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## A large-scale c-Fos brain mapping study on extinction of cocaine-primed reinstatement

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## **Abstract**

Individuals with cocaine addiction can experience many craving episodes and subsequent relapses, which represents the main obstacle to recovery. Craving is often favored when abstinent individuals ingest a small dose of cocaine, encounter cues associated with drug use or are exposed to stressors. Using a cocaine-primed reinstatement model in rat, we recently showed that cocaine-conditioned interoceptive cues can be extinguished with repeated cocaine priming in the absence of drug reinforcement, a phenomenon we called extinction of cocaine priming. Here, we applied a large-scale c-Fos brain mapping approach following extinction of cocaine priming in male rats to identify brain regions implicated in processing the conditioned interoceptive stimuli of cocaine priming. We found that cocaine-primed reinstatement is associated with increased c-Fos expression in key brain regions (e.g., dorsal and ventral striatum, several prefrontal areas and insular cortex), while its extinction mostly disengages them. Moreover, while reinstatement behavior was correlated with insular and accumbal activation, extinction of cocaine priming implicated parts of the ventral pallidum, the mediodorsal thalamus and the median raphe. These brain patterns of activation and inhibition suggest that after repeated priming, interoceptive signals lose their conditioned discriminative properties and that action-outcome associations systems are mobilized in search for new contingencies, a brain state that may predispose to rapid relapse.

## Introduction

On the way to recovery from cocaine addiction, one of the biggest threats is relapse [1,2]. Craving, and subsequent relapse, are often favored when abstinent individuals ingest a small dose of cocaine, encounter discrete and contextual cues associated with drug use or are exposed to stressors [3-7]. In laboratory animals, cocaine-induced craving is modeled using drug-primed reinstatement, typically tested following extinction training [8-10]. Interestingly, this model revealed discriminative capacities where cocaine priming selectively reinstates cocaine-, but not heroin- or food-seeking behavior [11-14], likely via specific interoceptive effects originating from the periphery [15]. Indeed, cocaine priming generates conditioned interoceptive cues sufficient to induce reinstatement of cocaine-seeking in cocaine-experienced rats [15] and trigger glutamatergic and dopaminergic activity in both nucleus accumbens (NAc) and ventral tegmental area (VTA) [16-18]. Of note, the conditioned interoceptive effects are also observed with other addictive drugs in drug-experienced individuals [17,19-21]. In support of interoceptive functions in reinstatement, we have shown in rats that cocaine-conditioned interoceptive cues can be extinguished, even after cocaine escalation, with repeated cocaine priming in the absence of cocaine reinforcement, a phenomenon we called extinction of cocaine priming [22,23].

The neural mechanisms of reinstatement are extensively studied and a common brain pathway for cocaine-, cue- or stress-induced has been identified. Briefly, the VTA, the medial prefrontal cortex (mPFC), the NAc core and ventral pallidum (VP) appear to be central for this behavior [24-31]. Additionally, other brain regions such as the shell of the NAc, the dorsolateral striatum (dLSTR), the central (CeA) and basolateral (BLA) nucleus of the amygdala, the granular insular cortex (GI), the ventral subiculum (vSUB) and the pedunculo-pontine tegmental nucleus (PPTg) are critical for reinstatement behavior [27]. Despite these findings, it remains difficult to disentangle how the central and the interoceptive information are processed in the brain. Using our model of extinction of cocaine priming, we previously found a progressive loss of neuronal responses to cocaine in the prelimbic (PL), and the anterior cingulate regions (Cg) of the mPFC, as well as in the NAc core [23]. This suggests that recruitment of the top-down mPFC-NAc pathway is somehow involved in the conditioned interoceptive effects of cocaine priming. However, it is unclear where exactly these interoceptive stimuli of cocaine priming are processed in the brain and how they contribute to reinstatement of cocaine seeking behavior.

In the present study, we applied a large-scale c-Fos brain mapping approach [32,33] following extinction of cocaine priming to identify brain regions implicated in processing the conditioned interoceptive stimuli of cocaine priming. To this end, we measured changes in c-Fos expression in 17 regions of interest, composed of 48 different brain subregions. We found

that cocaine-primed reinstatement is associated with increased c-Fos expression in key brain regions, while its extinction mostly disengages them. Moreover, while reinstatement behavior is correlated with insula and NAc activation, extinction of cocaine priming involves parts of the VP, the mediodorsal thalamus (MDT) and the median raphe (MR).

## **Material and methods**

We report here a comprehensive and large-scale Fos brain mapping of the neuronal correlates of extinction of cocaine priming in a cohort of rats from a previous published study from this laboratory [23]. For further information on Material and methods, see the **Supplemental Online Materials**.

### **Animals**

A total of 28 adult male Wistar-Han rats (220-260g) were used (Charles River, Lyon, France). Studies were conducted in accordance with institutional and international standards of care and use of laboratory animals [UK Animals (Scientific Procedures) Act, 1986; and associated guidelines; the European Communities Council Directive (2010/63/UE, 22 September 2010) and the French Directives concerning the use of laboratory animals (décret 2013-118, 1 February 2013)]; agreement number of the animal facility: A33-063-922 (Committee of the Veterinary Services Gironde).

### **Intravenous cocaine self-administration**

Rats were anesthetized with a mixture of ketamine-xylazine (110 mg/kg-15 mg/kg, i.p., Bayer Pharma and Merial, respectively, Lyon, France) and implanted with an indwelling silastic jugular venous catheter (Dow Corning Corporation, Michigan, USA), as in [34]. After surgery, rats were flushed daily with 0.2 ml of a sterile antibiotic solution containing heparinized saline (280 IU/ml) and ampicillin (0.1 g/ml) to maintain patency. After 7 days of recovery from surgery, rats were habituated to the experimental chambers during 2 consecutive 3-h daily sessions with no lever or light cue presented (30 x 40 x 36 cm, Imétron, Pessac, France, see [35] for additional information about the apparatus). Then intravenous cocaine self-administration training consisted in 18 days of cocaine self-administration under a fixed-ratio (FR) 1 schedule of reinforcement during 3-h daily sessions (0.25 mg/injection over 4s) followed by 12 days under a FR3 schedule of reinforcement under the same conditions (**Fig. 1a**) as in [23]. All self-administration sessions began with extension of the operant lever and ended with its retraction after 3 h. No inactive lever was used. Responses on the active lever elicited a 148- $\mu$ l cocaine

infusion and a 20-s illumination of the light cue above the lever. During this 20-s time out period, responses were recorded but had no programmed consequences. All self-administration sessions were run 6-7 days a week.

### **Extinction of cocaine-primed reinstatement**

Extinction testing began one day after the last session of cocaine self-administration by using a between-session procedure previously described [23]. Briefly, all rats were first tested for 3 consecutive daily sessions of extinction of operant responding. Each daily session lasted 135 min during which completion of the FR3 schedule resulted in the 20-s cue light presentation but no drug delivery. Each session was subdivided into three 45-min periods that were identical except that rats received one non-contingent intravenous injection of vehicle at the onset of the last 45-min period. The operant behavior measured after vehicle served as a control for subsequent cocaine priming (**Fig. 1a**). Rats were then distributed into 2 balanced groups, group C and V, with similar pre-extinction levels of cocaine intake (i.e., mean cocaine injections over the last 3 self-administration sessions) and similar levels of extinguished responding to vehicle. On the following day, rats were subjected to extinction of cocaine priming during 8 consecutive daily sessions. Each daily session was identical to the 3 previous sessions with vehicle except that rats from group C received a 1-mg dose of cocaine administered intravenously instead of vehicle during the last 45-minute period [22,23,36,37] while rats from the control group V continued to receive an intravenous vehicle injection (**Fig. 1a**). Priming doses occurred after 90 min of extinction to have also a within-session measure of reinstatement (i.e., by comparing responding 45 min before and 45 min after drug priming) [22,36,37].

### **Reinstatement test after extinction of cocaine-primed reinstatement**

After extinction of cocaine-primed reinstatement in group C, each group was subdivided into two subgroups during the final test session during which they received either cocaine or vehicle, leading to a total of 4 subgroups. Subgroup V-V (n=5) was initially repeatedly primed and tested during the final session with vehicle. This subgroup controlled for any changes in brain activity unrelated to cocaine-primed reinstatement or its extinction. Subgroup V-C (n=5) was initially repeatedly primed with vehicle and tested with cocaine, thereby allowing measurement of the neuronal correlates of cocaine-primed reinstatement. Subgroup C-V (n=5) was initially repeatedly primed with cocaine and tested with vehicle, thereby allowing assessment of any eventual neuronal correlates associated with past extinction of cocaine priming. Finally, subgroup C-C (n=4) was initially repeatedly primed with cocaine and tested with cocaine, thereby allowing assessment of the neuronal correlates associated with

extinction processes recruited to block expression of cocaine-primed reinstatement. The final test session was identical to sessions of extinction of cocaine priming, except that the last post-injection period lasted 60 minutes (**Fig. 1a**).

### **c-Fos immunohistochemistry**

At the end of the test session, rats were anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and perfused transcardially with 0.1 M PBS and 4% formaldehyde (VWR, France). We perfused rats 60 min after the onset of priming injection, as previously described [32,38-40]. Brains were then extracted, post-fixed, cryoprotected, flash-frozen with -55°C isopentane, sliced on a cryostat (Leica CM 3050S), and processed for Fos immunohistochemistry analysis [23,32,33,41]. Briefly, 50 µm free-floating coronal sections were incubated with H<sub>2</sub>O<sub>2</sub> 0.3 % for 30 min after being washed with PBS (0.1M, 3 times for 10 min). Sections were then treated with a blocking solution (3% normal goat serum in 0.1 M PBS containing 0.3% Triton X-100) for 1h at room temperature (RT), incubated with the primary rabbit polyclonal anti-c-Fos antibody for 48 h at 4°C (1:8000; sc-52, Santa-Cruz Biotechnology Santa Cruz, RRID:AB\_2106783), washed in PBS and incubated for 2h at RT with biotinylated goat anti-rabbit IgG (1:200; BA-1000, Vector Laboratories, Clinisciences, RRID:AB\_2313606). Tissue sections were further treated with avidin-biotin-peroxidase complex (ABC, 1:200 for 2h at RT; ABC Vectastain Elite Kit, PK-6100, Vector laboratories) and 3,3'-diaminobenzidine substrate (DAB, 0.05% w/v with 0.003% H<sub>2</sub>O<sub>2</sub>, 5-min reaction time, Sigma-Aldrich), then mounted onto gelatin-alum-coated slides and air-dried overnight. Sections were dehydrated in ascending series of ethanol concentrations and coverslipped with Eukitt mounting medium (Sigma-Aldrich).

### **Imaging and Fos counting**

We counted c-Fos positive (Fos+) cells in both hemispheres (50 µm thick, 400 µm apart) throughout the entire anteroposterior (AP) extent (3-6 sections) of each region of interest (ROI) (see refs. [32,33] and **Supplementary Table S1** for exact anatomical coordinates, shapes and extents of the sampled area, and **Supplemental Online Materials** for details on Imaging and Fos counting).

### **Data analysis**

Data are presented as mean values ± SEM. In some graphs, individual values were plotted to show the distribution range (**Figs. 1d, 2a, Supplementary Figs. S1b and S2b**). All statistical tests were performed using Statistica, version 7.1 (Statsoft, Inc., Maisons-Alfort, France) and

GraphPad, version 8.4.3. All behavioral data were subjected to two- or three-way mixed analysis of variance (ANOVA) followed by post hoc comparisons using the Tukey's HSD test. Statistics for c-Fos expression were performed using two-way mixed ANOVA, followed by Tukey's HSD post hoc test on raw data. Correction for multiple testing with a false discovery rate of 0.15 was applied using Benjamini–Hochberg procedure [42]. To facilitate the visualization of the variations in c-Fos levels across ROIs, **Fig. 2a, c** displays expression as z-score from the V-V subgroup. Similarly, **Fig. 3a** displays expression as z-score from the V-C subgroup. Normality was verified by using the Shapiro-Wilk test. Finally, Pearson's correlations were used to examine relationships between responses on the active lever during the final test session and c-Fos densities in different ROIs. Statistical difference was set at  $p \leq 0.05$ . For better clarity and because our analyses included many main effects and interactions, we only report significant effects that are critical for data interpretation. In some cases of statistically significant interactions, we only indicate the results of the post hoc analyses. However, detailed statistics are provided in **Supplementary Tables S2-S4**. Sample sizes were determined based on our previous studies [23,32,33].

## Results

### Extinction of cocaine-primed reinstatement

All animals were submitted to the same FR training procedure (**Fig. 1a**) but were later assigned to different subgroups for subsequent extinction of priming and final tests (subgroups: V-V, V-C, C-V and C-C, see Materials and Methods). These subgroups showed comparable acquisition of cocaine self-administration (**Supplementary Fig. S1a**) with similar total cocaine intake (**Supplementary Fig. S1b**) before extinction testing. Most groups showed behavioral extinction (Day:  $F_{(8, 81)} = 15.71$ ,  $p = 0.0001$ ) and showed similar levels of extinction for vehicle before repeated priming (Day x Subgroup:  $F_{(6, 45)} = 0.38$ ,  $p = 0.89$ , **Fig. 1b**). They also showed similar levels of operant extinction before repeated priming (Day x Subgroup:  $F_{(6, 45)} = 0.38$ ,  $p = 0.8880$ , **Fig. 1b**). In comparison to V-V and V-C subgroups that received repeated vehicle, C-V and C-C subgroups initially reinstated responding after cocaine priming, but this effect progressively decreased with repeated priming (Day x Subgroup interaction:  $F_{(7, 105)} = 8.26$ ,  $p = 0.0001$ ) and eventually dissipates ( $p > 0.05$ , Tukey post hoc HSD, **Fig. 1c**). During the Test day, as expected, only the V-C subgroup responded to cocaine priming ( $F_{(1, 15)} = 18.64$ ,  $p = 0.0006$ ;  $p < 0.01$ , Tukey post hoc HSD; **Fig. 1d**, adapted from ref. [23]); the C-C subgroup behaved as the two subgroups (i.e., C-V and V-V) that received vehicle during the Test day.



## Large scale mapping of c-Fos expression associated with extinction of cocaine-primed reinstatement

Following the last behavioral test, rats from all 4 different subgroups were perfused and stained for c-Fos (**Fig. 1a**). Four main potential patterns of Fos expression are of interest (**Fig. 2a**, activation pattern key). The first pattern corresponds to a common increase or decrease of Fos expression in C-V and C-C subgroups compared to both V-V and V-C subgroups. This pattern of expression reflects a main effect of extinction of cocaine priming (e.g., vmVP, **Fig. 2a, b** and **Supplementary Table S2**). Second, both V-C and C-C subgroups could have higher or lower c-Fos expression compared to both V-V and C-V subgroups, reflecting neural activity associated with cocaine-primed reinstatement (e.g., cVP). The third pattern corresponds to a c-Fos expression in the C-C subgroup similar to both V-V and C-V subgroups but different from the V-C subgroup, reflecting a disengagement of the structure after cocaine priming (e.g., DLO). Finally, a change of c-Fos expression specific to the C-C subgroup, compared to all other subgroups, suggests that the structure is engaged in extinction of cocaine priming (e.g., IMDT).

In the medial orbital (MO) area of the orbitofrontal cortex (OFC), a significant Priming Extinction x Test interaction ( $p= 0.03$ ) revealed that both the V-C and the C-C subgroups had higher c-Fos expression compared to the V-V group ( $p< 0.01$  at least) (**Fig. 2a**). In the dorsolateral (DLO) area, the Priming Extinction x Test interaction ( $p= 0.02$ ) indicated that the V-C subgroup was significantly different from all 3 subgroups ( $p< 0.01$ ; **Fig. 2a**). This pattern of expression was also seen in different part of the prefrontal cortex including the cingulate (Cg): Priming Extinction x Test ( $p= 0.02$ ; V-C:  $p< 0.05$  at least from all 3 subgroups) and the dorsal portion of the prelimbic (dPL) cortex: Priming Extinction x Test ( $p= 0.01$ ; V-C:  $p< 0.05$  at least from all 3 subgroups, **Fig. 2a**). In the insular complex, such pattern was observed in the dorsal portion of the agranular insular cortex (aid): Priming Extinction x Test ( $p= 0.02$ ; V-C:  $p< 0.05$  at least from all 3 subgroups), and the dysgranular insular cortex (DI): Priming Extinction x Test ( $p= 0.01$ ; V-C:  $p< 0.01$  at least from all 3 subgroups). In the granular portion (GI) of the insular cortex, only the V-C subgroup showed higher expression compared to the V-V subgroup: Priming Extinction x Test ( $p= 0.04$ ; Tukey  $p< 0.05$ , **Fig. 2a**).

In the dorsal striatum, cocaine infusion during the Test increased c-Fos expression in all subregions for the Priming Extinction x Test interaction: dmSTR:  $p= 0.045$ ; dlSTR:  $p=0.005$ ; vmSTR:  $p= 0.04$ ; vlSTR:  $p=0.0006$  and ceSTR:  $p=0.01$ . The corresponding posthoc tests indicated higher c-Fos expression in the V-C subgroup compared to all 3 subgroups ( $p< 0.05$  at least). Moreover, in the dmSTR, the C-C subgroup was significantly different from the 3 other subgroups ( $p< 0.05$ ) indicating that the C-C subgroup had higher c-fos levels compared to the V-V and the C-V subgroups but lower than the V-C subgroup (**Fig. 2a**). In the ventral

striatum the V-C subgroup showed significantly higher c-Fos expression compared to the 3 other subgroups for the Priming Extinction x Test interaction in the NAc core:  $p=0.02$ , the NAc vmSh:  $p=0.03$  and the NAc lSh:  $p=0.002$ , Tukey posthoc:  $p<0.05$  at least (**Fig. 2a**).

After the cocaine Test, the lateral portion of the mediodorsal thalamus (IMDT) exhibited high c-Fos expression: Priming Extinction x Test:  $p=0.04$ . C-Fos expression in the V-C subgroup was significantly higher compared to the V-V subgroup ( $p<0.05$ ), and interestingly the C-C subgroup displayed, this time, higher c-Fos expression compared to all 3 subgroups (Tukey,  $p<0.05$  at least; **Fig. 2a**). Finally, in the dorsal portion of the subiculum (dSub), the V-C subgroup had significantly greater c-Fos expression compared to the other 3 subgroups: Priming Extinction x Test:  $p=0.01$ , Tukey:  $p<0.05$  at least (**Fig. 2a**). Of note, some main effects of Priming Extinction condition or of the Test condition were observed for several brain regions (**Fig. 2b**, see also **Supplementary Fig. S2** for representative images). Detailed statistics and corrected p-values are available in **Supplementary Table S2**.

### **Extinction of cocaine-priming re-organizes the patterns of c-Fos expression**

When assessing c-Fos expression in the different subgroups compared to the V-V subgroup, the 10 regions with the highest expression levels in the V-C subgroup mostly belonged to the dorsal and ventral striatum (6/10 regions) with increased expressions ranging from 3- to 9.5-fold (**Fig. 2c**, top panel). This dominance of the striatal complex was absent in the C-V subgroup, with c-Fos levels largely comparable to the V-V subgroup (**Fig. 2c**, middle panel). Finally, in the C-C subgroup, the dorsal striatum was also highly represented (4/10 regions) but with drastically reduced expression levels, ranging from 1.7- to almost 4-fold. Interestingly, the IMDT was the second region with highest expression (2.7-fold increase) compared to the V-V subgroup (**Fig. 2c**, bottom panel).

In order to better visualize the differences in expression levels between the two subgroups that were tested with cocaine but had different priming extinction histories, we built a color mapping of all the brain regions for which the C-C subgroup significantly differed from the V-C subgroup (based on statistics from **Fig. 2a**, **Supplementary Table S2**). This allowed to highlight overall decreased expression levels in various prefrontal (-35 to -38%), insular (-36 to -45%) and striatal areas (-42 to -64%) as well as in the dSub (-49%; **Fig. 3a**). Interestingly, the IMDT was the only brain region in which c-Fos expression increased (+61%; **Fig. 3a**).

### **C-Fos expression shows both positive and negative correlations with active responding to cocaine priming**

Overall, the previous analysis allowed to extract general patterns of brain activation associated with cocaine priming as well as with its extinction. We however wanted to assess, in a brain-wide fashion, whether c-Fos expression patterns were related to simple pharmacological activation or to operant responding during cocaine priming. For that, we correlated c-Fos expression of both subgroups receiving saline (i.e., V-V and C-V) and both subgroups receiving cocaine priming (i.e., V-C and C-C) during the test day to the number of active responses during this session (**Fig. 3b**). For both subgroups receiving saline, we found that the pIC, the DI and the GI subregions of the insula as well as the IMDT, showed positive correlations: pIC:  $r = 0.37$ ,  $R^2 = 0.40$ ,  $p = 0.0491$ ; DI:  $r = 0.73$ ,  $R^2 = 0.53$ ,  $p = 0.0167$ ; GI:  $r = 0.86$ ,  $R^2 = 0.75$ ,  $p = 0.0013$ ; IMDT:  $r = 0.70$ ,  $R^2 = 0.49$ ,  $p = 0.0241$  (**Fig. 3b**, left panel).

For both V-C and C-C subgroups, all the subregions of the insular cortex showed significant positive correlations, indicating that higher active responses were associated with higher c-Fos expression: aIC:  $r = 0.87$ ,  $R^2 = 0.75$ ,  $p = 0.0118$ ; aid:  $r = 0.89$ ,  $R^2 = 0.80$ ,  $p = 0.0066$ ; aiv:  $r = 0.83$ ,  $R^2 = 0.69$ ,  $p = 0.0198$ ; pIC:  $r = 0.76$ ,  $R^2 = 0.58$ ,  $p = 0.0468$ ; DI:  $r = 0.87$ ,  $R^2 = 0.76$ ,  $p = 0.0109$ ; GI:  $r = 0.82$ ,  $R^2 = 0.67$ ,  $p = 0.0249$  (**Fig. 3b**, right panel). This pattern was also observed for 2 nucleus accumbens subregions: the NAc vmSh:  $r = 0.96$ ,  $R^2 = 0.93$ ,  $p = 0.0004$  and the NAc ISh:  $r = 0.81$ ,  $R^2 = 0.65$ ,  $p = 0.0279$ . Interestingly, negative correlations were found for the vmVP:  $r = -0.87$ ,  $R^2 = 0.76$ ,  $p = 0.0105$ , the IMDT:  $r = -0.86$ ,  $R^2 = 0.75$ ,  $p = 0.0121$  and the MR:  $r = -0.76$ ,  $R^2 = 0.58$ ,  $p = 0.0478$  rather indicating that lower active responses were associated with higher c-Fos expression. The complete list of correlations is available in **Supplementary Table S3**.

### **The insular cortex is differently correlated with active response to cocaine priming along the anteroposterior axis**

The insula forms a large complex spanning across approximately 6.5 mm of the rat brain and is composed of well-defined subregions based on cytoarchitecture and functional connectivity. Moreover, studies suggest distinct roles of these subregions along both the AP and DV axes and overall place the insula as a major hub for interoceptive signals [43,44]. Given these unique features, we performed a finer anteroposterior analysis in search for potential differences between conditions. First, we assessed whether the expression of c-Fos was uniform along the anteroposterior axis (**Supplementary Fig. S3a-f**). The mixed-effects analysis 3-way ANOVA showed a significant AP x Priming Extinction x Test interaction only for the aid subregion:  $F_{(9, 62)} = 2.03$ ,  $p = 0.05$  and the Tukey post hoc test highlighted higher expression in the V-C subgroup for AP +3, +2.6 and +1.8 mm compared to the V-V subgroup ( $p < 0.05$  at least; **Supplementary Fig. S3b**). Next, since c-Fos levels were associated with active responding to cocaine priming in all the different insular subregions (**Fig. 3b**, right panel),

we interrogated if c-Fos levels uniformly correlated to behavior along the anteroposterior axis. Overall, the aIC and its two components, the aid and the aiv, showed significant correlations with behavior mostly in their anterior portions (i.e., between AP +4.2 and +1.8 mm; **Supplementary Fig. S3g**). Similarly, the DI and GI showed positive correlations with active responses in the anterior sections (i.e., between approximately AP +2.6 and -0.2 mm; **Supplementary Fig. S3g**). Finally, for the pIC, only one posterior section (AP -1.8 mm) showed significant correlation (**Supplementary Fig. S3g**). Statistics for correlations are available in **Supplementary Table S4**.

## Discussion

Using large scale c-Fos mapping, this study highlighted the neural correlates of both cocaine-induced reinstatement and its extinction. While cocaine-induced reinstatement was associated with drastic increases in c-Fos levels in the dorsal and ventral striatum, several prefrontal area and subregions of the insular cortex, extinction of cocaine priming mostly reduced c-Fos expression in these areas. Additionally, positive correlations between c-Fos expression and responses during the reinstatement test were observed in different regions of the insula and the NAc shell while negative correlations involved the VP, the MDT and the MR. Here, we will restrict our discussion to our findings related to the extinction of cocaine priming, for a detailed discussion please see the **Supplementary Discussion**.

First, this study has some limitations that must be considered and could be addressed by future work. Indeed, we exclusively used male rats. Sex-differences are observed in initial stages of cocaine-related extinction behavior, but they usually homogenize following repeated sessions [45,46]. Although we do not expect drastically different behavioral output, studies report differences in cocaine-related neurotransmission and c-Fos expression between males and females [45,47]. Future studies will thus be important to map the c-Fos expression landscape of extinction of cocaine priming in females. Moreover, we used c-Fos expression to explore the involvement of brain regions during a final cocaine-primed reinstatement test. Given its expression kinetics, the efficiency of c-Fos to account for previous drug exposure and the different phases of our protocol is limited. Guided by the present findings, future work using in vivo neuronal recording will help to further dissect the differences in neuronal activation between groups.

### **Extinction of cocaine-priming disengages the fronto-striatal circuits**

Overall, following extinction of cocaine-primed reinstatement, c-Fos expression was significantly decreased, essentially to baseline levels, in most cortical regions. Interestingly,

the MO was the only cortical region showing increased c-Fos levels in both cocaine-primed subgroups (i.e., V-C and C-C). The MO participates to cocaine-induced reinstatement [48] and is suspected to code for the relative motivational value of anticipated rewards [49]. Similar MO activation independently from prior extinction of cocaine priming further suggests that in our setting, extinction of cocaine priming is not due to a mere loss of drug motivation, as suggested previously [23]. At the subcortical level, striatal c-Fos expression soared following cocaine-primed reinstatement, reflecting interoceptive integration of the cocaine effects and shaping of incentive motivation [50-52], consistently with somatosensory and sensorimotor functions of the lateral striatum [53]. Following extinction of cocaine-primed reinstatement, hypoactivation observed in the lateral striatum would illustrate the acquired disconnection between the interoceptive effects of cocaine priming and its unavailability for self-administration. Similarly, extinction of cocaine priming led to reduced c-Fos expression in the medial part of the dorsal striatum, usually described in goal directed actions [54]. Interestingly, goal-directed actions also require updating and self-monitoring of behavior [55]. Lower c-Fos levels after extinction of priming would thus represent the loss of relevant behavioral meaning associated with the interoceptive effects of cocaine. Of note, while c-Fos expression was reduced in the dmSTR following extinction of priming, levels remained elevated above control levels. In fact, extensive exposure to a task does not necessarily involve medial striatum disengagement and the dmSTR can still be activated by well-known stimuli, even after extended training [54]. While the precise role of the dmSTR in our task remains to be specified, basal dmSTR activation by cocaine could further contribute to the rapid return of goal-directed drug intake when cocaine is reintroduced [23]. Finally, c-Fos expression was reduced in the ceSTR after extinction of priming. The role of this striatal compartment is not well described in drug-related behavior, but it has been incriminated in compulsive-like behavior through its connections with the OFC [56,57], a circuit known to support repetitive behaviors [58]. While our task does not measure compulsion, decreased c-Fos expression in the ceSTR could reflect the loss of high lever-pressing behavior usually exhibited following cocaine priming. Interestingly, neuronal tracing suggests that the DLO projects to the ceSTR [57]. As we found decreased c-Fos in both DLO and ceSTR after extinction of drug-induced reinstatement, targeting the orbitofronto-striatal circuit could be effective in preventing or limiting relapse-like behaviors.

Unsurprisingly, we found high c-Fos levels in multiple accumbal subregions after cocaine-primed reinstatement [26,27]. Following extinction of cocaine priming, however, c-Fos expression was decreased in the NAc core, a subregion usually associated with the reinforcing effects of cocaine [59]. This would indicate that, despite the interoceptive effects of cocaine, lever-presses were no more reinforced. Extinction of priming also led to drastic reduction in c-Fos expression in the ISh, a subregion involved in reward [60] or in both reward and aversion,

overall suggesting a role in reward salience [61]. It would further indicate that the interoceptive effects of cocaine have lost their ability to become discriminative stimuli usable to reengage drug seeking. Of note, drug-primed reinstatement induced c-Fos expression in the vmSh. This was surprising as the vmSh is generally involved in aversion [62,63]. Non-contingent injections of cocaine can produce interoceptive sensations analogous to anxiety [64,65] and although passive cocaine infusion induces reinstatement, they are likely also associated with a transient unpleasant sensation. In this context, c-Fos expression in the vmSh might be put in regard to activation of insular area, a brain region important for interoception [43]. Interestingly, animals that extinguished reinstatement did not show vmSh (nor insular) c-Fos expression further suggesting that both rewarding and aversive interoceptive aspects of cocaine have been decoupled from the intake behavior. Altogether, our dorsal and ventral striatum data highlight interesting phenomena. Acutely, cocaine should have the same direct pharmacological action independently from prior extinction of cocaine priming. Moreover, we previously showed that tolerance is unlikely to play a confounding role in our paradigm [23]. Indeed, after extinction of cocaine priming, cocaine remained able to induce the same level of psychomotor stimulation as before extinction (within-subject comparison) and also in comparison to rats that did not undergo extinction of cocaine priming. We can thus postulate similar dopaminergic activation (as illustrated by the main drug effect in the VTA). However, mesolimbic activation and behavioral output were different, further suggesting either that cocaine priming does not produce sufficient neuronal activation to precipitate cocaine seeking following extinction of priming or that active learning mechanisms developed in order to uncouple the pharmacological action of cocaine from behavior.

### **Extinction of cocaine-priming decreases activation in anterior, but not posterior, insular subregions**

Next, we found distinct c-Fos expression patterns in the insula. While its role remains difficult to precisely apprehend [44], the insula regulates interoceptive processes representing bodily states associated with drug use and motivation driving addictive behaviors [66-68]. Most studies implicated the anterior, and sometimes the posterior, insula in cue- or context-induced reinstatement but its role in drug-induced reinstatement is less characterized [44,66,69,70]. Here, cocaine-primed reinstatement induced high c-Fos levels in the anterior portion of the insula such as the aI, the DI and GI and returned to baseline after extinction of cocaine priming. These findings align with studies showing that aIC lesions reduce cocaine-primed reinstatement ([71] but see also [69]). The aIC receives sensory information via thalamic inputs as well as intra-insular connections with the medial and posterior insula. The aIC is also bidirectionally connected to the amygdala, the hippocampus, and the mPFC [72] and projects

to the NAc, conferring the aIC a central role in guiding motivated behavior, as further supported by the absence of correlation in the saline-priming condition. Conversely, the pIC preferentially processes sensory information through inputs from visceral thalamic sensory nuclei and somatosensory cortex [73]. Decreased activity in anterior insular subregions after extinction of priming, but no change in the pIC, coupled with shared positive correlations between saline and cocaine priming, suggests once again interoceptive cocaine effects may be decoupled from their predicted value, thereby attenuating goal-directed cocaine seeking.

### **Extinction of cocaine-priming engages the cortico-thalamo-cortical loop**

Finally, c-Fos expression in the IMDT was elevated after cocaine-primed reinstatement and even higher after its extinction. Importantly, the IMDT was the only brain region showing increased c-Fos activity after extinction of priming (see **Fig. 3a**). While this contrasts with the ineffectiveness of MDT inhibition on cocaine-primed reinstatement [31], later studies showed that MDT inhibition alters the sensitivity to changes in action-outcome contingencies rather than responses to cues that are no longer rewarded [74]. This could explain higher MDT activation after repeated drug priming as it would reflect enduring, unpredictable, changes in action-outcome contingencies induced by non-contingent cocaine delivery. This also agrees with an “attention to learn” function, where MDT-PFC circuits use relevant sensory stimuli to adapt to changing rules [75]. Of note, the MDT has reciprocal connections with the aIC [72] and chemogenetic aIC stimulation that reduces alcohol consumption induces c-Fos expression in the MDT [76]. Here, such loop could provide the MDT with interoceptive information about reward but could also send the aIC task-related feedback to guide interoception-based behaviors. More work is however needed to get insights into the role of this cortico-thalamo-cortical loop in drug-induced reinstatement.

A potential involvement of cortico-subcortical loops was further suggested by our correlational analysis and allowed us to formulate hypotheses on the processes at play in extinction of drug priming. Overall, we identified positive correlations for the different insular sub-compartments as well as for the NAc shell, two interconnected systems [72,77]. We propose that the aIC uses homeostatic change from the drug-free period [43] to provide information to the NAc shell where it acquires salience and valence properties used for reinstatement of drug seeking. However, after extinction of priming, passive cocaine would not generate homeostatic changes any longer (illustrated by low insular c-Fos levels), inducing loss of salience and valence, and thus loss of responding. However, negative correlations further suggest that this process may not be completely passive. Consistent with the role of the MDT in processing contingency between action and outcome [74,78], we speculate that increased c-Fos expression in both groups reflects mechanisms searching to associate

contingencies to drug delivery, to generate appropriate behavioral strategies in response to dissociated outcome and action (i.e., uncued, passive cocaine injection) [75]. During reinstatement, this would simply lead to the return of lever pressing. Following extinction of priming however, temporal sequences of events and conditions predicting reward disappear, preventing normal vmVP processing [79]. Through its thalamic projections [80,81], the vmVP would send alternative “learning signals” to the IMDT in the attempt to associate new potential contingencies to drug delivery. These learning signals would also benefit from inputs from the MR [82] to include the negative value [83] elicited by the action-outcome mismatch.

Overall, we found that cocaine-primed reinstatement is associated with increased c-Fos expression in key brain regions (e.g., dorsal and ventral striatum, several prefrontal areas and insular cortex), while its extinction mostly disengages them. Moreover, while reinstatement behavior was correlated with insular and accumbal activation, extinction of cocaine priming implicated parts of the ventral pallidum, the mediodorsal thalamus and the median raphe. These brain patterns of activation and inhibition suggest that after repeated priming, interoceptive signals lose their conditioned discriminative properties and that action-outcome associations systems are mobilized in search for new contingencies, a brain state that may predispose to rapid relapse.

### **Data availability**

All raw c-Fos data are available in the Supplementary files. Other data is available upon reasonable request to the corresponding authors.

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### **Author contributions**

SHA conceived the project. SN, PG and SHA designed the experiments. SN, PG performed the experiments and collected the experimental data. ML, ME analyzed the data. ML and ME wrote the first draft of the paper. ML, ME and SHA revised and edited the paper. All authors reviewed content and approved the final version of the manuscript.



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## Competing Interests

The authors declare no competing interests.

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## Figure Legends

**Fig. 1:** Extinction of cocaine priming: **a.** Timeline of the experimental procedure: Following intravenous surgery and recovery, rats were habituated to the operant chambers. They then underwent 18 sessions of Fixed-Ratio (FR) 1 followed by 12 sessions of FR3 training (3h/session). Extinction was performed for 3 days. Each session was subdivided into three 45-min periods that were identical except that rats received one non-contingent intravenous injection of vehicle (V) at the onset of the last 45-min period. Next, extinction of priming consisted of 8 daily sessions similar to the ones used for extinction except that the last 45-minute block of extinction was initiated by cocaine delivery or vehicle (P). Finally, on the last day, rats underwent a final test identical to the extinction of priming session except that the last block lasted 60 minutes; **b.** Active responses made during the final 45-minute period of extinction initiated by one non-contingent injection of vehicle (V) were similar between animals of each subgroup (panel Extinction; NP: the non-primed level of active responding during the 45-min period preceding the priming dose, V: active responses during the third 45-min period initiated by vehicle injection). \*\*V-V:  $p=0.0012$ ; \*\*\*V-C:  $p=0.0001$ ; +C-V:  $p=0.051$ ; C-C:  $p=0.999$ ; **c.** Active responses made during the three 45-minute periods of extinction of cocaine priming (panel Extinction of Priming): Animals primed with cocaine showed progressive extinction of the priming effects of the drug (\*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to the V-V subgroup). **d.** Number of active responses made during the last Test day (bar graph adapted from [23]). Only the V-C subgroup showed reinstatement (\*\* $p < 0.001$  from all other subgroups). Insert, number of active responses of the V-C subgroup during the 3 periods of extinction of the Test.

V-V (n=5): repeatedly primed and tested with vehicle, V-C (n=5): repeatedly primed with vehicle and tested with cocaine, C-V (n=5): repeatedly primed with cocaine and tested with vehicle, C-C (n=4): repeatedly primed and tested with cocaine.

**Fig. 2:** Brain-wide mapping of c-Fos expression following extinction of cocaine priming: **a.** Heatmap of c-Fos expression levels across 48 subregions of the rat brain along the anteroposterior axis. Expression is presented as z-score from the V-V subgroup. \* $p < 0.05$  at least from the V-V subgroup, # $p < 0.05$  at least from the V-C subgroup, ° $p < 0.05$  at least from the C-C subgroup; **b.** Brain regions for which a significant main effect of either cocaine during extinction of priming or cocaine during the test was found; **c.** List of the 10 brain regions with the highest c-Fos expression for the different subgroups of rats (presented as z-score from the V-V subgroup), \* $p < 0.05$  at least from the V-V subgroup. Detailed statistics can be found in Supplementary Table S2.

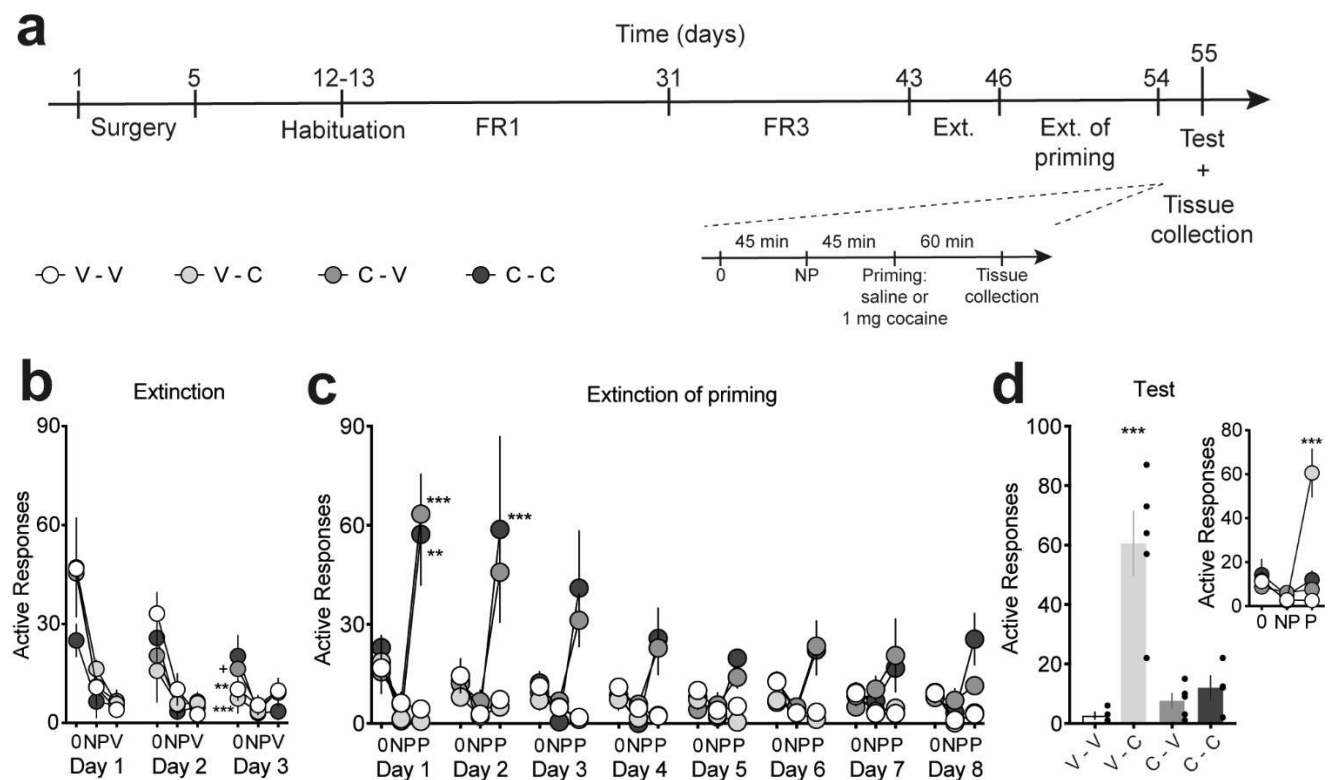
V-V (n=5): repeatedly primed and tested with vehicle, V-C (n=5): repeatedly primed with vehicle and tested with cocaine, C-V (n=5): repeatedly primed with cocaine and tested with vehicle, C-C (n=4): repeatedly primed and tested with cocaine.

**Fig. 3:** Changes in c-Fos expression pattern following extinction of cocaine priming and correlations between c-Fos levels and active responses during the last Test. **a.** Color mapping of the brain regions of the C-C subgroup with significantly different expression pattern compared to the V-C subgroup. Detailed statistics are presented in Supplementary Table S2; **b.** Heatmap representation of the Pearson's correlation coefficient between c-Fos expression and active responses during the last test in animals primed with saline (i.e., V-V and C-V, left panel) and with cocaine (i.e., V-C and C-C, right panel), \* $p < 0.05$  at least. Detailed statistics can be found in Supplementary Table S3.

V-V (n=5): repeatedly primed and tested with vehicle, V-C (n=5): repeatedly primed with vehicle and tested with cocaine, C-V (n=5): repeatedly primed with cocaine and tested with vehicle, C-C (n=4): repeatedly primed and tested with cocaine.



**Figure 1**



**Figure 2**

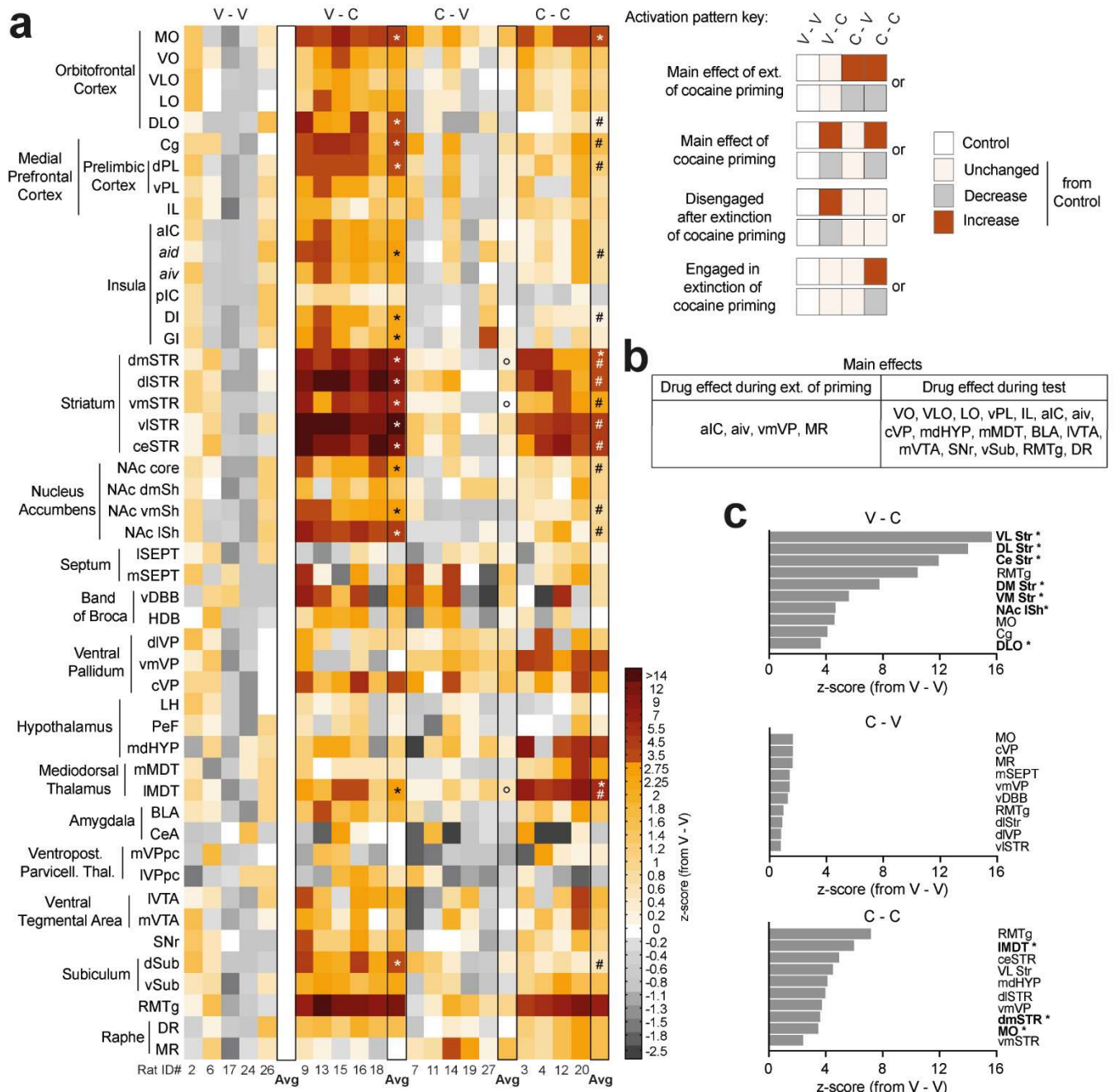


Figure 3

