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Cocaine conditioned place preference: unexpected suppression of preference due to testing combined with strong conditioning conditions

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ABSTRACT

Conditioned place preference (CPP) is widely used for evaluating the rewarding effects of drugs. Like other memories, CPP is proposed to undergo reconsolidation during which it is unstable and sensitive to pharmacological inhibition. Previous studies have shown that cocaine-CPP can be apparently erased by extracellular signal-regulated kinase (ERK) pathway inhibition during cocaine reconditioning (re-exposure to the drug-paired environment in the presence of the drug). Here we show that blockade of D1 receptors during reconditioning prevented ERK activation and induced a loss of CPP. However, we also unexpectedly observed a CPP disappearance in mice that underwent testing and reconditioning with cocaine alone, specifically in strong conditioning conditions. The loss was due to the intermediate test. CPP was not recovered with reconditioning or priming in the short term, but it spontaneously reappeared after a month. When we challenged the D1 antagonist-mediated erasure, we observed that both a high dose of cocaine and a first CPP test were required for this effect. Our results also suggest a balance between D1-dependent ERK pathway activation and an A2a-dependent mechanism in D2 striatal neurons in controlling CPP expression. Our data reveal that, paradoxically, a simple CPP test can induce a complete (but transient) loss of place preference following strong but not weak cocaine conditioning. They emphasize the complex nature of CPP memory and the importance of multiple parameters that must be taken into consideration when investigating reconsolidation.

INTRODUCTION

The discovery that memory can return to a labile state after memory recall revealed that memory is in fact malleable (Nader et al., 2000b; Przybylski and Sara, 1997). If not destabilized during a period of time after retrieval termed “reconsolidation window”, the memory can be consolidated again and strengthened, a phenomenon referred to as reconsolidation (Lee, 2008). During the reconsolidation window the memory can also be modified to allow incorporation of new information (Choi et al., 2010; Lee, 2009). The occurrence of memory erasure following recall has been supported by a growing amount of studies, using different paradigms and pro-amnestic procedures, including electroconvulsive shock (Misanin et al., 1968), protein synthesis inhibitors (Nader et al., 2000a), extracellular signal-regulated kinase (ERK) pathway inhibitors (Cestari et al., 2014; Miller and Marshall, 2005; Valjent et al., 2006), or other types of manipulations (Mactutus et al., 1979; Reichelt and Lee, 2013).

Most studies on memory reconsolidation have focused on aversive memories and less is known about appetitive conditioning and reward-associated memories. The latter can be implicated in pathological learning associations as in the case of drug abuse (Torregrossa et al., 2011). Long-lasting associations between a drug and a specific context can trigger relapse after withdrawal (Milton and Everitt, 2012). Therefore, the manipulation of drug-associated memories could have potential therapeutic implications in the context of addiction. The most widely used paradigm in studies of appetitive memory is conditioned place preference (CPP), which can be performed in various animal species (Bardo and Bevins, 2000). The CPP paradigm has been used to show memory disruption by various procedures (Milekic et al., 2006; Miller and Marshall, 2005; Valjent et al., 2006; Xue et al., 2012).

Cocaine is the most frequently illegal drug reported to the Drug Abuse Warning Network by emergency departments in the United States (Substance Abuse and Mental Health Services Administration, 2016) and is also the second most commonly used illegal drug in Europe, according to the European Monitoring Center on Drugs and Drug Addiction (EMCDDA, 2016). A wide variety of studies have shown the importance of ERK in memory formation across several species and brain areas (Peng et al., 2010). Our group and others have shown that a cocaine-associated memory can be apparently erased by inhibiting the ERK pathway during recall (Miller and Marshall, 2005; Valjent et al., 2006). However, as ERK is a ubiquitous pathway, identification of more specific targets and a better understanding of the apparent erasure are required before considering any potential therapeutic application. Therefore, in the present study we aimed at finding a more specific target and defining the conditions that allow cocaine CPP erasure. This series of experiments unexpectedly led us to discover that testing the mice once, specifically after conditioning with a high dose of cocaine, can cause by itself a complete loss of preference. To our knowledge, this effect has never been reported before and can have important implications in the interpretation of studies which use CPP as a model and for deciphering the underlying mechanisms.

MATERIALS AND METHODS

Animals

All the experiments were conducted with 8-12 week old male C57Bl/6J mice (Janvier, France), except for immunofluorescence experiments in which we used mutant adult male mice aged 2-6 months: *drd1::EGFP* and *drd2::EGFP* BAC transgenic mice (expressing EGFP under the control of dopamine D1 and D2 receptor promoter, respectively) generated by GENSAT (Gene Expression Nervous System Atlas; (Gong et al., 2003)). The animals were housed 4 per cage, in a 12-h light-dark cycle, in stable conditions of temperature, with food and water *ad libitum*. They were acclimatized at least one week, before being handled for 3 days prior to behavioral conditioning. The procedures were always performed during the light phase of the diurnal cycle. All the experiments were in accordance with the European Community Council Directive 2010/63/EU and approved (Ce5/2012/095) by the *Comité d'éthique pour l'expérimentation animale Charles Darwin* (Paris, France) #05.

Behavioral experiment setup

Conditioned place preference (CPP) was performed in two compartments of a Y-shaped apparatus (Imetronic, Pessac, France) with different wall textures and different visual cues (**Fig. 1a & b**), in a low luminosity environment (10 lux). The two types of cues were used simultaneously, unless otherwise indicated. The two different textures and visual cues (**Fig. 1c**) were chosen so that naïve C57Bl/6J mice did not show any preference on average for either compartment. The floor was kept completely smooth because we observed a strong interaction between floor texture and increase in place preference after cocaine conditioning (data not shown), similarly to Rakover-Atar and Weller (1997). After each mouse the different parts were cleaned with demineralized water and H₂O₂ 2% (vol/vol), before being carefully dried. Hydrogen peroxide is a strong oxidizer and therefore degrades odorant molecules.

Behavioral procedure

The typical procedure was as follows (**Fig. 1d**). (i) Pretest: on the first day, mice were placed in the center of the apparatus (**Fig. 1a**) and allowed to explore freely both compartments for 15 min. The time spent in each compartment was automatically recorded. Then mice were assigned to one or the other compartment in an unbiased counterbalanced way for each group, to avoid any initial bias in preference prior to conditioning. (ii) Conditioning: the second day mice were injected with cocaine and placed immediately in the assigned closed compartment (**Fig. 1b**) for 20 min. The next day they were placed in the other closed compartment after saline injection (10 mL/kg). This was repeated twice (3 cocaine-pairings, 3 saline, in total) unless otherwise specified. (iii) Test 1: CPP was tested the following day during 15 min to assess the efficiency of conditioning. (iv) Re-exposure: the mice treated or not with various inhibitors at the indicated times (see below for each experiment) were injected with cocaine and placed in the cocaine-paired compartment as during the initial conditioning phase. (v) Test 2: the disappearance or not of the preference was tested on day 10. (vi) Drug priming: in some experiments, a priming dose of 10 mg/kg of cocaine was injected just before CPP testing, in order to evaluate if it could restore the place preference.

During testing sessions, the experimenter was unaware of which treatment and which compartment had been previously assigned to the mice.

Drugs

Cocaine hydrochloride (Cooper, Melun, France) was dissolved in 9 g/L NaCl solution (saline) and injected i.p. (10 mL/kg). Solutions were made fresh every day as cocaine can undergo spontaneous hydrolysis (Isenschmid et al., 1989). SCH23390 hydrochloride (Tocris Bioscience, Bristol, United Kingdom) was dissolved in saline and injected i.p. 0.25 mg/kg (Pascoli et al., 2005), 10 minutes before the animal was placed in the apparatus. SCH58261 (Tocris Bioscience, Bristol, United Kingdom) was dissolved in DMSO (final dose 2.5 mL/kg) and injected i.p. 10 mg/kg (Beeler et al., 2012) 30 minutes before the animal was placed in the apparatus. Raclopride (Tocris Bioscience, Bristol, United Kingdom) was dissolved in saline and injected i.p. 0.3 mg/kg (6.67 mL/kg), 15 minutes before the animal was placed in the apparatus (Valjent et al., 2000).

Statistical analysis

After each test, the percentage of “preference for the cocaine-paired compartment” for each mouse was calculated as follows:

$$\frac{\textit{time spent in the cocaine paired compartment}}{(\textit{time spent in the cocaine paired compartment}) + (\textit{time spent in the saline paired compartment})}$$

The time spent in the small central zone was negligible and not taken into account in the calculation. Results were expressed as mean \pm SEM. Results were analyzed by two-way repeated measures ANOVA with matching between consecutive tests within mice, followed by Bonferroni post hoc tests, using the GraphPad Prism program. The “matching” F value evaluates whether the matching was effective, i.e. the chance of randomly observing an effect this big (or bigger) in an experiment of this size with unmatched samples.

Tissue preparation and immunofluorescence

Drd1a::EGFP and *drd2::EGFP* mice were quickly and deeply anesthetized with pentobarbital (500 mg/kg, i.p.) and transcardially perfused with 40 g/L formaldehyde in PBS for 5 min. Brains were post-fixed overnight at 4°C before being cut in 30 μ m sections using a vibratome (Leica, France). Sections were stored at -20°C in a cryoprotective solution containing glycerol: ethylene glycol: PBS 30:30:40 (vol/vol). Selected free-floating sections were processed for immunolabeling in 48-well plates. They were rinsed with Tris-buffered saline (TBS) pH 7.5 containing 50 mM NaF (as phosphatase inhibitor). Sections were first permeabilized with 10% (vol/vol) methanol in TBS containing 50 mM NaF, for 5 min. After being rinsed three times, they underwent a second permeabilization with 0.2% (vol/vol) Triton X-100 in the same buffer, for 20 min. They were then rinsed again 3 times before being incubated overnight at 4°C with the anti-phospho-ERK1/2 (pERK) mouse IgG (Cell Signaling; ref 5726, 150 ng/mL final). After 3 10-min rinses in TBS, sections were incubated with a Cy3-coupled goat anti-mouse secondary antibody (Jackson Laboratory, 1/400) for 45 min at room temperature. Finally, they were rinsed twice in TBS, twice

in 50 mM Tris pH 7.5 and mounted in Vectashield with 4,6-diamidino-2-phenylindole (DAPI), (Vector Laboratories, USA).

Confocal microscopy

A laser scanning confocal microscope (TCS SP2, Leica) with a 40X objective was used for image acquisition. It was carried out at the *Institut du Fer à Moulin* Cell and Tissue Imaging Facility.

RESULTS

ERK is selectively activated in D1 striatal neurons during cocaine CPP re-exposure session

Previous studies have shown that MEK (MAP-kinase/ERK-kinase) inhibition during re-exposure of mice to the environment in which they were initially conditioned (Miller and Marshall, 2005), or during a reconditioning session (Valjent et al., 2006) was sufficient to trigger an apparent erasure of the previously learned cocaine CPP. Local infusion of MEK inhibitors in the nucleus accumbens triggered this erasure (Miller and Marshall, 2005), suggesting that target cells of the inhibitors are located in this brain region. With the experimental settings that we had established in our laboratory, both the conditioned stimulus (the context) and the unconditioned stimulus (cocaine) were necessary to trigger a reconsolidation window sensitive to MEK inhibitor (Valjent et al., 2006). To determine in which neurons the ERK pathway is activated at the time of action of MEK inhibitors, we immuno-labeled for pERK (activated form of ERK) brain sections of mice which were formaldehyde-fixed just after a reconditioning session (**Fig. 2a**). Since the striatal projection neurons (SPNs) are mainly divided in two approximately equal populations, expressing either D1 or D2 dopamine receptors (D1- and D2-SPNs, respectively) (Gagnon et al., 2017), we used *drd1a::EGFP* and *drd2::EGFP* reporter mouse lines to identify in which neurons ERK was activated. Study of sections in both lines of mice showed that virtually all pERK immunoreactive cells were D1-SPNs in both the dorsal striatum (DS) and nucleus accumbens (NAc) when mice were reconditioned to cocaine (DS $99 \pm 1\%$, NAc core $91 \pm 6\%$, NAc shell $97 \pm 3\%$, $n = 6$ mice) (**Fig. 2a**). These results show that activation of the ERK pathway is restricted to D1-SPNs during cocaine re-exposure in the associated context, at a time when CPP memory is sensitive to MEK inhibitors.

A D1R antagonist during re-exposure erases previously learned CPP

We then explored if the D1R antagonist SCH23390, which can prevent cocaine-induced ERK activation in the striatum (Valjent et al., 2000), could recapitulate the effects of MEK inhibition during re-exposure (cocaine + context). We first verified that in our experimental conditions SCH23390 (0.25 mg/kg) completely prevented ERK phosphorylation (in the DS and the NAc) when injected before the re-exposure session with cocaine (**Fig. 2b**). To evaluate the effects of SCH23390 on previously acquired CPP we used the same procedure as previously described for MEK inhibitors (Valjent et al., 2006). We conditioned mice with cocaine (20 mg/kg, 3 cocaine pairings in one compartment, alternated with 3 saline pairings in the other; in this experiment the

two compartments differed by tactile cues only) and tested them subsequently to assess their preference for the cocaine-paired compartment (Test 1, **Fig. 2c**). The next day, these mice received an injection of either SCH23390 (0.25 mg/kg) or vehicle 10 min before being reconditioned with an injection of 20 mg/kg cocaine in the previously cocaine-paired compartment. The following day they were subjected to a second test (Test 2) (**Fig. 2c**). Re-exposure to both cocaine and context in the presence of SCH23390 completely abolished CPP (**Fig. 2c**). Thus, the specific blockade of D1R was sufficient to inhibit the ERK pathway in the striatum during the re-exposure and to mimic the erasure of the cocaine-place preference obtained with MEK inhibition. Although we did not previously observe it (Valjent et al., 2006), in a new series of experiments we noticed that the mice undergoing re-exposure without SCH23390 (equivalent to “reconditioning”) displayed a significantly decreased CPP when tested again (Test 2, **Fig. 2c**). Although behavioral results are known to be quantitatively variable across experimenters (Lewejohann et al., 2006), we were intrigued by this difference and we investigated it further.

Loss of CPP following simple reconditioning is restricted to high doses of cocaine and is resistant to priming

We explored the parameters which might explain the existence of a variable decrease in CPP following a reconditioning session. First, we hypothesized that the conditioning was not robust enough, so we added visual cues to the walls and floors of compartments, in addition to the tactile cues (**Fig. 1c**), and repeated the procedure. In contrast to our anticipations, the mice lost their place preference between Test 1 and Test 2 (**Fig. 3a**) with all experimenters. So, reinforcing the conditioning cues did not prevent the loss of CPP between Test 1 and Test 2, but rather facilitated it.

It is known that repeated testing without reward can extinguish a previously learned place preference (Mueller and Stewart, 2000), but not as rapidly as we observed in our experiments. After extinction, further conditioning (Brown et al., 2008) or cocaine priming (i.e. injecting a low dose of cocaine just before testing) (Mueller and Stewart, 2000) have been shown to reinstate the place preference. However, in our experimental conditions, an additional cocaine conditioning session between the two tests did not prevent the loss of CPP. Moreover, cocaine priming (10 mg/kg just before the test) did not restore the place preference (**Fig. 3a**). This indicates that the loss of CPP between the two tests cannot be considered as a classical extinction.

We next investigated whether the dose of cocaine would influence the loss of CPP observed in our experimental conditions. We repeated the experiment as in Figure 2c (without SCH23390 during reconditioning) with 10, 15, and 20 mg/kg of cocaine. Within this dose range, the mice developed a comparable place preference, spending about two thirds of their time in the cocaine-paired compartment during the first testing (Test 1) indicating the existence of a dose ceiling effect (**Fig. 3b**). However, when mice were tested after reconditioning (Test 2) a difference appeared between the 3 groups. The mice conditioned with 20 mg/kg consistently and significantly lost their place preference at Test 2, whereas the mice receiving only 10 mg/kg kept the same level of preference between Test 1 and Test 2 (**Fig. 3b**). Mice receiving 15 mg/kg had an intermediate

response (**Fig. 3b**). Thus, the loss of cocaine CPP was grossly dose-dependent, being clearly apparent with a high dose.

Testing once CPP learned with a high dose of cocaine and 3 pairings is sufficient to trigger a complete loss of place preference

Since the disappearance of place preference occurred when the dose of cocaine was high and since such doses can mediate aversion (Riley, 2011), we reasoned that it was perhaps the repeated exposure to a high dose of cocaine, which was responsible for the erasure of CPP. To test this hypothesis we assessed the respective contributions of Test 1 and of the subsequent 4th cocaine injection paired with the conditioned context (reconditioning), using 20 mg/kg of cocaine. To our surprise the mice which underwent Test 1 but no reconditioning consistently lost their preference for the cocaine-paired compartment at Test 2 (**Fig. 4a**). In contrast, the mice for which Test 1 was omitted had a normal preference at the time of Test 2, whether or not they had received the 4th cocaine-pairing (**Fig. 4a**). This result clearly demonstrated that Test 1 was the main step responsible for the subsequent loss of CPP.

We then explored whether the apparent erasure due to Test 1 would also occur with a weaker conditioning, i.e. a reduced number of pairings. We therefore performed conditioning with the same experimental setup, but using only one injection of cocaine 20 mg/kg and one saline injection in the other compartment. With 16 mice per group, this single pairing was sufficient to induce a highly significant place preference, although the increased time spent in the cocaine-paired compartment was less pronounced than after 3 repeated cocaine pairings (**Fig. 4b**). When we carried out a second test (Test 2) the mice still showed a significant preference for the cocaine-paired compartment (**Fig. 4b**). So the loss of place preference after Test 1 did not occur when the conditioning was done with only one pairing. This latter result was in line with the previous findings and indicates that, somehow paradoxically, testing the mice once (Test 1) is responsible by itself for the complete loss of place preference observed after strong conditioning (i.e. increased visual cues, high dose of cocaine, high number of pairings).

Test-induced loss of place preference following strong conditioning is transient and CPP is spontaneously restored after 1 month

To complete the characterization of the test-induced loss of place preference, we investigated whether the CPP induced by repeated pairings with 20 mg/kg cocaine and apparently erased by Test 1 could spontaneously be re-expressed at a later time. After conditioning with 3 pairings of cocaine 20 mg/kg and a first post-conditioning test (Test 1), we re-tested the mice (Test 2) either the next day or 4 weeks later (**Fig. 5**). Whereas the animals tested the next day consistently showed a lack of preference for the cocaine-paired compartment, the group tested after a month interval still displayed a strong place preference (**Fig. 5**). So, even if the loss of preference was not reinstated in the short term by further conditioning or cocaine priming, the mice spontaneously and completely re-expressed their conditioned place preference in the longer term.

A high dose of cocaine and the existence of a first test are necessary for D1R-antagonist-induced apparent erasure of CPP

After having identified this unexpected effect of Test 1 observed with a conditioning cocaine dose of 20 mg/kg but not 10 mg/kg, we examined the role of these parameters in the D1R antagonist-mediated erasure of place preference. First we used a dose of 10 mg/kg instead of 20 mg/kg with the same procedure as above (Pretest/Conditioning/Test 1/Re-exposure/Test 2) with SCH23390 (0.25 mg/kg) or vehicle injected 10 min before re-exposure (cocaine + context) (similarly to Figure 2c). In this experiment, D1R antagonist-mediated erasure of the place preference did not occur (**Fig. 6a**). This experiment indicated that the apparent erasure of CPP by SCH23390 was only observed following conditioning with a high dose of cocaine, not a lower one.

We then evaluated if Test 1 (just after the initial conditioning) was necessary. Initially, this intermediate test was implemented only to make sure that the animals were correctly conditioned and it is widely used in “erasure” procedures. The mice were conditioned with 3 pairings of cocaine 20 mg/kg and subsequently underwent the re-exposure (cocaine + context) in the presence or absence of SCH23390, before finally being tested for their preference, without any intermediate test. The injection of SCH23390 just before re-exposure did not have any effect on the final preference (**Fig. 6b**). Our results show that the D1-antagonist SCH23390 does not mediate the disappearance of the place preference when the intermediate test is omitted. Both the intermediate test and the dose of cocaine are necessary conditions required for the D1R-antagonist place preference-mediated erasure to occur (see **Fig. 2c**).

D1R-antagonist-induced CPP erasure is prevented by an A2aR antagonist, indicating a role of D2 SPNs

The transient character of the disappearance of CPP following Test 1 with a reappearance when Test 2 was carried out a month later, suggested that conditioning was not fully reversed or “erased” but rather masked by another process. Since D1R- and D2R-expressing striatal neurons have opposite effects on the induction of CPP (Kravitz et al., 2010), we hypothesized that the D2 neurons might play a role in the loss of CPP we observed in our experimental conditions. Dopamine receptors 2 (D2R) and adenosine 2A receptors (A2aR) are mostly localized in D2 SPNs (Schiffmann et al., 1991), have an opposite effect on adenylyl cyclase (Kull et al., 1999), and can potentially form heteromers (Fuxe et al., 2005). We used raclopride and SCH58261, antagonists of D2R and A2aR, respectively, to determine whether these drugs could modify the loss of CPP induced by reconditioning in the presence of the D1R antagonist. We investigated the effects of these compounds on the D1R-antagonist place preference mediated-erasure, within the conditions in which it does occur (i.e. as in Figure 2c). We conditioned mice with 3 pairings of cocaine (20 mg/kg), tested them just after conditioning (Test 1), before performing the re-exposure session with the D1R antagonist injection (exactly as in Figure 2c). The mice injected with the D1R antagonist before re-exposure (cocaine + context) did not show any preference the next day in Test 2 (**Fig. 7**), as reported above. The mice which were injected with the D2R antagonist also lost the previous CPP (**Fig. 7**). In contrast, the mice which received the A2aR antagonist during reconditioning in the presence of D1R antagonist, displayed a strong preference for the cocaine-paired compartment (**Fig. 7**). These results revealed that following blockade of A2aR during the

reconditioning step the erasing effects of the D1R antagonist combined with the intermediate Test1 were not observed. In contrast, the D2R antagonist appeared to increase these effects, possibly in relation with the opposing actions of D2R and A2aR on signaling in D2 neurons. These experiments strongly suggest that in addition to D1 neurons, the D2 neurons play a key role in the place preference disappearance.

DISCUSSION

In the present study we show that a D1R antagonist can contribute to the apparent loss of cocaine-induced CPP, using similar experimental settings as in a previous study showing that a MEK inhibitor could erase cocaine-induced CPP (Valjent et al., 2006). However, our study revealed a regulation of CPP expression, which was more complex than anticipated and highly dependent on the overall experimental conditions. Further investigation revealed the existence of a transient blockade of CPP that can follow its simple evaluation and paradoxically appears only following strong conditioning.

The loss of CPP after a strong conditioning (strong cues, high dose, repeated pairings) is surprising and may reveal important features of this cocaine-induced behavioral modification. It is known that letting the animal freely access the previously cocaine-paired environment in the absence of cocaine, as done during the test sessions, results in a small extinction (Mueller and Stewart, 2000). To completely extinguish CPP expression, this exposure has to be repeated several times (Mueller and Stewart, 2000). In our conditions the loss of CPP was complete after only one session of “test” and strikingly occurred specifically in “strong conditioning conditions”, whereas this effect was absent if the conditioning was weaker (3 pairings 10 mg/kg, or only one pairing 20 mg/kg). This suggests that such CPP loss may result from mechanisms at least partially different from those of standard extinction or that our experimental conditions triggered a particularly strong extinction mechanism. Indeed, in contrast to extinction, the loss of CPP we observed was not prevented by cocaine reconditioning and was also not rescued by cocaine priming before Test 2. However, the loss of CPP we observed was transient since place preference was fully restored when Test 2 was carried out a month after initial conditioning, suggesting that the initial learning was preserved.

How can only one test trigger a complete but transient loss of preference behavior? And why does it appear only when the conditioning has been strong? Several levels of explanation can be envisioned. Dopamine response encodes a reward prediction error rather than the reward itself (Schultz, 1986). Recent data indicate that it may also signal reward value through regulation of different aspects of dopamine neurons firing and dopamine release (Hamid et al., 2016). Formal models of learning used to predict behavior derived from Rescorla and Wagner (1972), propose that there is a predictive value associated to a given action. This value depends on the history of previous rewards and is updated after each trial depending on the prediction error. In our experimental conditions, we could hypothesize that the stronger the conditioning, the higher the expectation during the test, possibly leading to a strong negative prediction error. This negative error would be less pronounced when the conditioning was weaker. Another aspect of the

experimental conditions that could contribute to the loss of CPP in this paradigm is the difference between the testing conditions and the conditioning conditions. During the test, mice have free access to both compartments, whereas during conditioning they are confined in one compartment. It is possible that mice discriminate the two situations and that the more they trained in the first situation (conditioning), the better they make the difference with the second situation (testing). In this case, the animal would finally expect a reward in the conditioning situation but not in the testing situation, and thus would not show place preference anymore. This would be consistent with the fact that an extra conditioning session after the test would not improve the performance of the mice. Nevertheless, these two lines of explanation do not account for the reappearance of CPP when Test 2 was carried out 4 weeks after conditioning (see Fig. 5). This suggests that, as in the case of extinction, disappearance of the behavior involves the recruitment of separate inhibitory pathway(s).

Even though our study is phenomenological and does not allow to precisely identify the underlying mechanisms at the molecular and cellular levels, the effects of pharmacological agents provide some interesting clues. Apparent erasure of CPP is triggered or enhanced by the blockade of the ERK pathway or of D1R, which triggers the activation of this pathway. ERK activation in the striatum following cocaine injection is restricted to D1 SPNs and depends on D1R stimulation ((Valjent et al., 2000), and this study). Although the precise site of action of the D1R antagonist SCH23390 was not investigated in this study, striatal neurons are good candidates, although involvement of other brain regions including amygdala, hippocampus and prefrontal cortex is also possible, as suggested in distinct but related paradigms (Alleweireldt et al., 2006; Cosme et al., 2016) . In addition, we found evidence for an opposite role of D2 SPNs. The blockade of A2aR by an antagonist counteracted the loss of CPP, whereas the blockade of D2R if anything further decreased the preference. Further work will be needed to address the precise cellular bases of these effects. Synaptic plasticity in the form of long term potentiation of corticostriatal synapses is known to depend on cAMP and ERK stimulation by D1R in D1 SPNs and on cAMP stimulated by A2aR and inhibited by D2R in D2 SPNs (Pascoli et al., 2011; Shen et al., 2008). The effects of antagonists of these various receptors in our experimental conditions are compatible with a positive contribution of cAMP/ERK-dependent plasticity in D1 SPNs to the CPP expression, as previously shown for its induction. Conversely A2aR-mediated cAMP-dependent plasticity in D2 SPNs might reinforce an opposite behavioral regulation, which may lead to an apparently decreased preference or even an avoidance, as suggested by the effects of D2R blockade during reconditioning. Thus, a hypothesis that could account for our observations is that apparent erasure of CPP could result from its masking through increased cAMP-dependent plasticity and gain of function of D2 SPNs. The recruitment of this putative negative mechanism might be facilitated by strong dose and/or cues. A variable balance between striatal circuits comprising D1 and D2 SPNs may thus regulate the behavioral output of preference for a particular place. The modifications in the behavioral choices observed after the initial conditioning suggest that this balance is not definitely blocked but remains dynamic. In rat it was shown that blockade of D1 receptors with SCH23390 12 h after the test session delayed extinction, suggesting that a D1 dependent mechanism might also be operating (Fricks-Gleason et al., 2012). In that study as in ours the antagonist was administered systematically and it may have exerted its effects at various levels, including the dorsal hippocampus, in which stimulation of D1-like receptors has been reported to

enhance extinction (Abraham et al., 2016). Such an effect is unlikely to account for our results which rather correspond to an enhancement of an extinction-like response.

In the erasure protocols, intermediate tests are usually performed to check the preference of the animals, but their effects are neglected. Here we show that an apparent erasure of place preference with D1R antagonist during a recall does not occur in the absence of the intermediate test, meaning that these tests should be more globally taken into consideration in the design of “erasure” protocols and their interpretations. The erasure phenomena are probably highly complex and their understanding is far from being complete. Many inconsistencies are apparent in the published reports and subtle boundary conditions are suspected to be at stake (Nader and Einarsson, 2010). Even with fear conditioning which is the most widely used paradigm, boundary conditions are being discussed to elucidate discrepancies and sometimes opposite results using similar protocols (Auber et al., 2013). Conflicting results are difficult to decipher (Lee et al., 2009; Pineyro et al., 2013) and boundary conditions are still not clearly defined. Besides, some studies suggest that memory erasure is not as long lasting as previously thought (Alberini, 2008; Eisenberg and Dudai, 2004; Rudy et al., 2006). Erasure is considered as an absence of recovery of the behavior but different parameters and different reinstatement procedures can be used, and they are rarely all tested. Therefore, it is never to exclude that an apparently erased memory could be reinstated by another appropriate procedure. The frontier between extinction (formation a new memory which hides the expression of the initial learning) and reconsolidation is narrow (Monfils et al., 2009).

In summary the present results suggest the existence of a balance between D1R- and A2aR -regulated mechanisms that control the compartment preference in the CPP paradigm, which can be shifted depending on the details of the experimental conditions. The present work points out some potential caveats in “erasure” studies, and resulting difficulties to interpret and compare different studies. It also raises the question of the vocabulary used, which influences the way we comprehend these phenomena. We hope the present findings will facilitate the design and interpretation of future studies aiming at understanding the mechanisms of memory erasure, which could have many therapeutic implications, including for drug addiction.

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Author Contributions

JAG and DH conceived and supervised the project. JAG, DH and LMP designed the experiments. LMP, EV, AB and SL performed experiments and analyzed data. OE provided experimental support and conceptual advice. JAG, DH, LMP, JC, and AB interpreted and discussed the data. LMP and JAG wrote the manuscript, with critical revision of the article from AB, DH, EV, JC, and OE. All the authors approved the final version of the manuscript.

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Figure legends

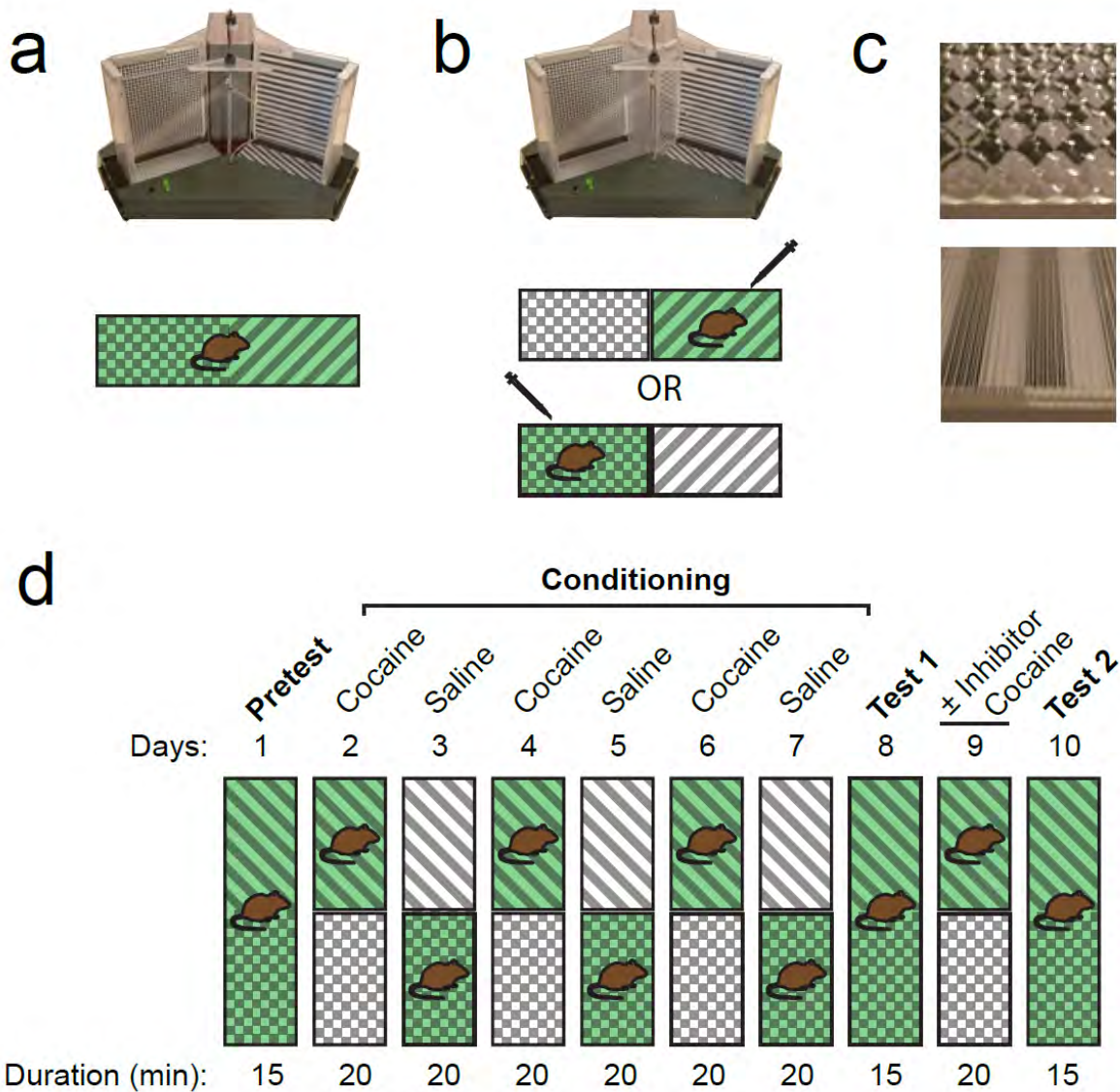


Figure 1: Experimental setup and outline. (a) Testing configuration and the corresponding simplified scheme. (b) Conditioning configuration and simplified schemes. (c) The two different combinations of tactile and visual cues on the compartment walls. (d) Typical experimental outline.

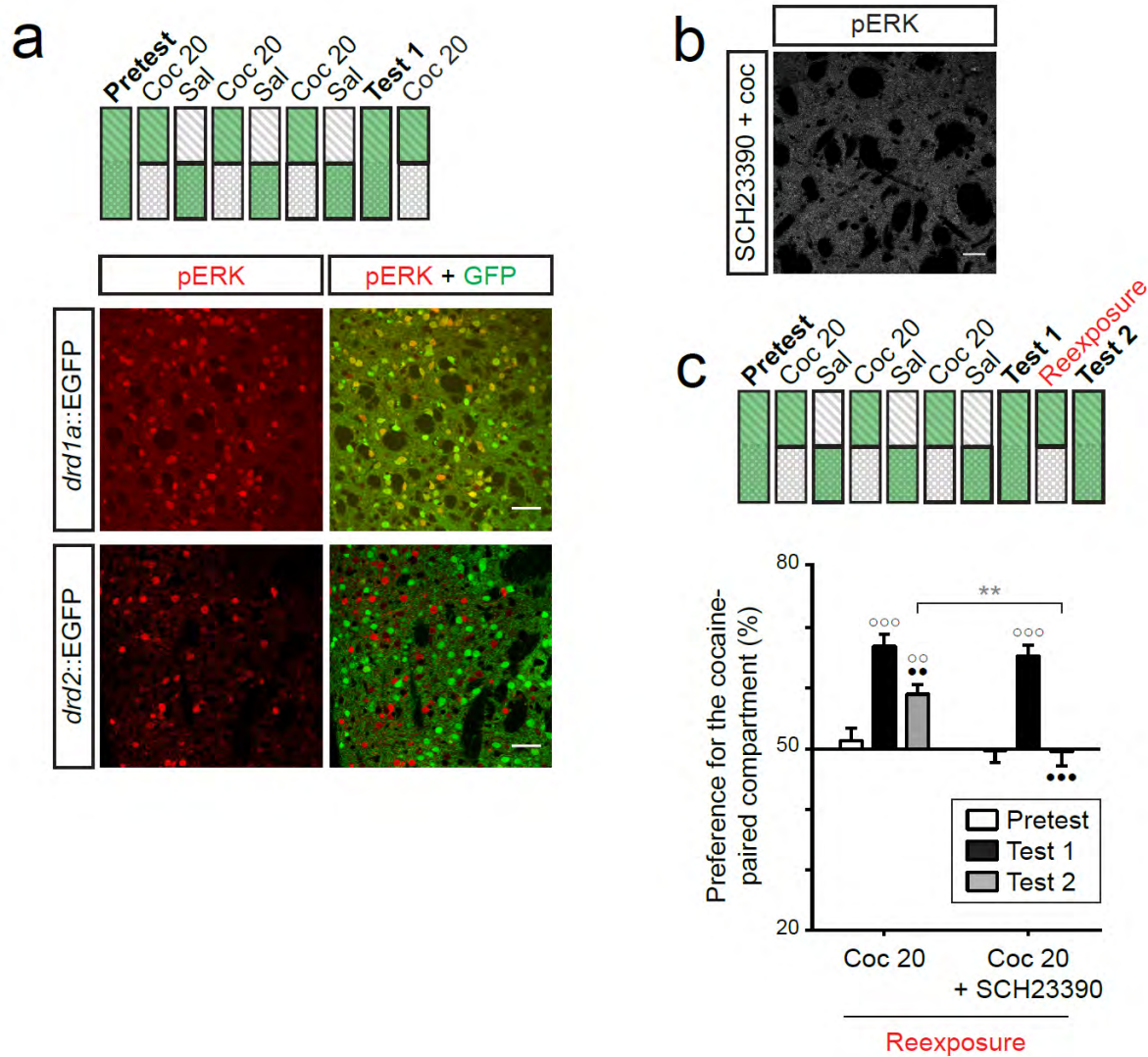


Figure 2: Role of D1 striatal neurons in CPP re-consolidation. (a) Confocal sections showing phospho-ERK (pERK) immunofluorescence in the dorsal striatum were generated from *drd1a::EGFP* and *drd2::EGFP* mice. Animals were sacrificed and perfused at the end of the 20 min reconditioning session. (b) Mice were treated as in A except that they received SCH23390 (0.25 mg/kg i.p.) 10 min before cocaine and re-exposure session. Confocal section showing phospho-ERK immunofluorescence in the striatum. (A and B), scale bar 50 μ m. (c) The day after Test 1, the mice were subjected to a “reexposure” (cocaine and paired-context) session. Ten min before cocaine injection and placement in the apparatus, mice received a D1R antagonist SCH23390 (0.25 mg/kg i.p.) or vehicle. Mice were subjected to a new test the following day (Test 2). Data are means \pm SEM (n= 22-24 WT mice per group). Two-way repeated-measures ANOVA: tests effect, $F_{(2,88)}=52.2$, $P<0.0001$; treatment effect $F_{(1,44)}=4.09$, $P=0.049$; interaction, $F_{(2,88)}=4.02$, $P=0.021$; matching, $F_{(44,88)}=2.56$, $P<0.0001$. Bonferoni *post hoc* test: $^{\circ\circ}P<0.01$, $^{\circ\circ\circ}P<0.001$ (Test 1 vs. Pretest); $^{\bullet\bullet}P<0.01$, $^{\bullet\bullet\bullet}P<0.001$ (Test 2 vs. Test 1); $^{**}P<0.01$.

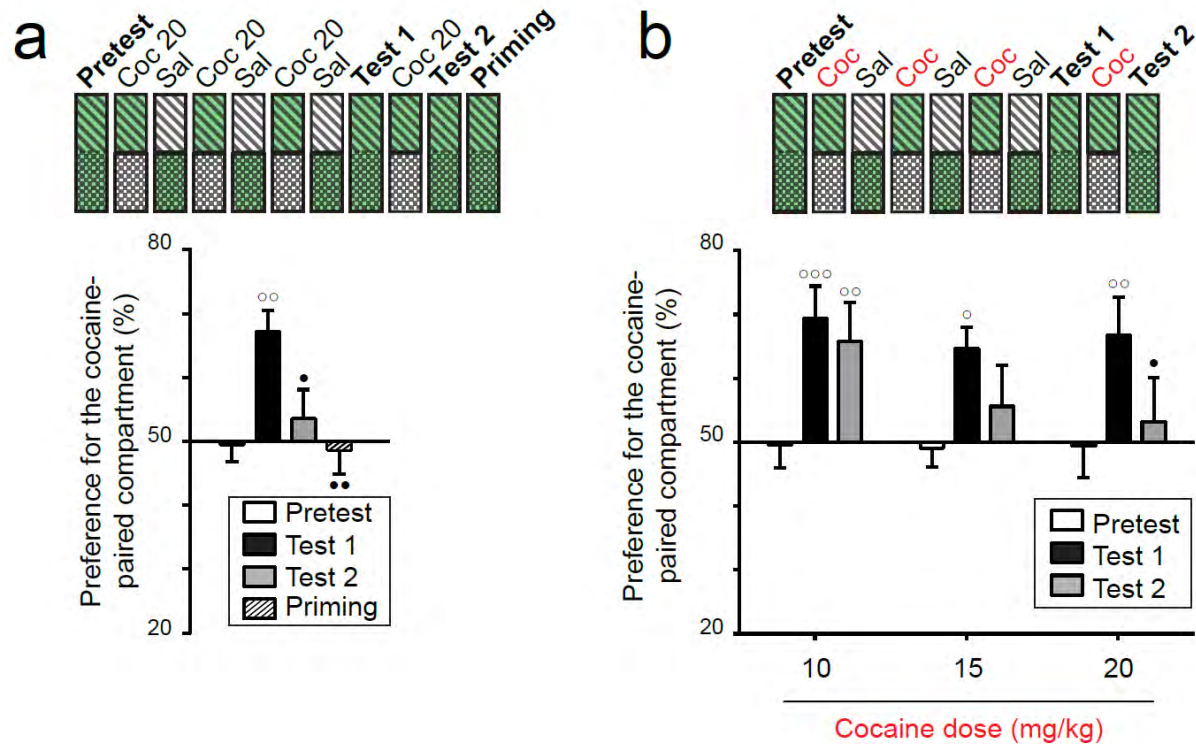


Figure 3: Disappearance of CPP: lack of effect of priming and dependence on cocaine doses. Mice were subjected to a cocaine CPP protocol, similarly to Fig. 2, but with additional visual cues on the compartment walls (see Methods and Results). (a) In these conditions, the CPP response observed in Test 1 was no longer detected in Test 2. The day after Test 2 they received a priming cocaine injection (10 mg/kg, i.p.) and were tested for preference. (b) Mice were conditioned with the indicated doses of cocaine. Note that the CPP was similar in Tests 1 and 2 when the dose of 10 mg/kg cocaine was used for conditioning. (a) Data are means \pm SEM (n= 20 mice per group). One-way repeated-measures ANOVA: tests effect, $F_{(3,57)}=6.0$, $P=0.0013$; matching, $F_{(19,57)}=1.36$, $P=0.19$. Bonferoni *post hoc* test: ° $P<0.05$, °° $P<0.01$, °°° $P<0.001$ (Test 1 vs. Pretest); • $P<0.05$, •• $P<0.01$ (Test 2 vs. Test 1). (b) Data are means \pm SEM (n= 16-20 mice per group). Two-way repeated-measures ANOVA: tests effect, $F_{(2,98)}=16.3$, $P<0.0001$; treatment effect $F_{(2,98)}=0.60$, $P=0.51$; interaction, $F_{(4,98)}=0.90$, $P=0.47$; matching, $F_{(49,98)}=3.90$, $P<0.0001$. Bonferoni *post hoc* test: ° $P<0.05$, °° $P<0.01$, °°° $P<0.001$ (Test 1 vs. Pretest); • $P<0.05$, •• $P<0.01$ (Test 2 vs. Test 1).

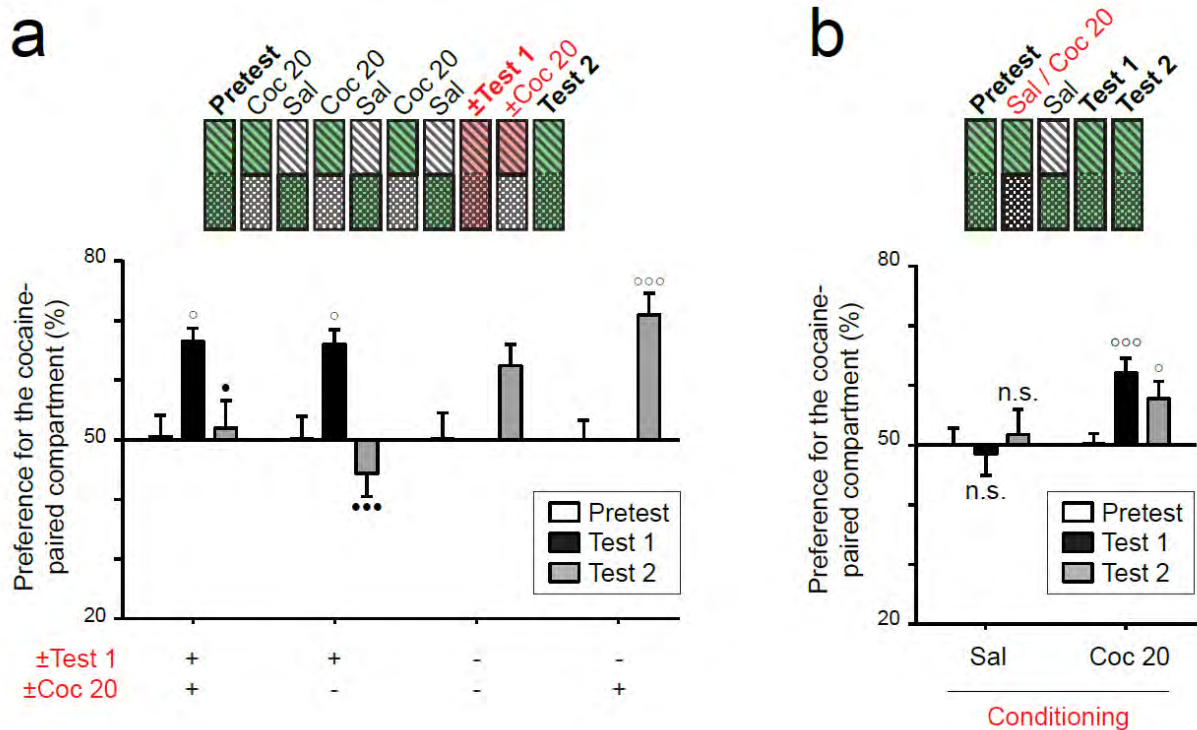


Figure 4: A first CPP test (Test 1) can be necessary and sufficient to trigger the disappearance of CPP to 20 mg/kg cocaine in subsequent test. Mice were subjected to a cocaine CPP protocol, similar to that in Fig. 3. When Test 1 or cocaine re-exposure were omitted, mice simply stayed in their home cage on that day. The actual day number of each manipulation is indicated below the bars. (a) Data are means \pm SEM ($n = 12$ mice per group). One-way ANOVA: $F_{(9,110)}=6.62$, $P<0.0001$. Bonferoni's multiple comparison *post hoc* test: $^{\circ}P<0.05$, $^{\circ\circ}P<0.001$ (Test 1 vs. Pretest); $^{\bullet}P<0.05$, $^{\bullet\bullet}P<0.001$ (Test 2 vs. Test 1). (b) Data are means \pm SEM ($n_{\text{saline}} = 8$, $n_{\text{cocaine}} = 16$). Two-way repeated-measures ANOVA: tests effect, $F_{(2,44)}=2.85$, $P=0.068$; treatment effect $F_{(1,22)}=3.99$, $P=0.058$; interaction, $F_{(2,44)}=4.113$, $P=0.023$; matching, $F_{(22,44)}=2.87$, $P=0.0014$. Bonferoni *post hoc* test: n.s. non-significant, $^{\circ}P<0.05$, $^{\circ\circ}P<0.001$ compared to Pretest.

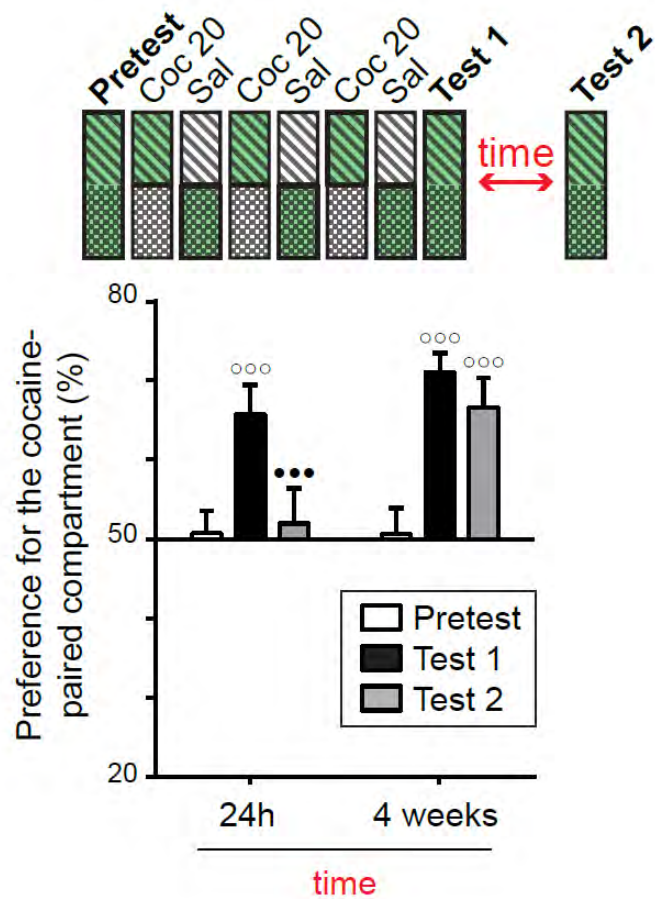


Figure 5: Spontaneous CPP recovery when Test 2 is delayed. Mice were conditioned to 20 mg/kg cocaine similarly to Fig. 3, except that Test 2 was carried out 1 day or 1 month later. Data are means + SEM (n= 12 mice per group). Two-way repeated-measures ANOVA: tests effect, $F_{(2,44)}=29.2$, $P<0.0001$; treatment effect $F_{(1,44)}=2.60$, $P=0.12$; interaction, $F_{(2,44)}=5.18$, $P=0.0096$; matching, $F_{(22,44)}=4.71$, $P<0.0001$. Bonferoni *post hoc* test: ^{ooo} $P<0.001$ (Test 1 vs. Pretest), ^{•••} $P<0.001$ (Test 2 vs. Test 1).

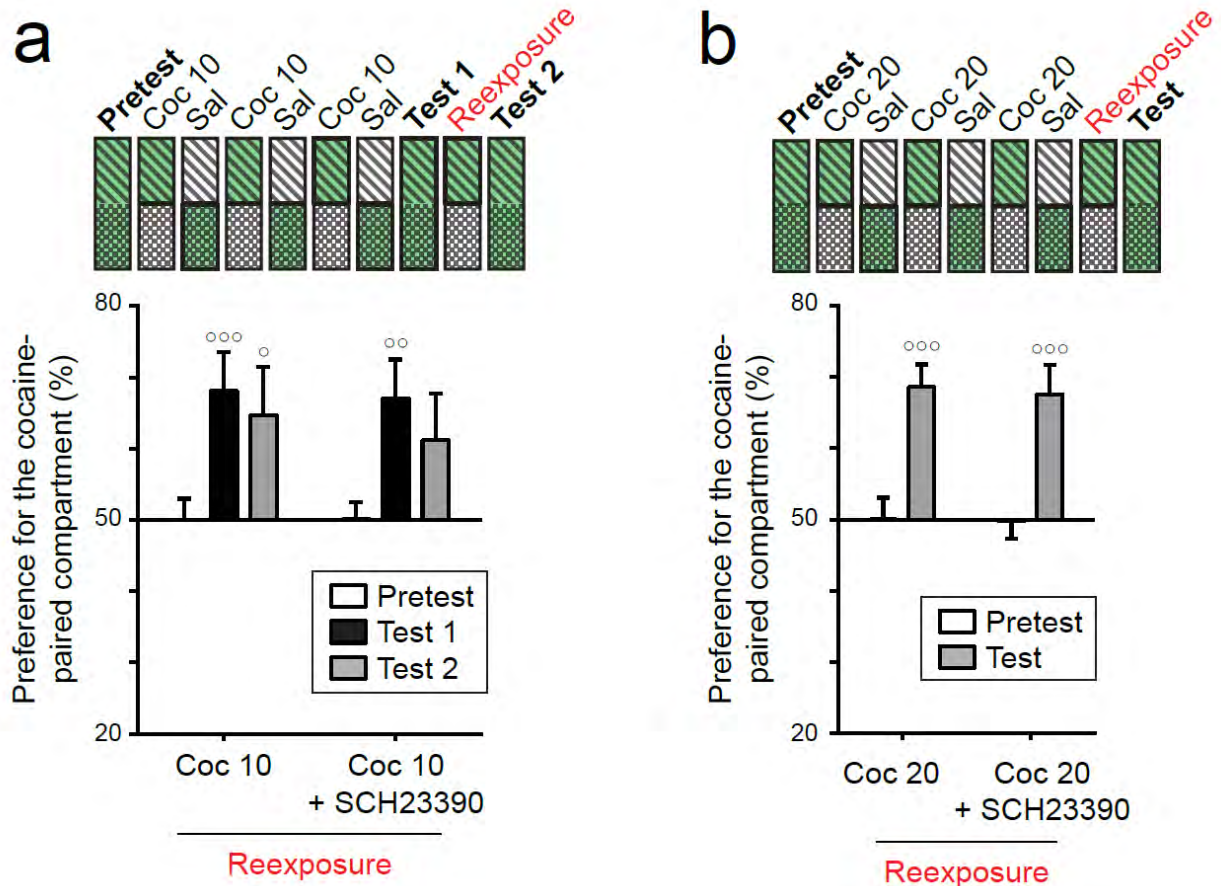


Figure 6: SCH23390-mediated erasure depends on experimental conditions. (a) SCH23390 was ineffective to change CPP response between Test 1 and Test 2 when animals were conditioned with 10 mg/kg cocaine, in contrast to what observed after conditioning with 20 mg/kg cocaine (see Fig. 3a). Data are means + SEM (n= 12 mice per group). Two-way repeated-measures ANOVA: test effect, $F_{(2,44)}=10.33$, $P=0.0002$; effect $F_{(1,44)}=0.06$, $P=0.80$, interaction, $F_{(2,44)}=0.11$, $P=0.90$; matching, $F_{(22,44)}=3.20$, $P=0.0005$. Bonferoni *post hoc* test: $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$, $^{\circ\circ\circ}P<0.001$ compared to Pretest (b) SCH23390-induced disappearance of CPP requires Test 1. Data are means \pm SEM (n= 12 mice per group). Two-way repeated-measures ANOVA: tests effect, $F_{(1,22)}=45.86$, $P<0.0001$; treatment effect $F_{(1,22)}=0.03$, $P=0.86$; interaction, $F_{(1,22)}=0.02$, $P=0.89$; matching, $F_{(22,22)}=1.95$, $P=0.063$. Bonferoni *post hoc* test: $^{\circ\circ\circ}P<0.001$ compared to Pretest.

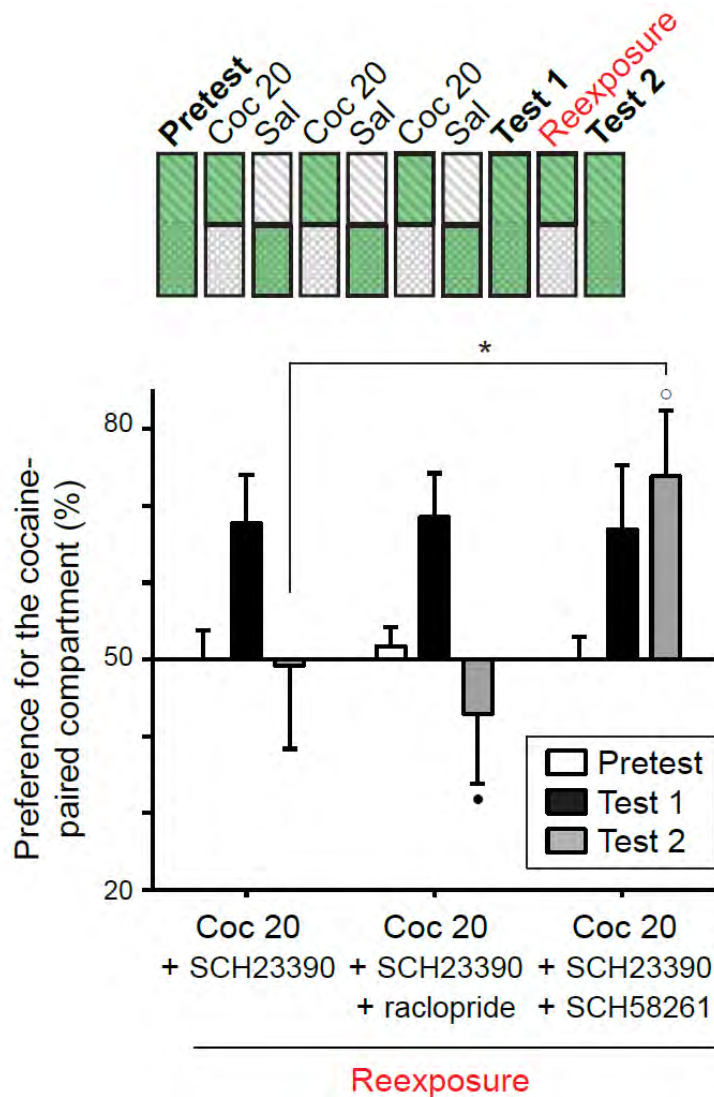


Figure 7: SCH23390-induced disappearance of CPP is prevented by an A2aR antagonist and increased by a D2R antagonist. Mice were treated as in Fig. 2B, SCH23390 was administered 10 min before the reconditioning session without or with raclopride (0.3 mg/kg, D2R antagonist -20 min before SCH23390-) or SCH58261 (10 mg/kg, A2aR antagonist -5 min before SCH23390-). Data are means \pm SEM ($n = 8$ mice per group). Two-way repeated-measures ANOVA: tests effect, $F_{(2,42)}=8.02$, $P=0.011$; treatment effect $F_{(2,21)}=0.86$, $P=0.44$; interaction, $F_{(4,42)}=3.11$, $P=0.02$; matching, $F_{(21,42)}=2.99$, $P=0.012$. Bonferoni *post hoc* test: $^{\circ}P<0.05$ (Test 1 vs. Pretest), $^{\bullet}P<0.05$ (Test 2 vs. Test 1), $^*P<0.05$.

