction between factors [$F(2, 38)=2.121$, $P=0.1339$]. For animals treated with saline on R2, a two-way repeated measures ANOVA displayed a non-significant effect of the test factor [$F(1, 38)=0.03306$, $P=0.8567$], non-significant effect of the CLE factor [$F(2,38)=2.382$, $P=0.106$] nor the interaction between factors [$F(2, 38)=1.472$, $P=0.2422$]. These outcomes demonstrate that cocaine and also saline were able to recover the conditioned response, but cocaine group exposed a greater time on CS+ than saline group. In any of these two cases, CLE treatment during extinction modulates the reinstatement of cocaine-CPP.

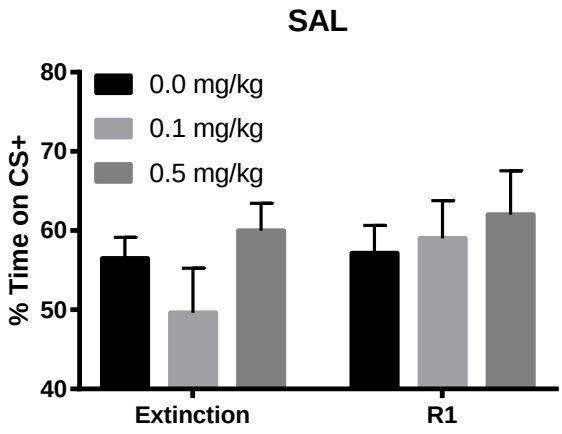

Fig.13A

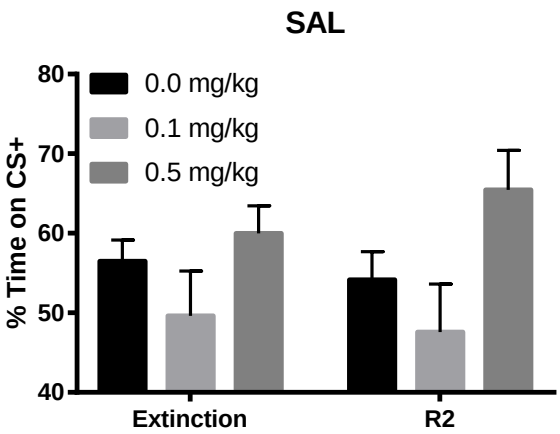

Fig.13B

Fig. 13B. Mean±SEM percentage time on CS+ on last extinction test and R1 of animals treated with saline before the reinstatement test. Black bars represent the dose of 0.0 mg/kg CLE during extinction, light grey 0.1 mg/kg and dark grey 0.5 mg/kg of CLE. **13B.**Mean ± SEM percentage time on CS+ on last extinction test and R2 on animals treated with saline before R2 test. Black bars refer to 0.0 mg/kg of CLE during extinction, light grey 0.1 mg/kg and dark grey 0.5 mg/kg of CLE.

DISCUSSION

These experiments demonstrate that, two different apparatus preparation; A1 or A2 configuration, are able to induce reliable cocaine-induced CPP in C57BL/6 mice. Our data denotes, however, a lack of significant differences in the magnitude or the number of animals expressing preference on T1 between procedures. Overall, these results suggest that both CPP configurations modulate the acquisition of conditioned memory in similar ways. Our data also indicates that these two apparatus configurations produce similar extinction of cocaine-induced CPP. These results were found when, after acquisition, we followed a forced (FE) or a choice (CE) extinction procedure. FE and CE, produce a progressive decrease in the time that animals spent on the CS+ compartment. Because we found no differences between A1 and A2 procedures, we collapsed the data and analyzed the differences between FE and CE. The general analysis demonstrated no differences between the two extinction procedures. A more detailed follow-up analyses, using Pairwise comparisons, revealed a slight difference in the ratio of extinction. Thus, while animals trained following CE show preference on first extinction test, the FE group was already extinguished. Interestingly, when animals were challenged with cocaine (10 mg/kg) as a priming stimulus, only animals that were exposed to a A2 were able to reinstate the conditioned response on R1. Nevertheless, our results indicated that in FE animals, only cocaine was able to reinstate the response in R1, whereas in CE animals we did not find differences between saline and cocaine on R2 and R3, suggesting that, in this case, context exposure could also reinstate the extinguished response after R1. These results suggest that under certain procedural circumstances, cocaine-priming but also context exposure and saline administration could reinstate CPP. Regarding the effects of CLE on extinction of CPP, we did not find any effects on the ratio of extinction at any of the doses

tested. Our analyses also showed no impact of any of the CLE doses on reinstatement of cocaine CPP.

Our results regarding acquisition of CPP, following two different compartment configurations, are supported by Cunningham & Zerizef (2014) and Hitchcock & Lattal (2018). These studies proved that either A1 or A2 configurations produce consistent cocaine-induced CPP with similar magnitude. A different study by Hitchcock, Cunningham & Lattal (2014) demonstrated a greater magnitude of cocaine CPP in animals trained in A2 configuration than in A1. This different result is not explained by different methodological procedures. The strain and the age of mice, conditioned stimulus, cocaine dose, time spent on tests and conditioning trials are exactly the same as our experimental procedures. Thus, both CPP configurations affect acquisition memory in the same way under our experimental conditions.

Our results also differ from Hitchcock, Cunningham & Lattal (2014) results in that they showed a greater extinction in animals trained in A1 configuration on the extinction phase. However, their procedure differs from our. They exposed animals during 30 minutes to CS+ without cocaine injection two days in a row and 24 hours later was the test. Contrary, the results of our experiments showed no differences in extinction between A1 or A2 following either FE or CE procedures. Furthermore, these two extinction procedures were compared using A1 or A2 configurations. As our results showed, no differences between two compartment configurations, data of A1 and A2 were collapsed to compare both extinction procedures regardless CPP configuration. Other studies support the idea that both extinction procedures produce a decrease in drug-induced CPP. FE procedure produce a reliable extinction of ethanol-induced CPP (Cunningham & Henderson, 2000) and CE procedure also a consistent extinction of ethanol- (Cunningham, Henderson & Bormann, 1998), morfine- and cocaine-induce CPP (Sakoori & Murphy, 2005) in mice. Also, Sakoori & Murphy (2005) suggest that CE procedure produce a slower extinction maintaining CPP longer. This supports our data showing a greater extinction magnitude on first extinction test following FE compared with CE. All these data indicates that both CPP configurations had an effect on memory extinction. In addition, these outcomes may suggest a slower extinction following CE.

Neither A1-FE nor A1-CE groups reinstate CPP after any treatment (saline or cocaine-priming). These results suggest a weak acquisition memory following A1 configuration because there was no effect of cocaine-priming on reinstate CPP in any reinstatement test following FE or CE. On the other hand, A2-FE group reinstate CPP after cocaine-priming in all reinstatement tests, but also reinstate CPP animals injected with saline on R2 and R3. A2-CE group reinstate CPP on R2 and R3 after being injected with saline or cocaine-priming. These outcomes suggest that acquisition memory formed with A2 compartment configuration is solid and lasting due to cocaine-priming but also saline had an effect on reinstate CPP short- and long-term. However, there is no previous evidence supporting our results on reinstatement following training on A1 or A2. Taking all results together suggest that A2 compartment configuration lead a more stable and enduring acquisition memory than A1 configuration under our experimental conditions. Zavala et al. (2003) found that rats trained on A2 compartment configuration and FE procedure, exposing animals to CS+ and CS- on alternative days without US, reinstate cocaine-induced CPP after a cocaine-priming injection (5 mg/kg and 10mg/kg). This outcome supports our result of A2-FE group on R1. Contrary to our results showing no reinstatement on

R1 following A2-CE, other studies showed that rats trained under a CE procedure reinstate cocaine-induced CPP after cocaine-priming (5 mg/kg) (Mueller & Stewart, 2000) and C57BL/6J mice reinstate morphine-induced CPP after a morphine challenge on first reinstatement test (Shoblock, Wichmann & Maidment, 2005). Other procedural differences could account for these discrepancies. For example, Mueller & Stewart, 2000 trained rats instead of C57BL/6J mice. Conditioning trials were for 20 minutes. Also, extinction tests were for 15 minutes instead of 5 minutes as in our experiments. Shoblock, Wichmann & Maidment (2005) used morphine instead of cocaine, a three-chamber apparatus, the conditioning trials were for 30 minutes and tests were for 15 minutes.

On the other hand, and considering the pharmacological results with clenbuterol, our analysis demonstrated no effect of CLE on extinction performance. Any of the CLE doses tested facilitate the extinction of CPP. Animals treated with any of the CLE doses on the extinction phase were not able to reinstated CPP after cocaine-priming. This suggests that pretreatment with CLE did not prevent reinstatement following a cocaine-priming. It is possible that CLE ineffectiveness were due to the selection of doses chosen. Some studies showed an inverted U dose-response effect showing that CLE can improve or impair retention depending on the dose administrated. They found that 0.01 and 0.03 mg/kg of clenbuterol injected post-training enhanced performance on inhibitory avoidance task but 0.3 and 1 mg/kg impaired it. 0.003 and 0.1 mg/kg had no effect in CD1 male mice (Introini-Collison & Baratti, 1986, 1992; Introini-Collison, Castellano & McGaugh, 1994). NA system appears to influence memory through interaction with opiate, GABAergic and cholinergic system in the amygdala (Castellano, Introini-Collison & McGaugh, 1993; Dalmaz, Introini-Collison & McGaugh, 1993; Introini-Collison, Castellano & McGaugh, 1994). This evidence suggests highest dose of CLE could to interact with other systems producing memory impairment. However, Chai et al. (2016) found an improvement on memory on Water Morris test performance in APP/PS1 mice after being treated with 2mg/kg of clenbuterol. Nevertheless, our results showed no effect of any CLE dose, but it didn't display an impaired performance through showing an increase conditioned response compared with control animals. It could be because CLE dose needed to produce improvement on learning is not the same for aversive and appetitive memories.

CLE ineffectiveness could be explained by differences in the timing of injection across experiments. CLE injected intraperitoneally (i.p.) 30 minutes before training in a 8-radial maze improved performance decreasing number of errors to found food-reward in rats (Sáez-Briones et al., 2015). Additionally, CLE infused on the prefrontal cortex 30 minutes before training increased the percentage of correct trials to get chocolate-reward in rats trained on a T-maze (Ramos, Colgan, Nou & Arnsten, 2008). LaLumiere, Niehoff & Kalivas (2010) showed improve on extinction learning in rats infused CLE intra-infralimbic cortex immediately post-training in self-administration paradigm. CLE also worked on task that implicates aversive learning. CLE injected immediately post-training in mice and rats improved performance on inhibitory avoidance task (Introini-Collison & Baratti, 1992; Introini-Collison, Dalmaz & McGaugh, 1996).

Another reason to the lack effect of CLE could be the duration of the extinction test. Suzuki et al. (2004) indicated that the time of exposure to the CS is important to determinate what memory will be affected and then, what behavior will be reinforced. In their experiments

demonstrated that extinction learning occurs after prolonged exposure to CS without US. However, after short exposure to the CS, memory reconsolidation can be targeted. It is possible that 5 min tests were insufficient to trigger extinction memory. LaLumiere, Niehoff & Kalivas (2010) demonstrated enhance on extinction memory in rats infused with CLE intra-infralimbic cortex immediately post-training of extinction trial (15 minutes) in self-administration paradigm. Altogether suggest that the time window to act on extinction memory is longer than 5 minutes.

Furthermore, a great deal of studies demonstrate the implication of the NA system in drug-related memory formation. Brenhouse, Dumais & Andersen (2010) demonstrated NA role on extinction learning increasing NE levels through a different action mechanism. Rats injected with atomoxetine, NE reuptake inhibitor, 25 minutes before extinction trials prevented cocaine-priming reinstatement CPP. This study proved that blocking NA system during acquisition memory or enhancing it during extinction memory produce a decrement of conditioned response. Taking all together demonstrates implication of NA system in both acquisition and extinction of drug-related memories on CPP paradigm.

Our results could be explained by β_2-AR are only involved in acquisition memory but not in extinction memory. Nevertheless, LaLumiere, Niehoff & Kalivas (2010) demonstrated β_2-AR role in extinction learning injecting CLE intra-infralimbic immediately after extinction trials in rats. Animals decreased number of lever presses in order to get cocaine suggesting enhancement on extinction learning as animals decreased conditioned response. That outcome support noradrenergic implications and β_2-AR role in memory extinction consolidation in self-administration paradigm. Nevertheless, neurobiological mechanisms underlying operant memories created in self-administration paradigm are different from conditioned memories formatted in CPP paradigm (Aguilar, Rodríguez-Arias & Miñarro, 2009). Thus it could be that β_2-AR doesn't participate in drug-conditioned-memories extinction consolidation.

To summarize, A2 CPP configuration produce strong and lasting acquisition memory considering reinstatement results in which animals trained in A2 reinstate CPP but no animals trained in A1. FE procedure produce quicker extinction memory formation than CE one in view of the fact that CPP stilled until second extinction test on CE but not on FE procedure. Thus, as our target was enhancing extinction memory, extinction procedure should produce weak extinction memory in order to accelerate its formation and do it stronger. For these reasons the protocol chosen to modulate extinction memory with CLE was A2-CE. On the other hand, despite findings that CLE works in several tasks, dose and at different time point our results had shown no CLE effect on drug-seeking behavior neither extinction phase nor reinstatement sessions. These results suggest no β_2-AR implication in cocaine-associated extinction memory under our experimental conditions.

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