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Context-induced relapse to cocaine seeking after punishment-imposed abstinence is associated with activation of cortical and subcortical brain regions

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Abstract

We recently developed a rat model of context-induced relapse to alcohol seeking after punishment-imposed abstinence to mimic relapse after self-imposed abstinence due to adverse consequences of drug use. Here, we determined the model's generality to cocaine and have begun to explore brain mechanisms of context-induced relapse to cocaine seeking after punishment-imposed abstinence, using the activity marker Fos. In exp. 1, we trained rats to self-administer cocaine (0.75 mg/kg/infusion, 6 hours/day, 12 days) in context A. Next, we transferred them to context B where for the paired group, but not unpaired group, 50 percent of cocaine-reinforced lever presses caused aversive footshock. We then tested the rats for cocaine seeking under extinction conditions in contexts A and B. We also retested them for relapse after retraining in context A and repunishment in context B. In exp. 2, we used Fos immunoreactivity to determine relapse-associated neuronal activation in brain regions of rats exposed to context A, context B or neither context. Results showed the selective shock-induced suppression of cocaine self-administration and context-induced relapse after punishment-imposed abstinence in rats exposed to paired, but not unpaired, footshock. Additionally, context-induced relapse was associated with selective activation of dorsal and ventral medial prefrontal cortex, anterior insula, dorsal striatum, basolateral amygdala, paraventricular nucleus of the thalamus, lateral habenula, substantia nigra, ventral subiculum, and dorsal raphe, but not nucleus accumbens, central amygdala, lateral hypothalamus, ventral tegmental area and other brain regions. Together, context-induced relapse after punishment-imposed abstinence generalizes to rats with a history of cocaine self-administration and is associated with selective activation of cortical and subcortical regions.

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Authors Contribution

YP, JMB, and YS: designed the experiments and wrote the paper; YP, JMB, JH, SA, JM, and AMT: ran the behavioral and immunohistochemistry experiments and were involved in data management and analysis of the behavioral and Fos data; CC: analyzed the Fos data

Introduction

In humans, environments or contexts previously associated with drug use often provoke relapse during abstinence (Wikler 1973; Staiger & White 1991; O'Brien *et al.* 1992; Collins & Brandon 2002). In rats, studies using the ABA renewal procedure (Bouton & Bolles 1979) have demonstrated that exposure to the drug self-administration context (context A) after extinction of the drug-reinforced responding in a different context (context B) reinstates drug seeking (Crombag & Shaham 2002; Crombag *et al.* 2008; Lasseter *et al.* 2010; Khoo *et al.* 2017). However, from a clinical perspective, a limitation of the extinction-reinstatement model is the use of operant extinction to achieve abstinence; in humans, abstinence rarely involves overt extinction of the drug-seeking response (Marlatt 2002; Epstein *et al.* 2006). Instead, abstinence is typically self-imposed, despite drug availability, because the adverse consequences of drug use outweigh the drug's rewarding effects (Klingemann 1991; Waldorf, Reinarman & Murphy 1991; Vanderschuren *et al.* 2017). To address the lack of homology between the human condition and the animal model, we recently developed a relapse model in which alcohol taking is suppressed by adverse consequences (punishment) (Marchant *et al.* 2013). The new model consists of a modified ABA renewal procedure in alcohol-preferring rats in which abstinence is achieved in context B, despite alcohol availability, by a punishment manipulation consisting of response-contingent footshocks. Using this model, we have demonstrated context-induced relapse to alcohol seeking when rats were tested in context A after punishment-imposed abstinence in context B (Marchant *et al.* 2013; Marchant *et al.* 2014; Marchant *et al.* 2016).

In the present study, we addressed four issues. First, we determined whether context-induced relapse after punishment-imposed abstinence generalizes to the psychostimulant drug cocaine. Second, we determined whether under our experimental conditions, punishment contingencies rather than non-selective fear-like responses to aversive footshock, control inhibition of drug seeking in context B, as is the case with food and alcohol rewards (Marchant *et al.* 2013; Bouton & Schepers 2015). Third, we determined whether relapse to cocaine seeking after punishment-imposed abstinence is observed in a repeated testing procedure in which after the initial relapse test, the rats are retrained in context A, repunished in context B and retested for relapse in context A (Marchant *et al.* 2016). Fourth, we have begun to study the brain mechanisms of context-induced relapse to cocaine seeking after punishment-imposed abstinence by using the activity marker Fos (Morgan & Curran 1991) to identify brain regions selectively activated during the relapse tests in context A. We studied relapse-associated Fos induction in paraventricular nucleus of the thalamus (PVT), lateral hypothalamus (LH), ventral subiculum (vSub) and nucleus accumbens (NAc) core and shell, because of their role in context-induced relapse to alcohol seeking after punishment-imposed abstinence (Marchant *et al.* 2013; Marchant *et al.* 2014; Marchant *et al.* 2016). We also studied relapse-associated Fos induction in dorsal and ventral medial prefrontal cortex (dmPFC, vmPFC), orbitofrontal cortex (OFC), anterior insula cortex (AI), bed nucleus of stria terminalis (BNST), ventral pallidum (VP), lateral and medial septum (LS, MS), central and basolateral amygdala (CeA, BLA), lateral and medial habenula (LHb, MHb), ventral tegmental area (VTA), substantia nigra (SN) and dorsal and median raphe (DRN, MRN). We studied these regions, because of their role in context-induced and stress-

induced reinstatement after extinction (Lasseter *et al.* 2010; Bossert *et al.* 2013; Peters, Pattij & De Vries 2013; Mantsch *et al.* 2016; Khoo *et al.* 2017), punishment-induced suppression of cocaine and food seeking (Jonkman, Pelloux & Everitt 2012; Pelloux *et al.* 2012; Pelloux, Murray & Everitt 2013; Jean-Richard-Dit-Bressel & McNally 2015), and the aversive effects of cocaine (Jhou *et al.* 2013).

Materials and Methods

Subjects

We used male Sprague–Dawley rats (Charles River, total $n = 33$), weighing 250–350 g prior to surgery. We maintained the rats under a reverse 12:12-hour light/dark cycle (lights off at 8:00 AM) with food and water freely available. We housed two rats per cage prior to surgery and then individually after surgery. We performed all experiments in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (eighth edition), under the protocols approved by the Animal Care and Use Committee. We excluded three rats due to failure of catheter patency.

Intravenous surgery

We anesthetized the rats with isoflurane (5 percent induction; 2–3 percent maintenance). We then inserted silastic catheters into the jugular vein that were passed subcutaneously to the mid-scapular region and attached to a modified 22-gauge cannula cemented to polypropylene mesh (Small Parts) as described previously (Adhikary *et al.* 2016; Bossert *et al.* 2016; Caprioli *et al.* 2017; Venniro *et al.* 2017). We injected the rats with ketoprofen (2.5 mg/kg, s.c., Butler Schein) after surgery and the following day to relieve pain and decrease inflammation and allowed the rats to recover for 5–7 days before cocaine self-administration training. During the recovery and training phases, we flushed the catheters every day with gentamicin (4.25 mg/ml, APP Pharmaceuticals) dissolved in sterile saline.

Apparatus

We trained and tested the rats in standard Med Associates (Fairfax, VT) self-administration chambers located inside sound-attenuating cabinets. Each chamber had two levers located 7.5–8.0 cm above the grid floor on opposing walls. Lever presses on the active retractable lever activated the infusion pump, whereas lever presses on the inactive non-retractable lever had no programmed consequences; the grid floors were connected to shockers. We modified the self-administration chambers to two contexts (A and B) that differed from each other in terms of their auditory, visual and tactile features, using procedures like those described in our previous studies (Bossert *et al.* 2004; Marchant *et al.* 2013; Adhikary *et al.* 2016). In one context, the doors of the sound-attenuating cabinet remained closed during the session, illumination was provided by a red houselight, the fan was turned on, the floor consisted of 19 stainless steel rods (4.8-mm diameter) spaced 16 mm apart, and there was an empty feeder in the chamber. In the other context, we kept the cabinet doors open, a white houselight provided illumination, the fan was turned off, the floor consisted of 26 stainless steel rods (3.2-mm diameter) spaced 11 mm apart, and there was no feeder in the chamber. The contexts are referred to as A and B, where A is the cocaine self-administration (training)

context and B is the punishment context. We counterbalanced the physical environments of contexts A and B.

Procedures

The experimental parameters for the self-administration, punishment and relapse phases were based on those procedures used in our previous studies that demonstrated context-induced relapse of alcohol seeking after punishment-imposed abstinence (Marchant *et al.* 2013; Marchant *et al.* 2014; Marchant *et al.* 2016). The experiments consisted of six (exp. 1) or three (exp. 2) phases. For exp. 1, the phases were self-administration training (context A; 12 days), punishment training (context B; 8 days), tests for context-induced relapse of cocaine seeking (contexts A and B, 2 days), self-administration retraining (context A; 6 days), punishment retraining (context B; 8 days), and retests for context-induced relapse of cocaine seeking (contexts A and B, 2 days). The timeline for exp. 1 is shown in Fig. 1a. For exp. 2, the procedure is the same as the first three phases of exp. 1, with the exception that we tested the rats in either context A or B or not tested them (refer to details in the succeeding texts). The timeline for exp. 2 is shown in Fig. 2a.

Phase 1: cocaine self-administration in context A

We trained the rats (exp. 1 $n = 14$; exp. 2 $n = 19$) to self-administer cocaine-HCl (supplied by the National Institute on Drug Abuse) dissolved in sterile saline for 6 hours/day for 12 days. The sessions began with the extension of the active lever and the illumination of the houselight, which remained on for the duration of the 6-hour daily session. We used a 'standard' extended daily access cocaine self-administration training procedure, because this procedure mimics human drug use characterized by escalation of drug intake over time (Ahmed & Koob 1998). Active lever presses led to the delivery of a cocaine infusion (0.75 mg/kg per infusion; 0.10 ml/infusion over 5 seconds) and a compound tone–light cue. During the first six sessions, we trained the rats using a fixed ratio-1 20-second timeout reinforcement schedule, and during the last six sessions, we trained the rats using a variable interval 30-second (VI-30) reinforcement schedule for six sessions. During these sessions, cocaine delivery was available after an active lever press at random intervals (range: 1 to 59 seconds) after the preceding cocaine delivery. We recorded lever presses during the timeout intervals, but the lever presses had no consequences. We used a VI-30 reinforcement schedule during the last six training sessions and during the punishment and relapse test phases, because this was the reinforcement schedule we previously used in our alcohol studies (Marchant *et al.* 2014; Marchant *et al.* 2016).

Phase 2: punishment in context B

During this phase, the rats continued to self-administer cocaine for 6 hours/day under the VI-30 reinforcement schedule. In exp. 1, we divided the rats into two groups: paired ($n = 8$) and unpaired ($n = 6$). For the paired group, 50 percent of the reinforced lever presses, which occurred after the VI-30 response requirement was met, delivered a 0.5-second footshock through the grid floor. During the punished response, the tone–light cue was presented, and 0.1 ml of cocaine was delivered. For the unpaired group, the 0.5-second footshock was delivered based on a rat's response in the paired group (i.e. yoked footshock delivery). The first session began in context B without any shock (0.0 mA), and thereafter, we began with

0.1 mA shock and increased the intensity by 0.1 mA every day until the last day (0.7 mA). In exp. 2, all rats received contingent shock pairings for 50 percent of the reinforced lever presses.

Phase 3: relapse tests

Exp. 1: We tested all rats for cocaine seeking (operationally defined as active lever presses under extinction conditions) without footshock punishment in both contexts A and B in 60-minute extinction sessions over 2 days; we counterbalanced the order of testing for both groups. We performed the first 24 hours after the final context B training session and separated the tests by 24 hours. The duration of the test session was 60 minutes to minimize a potential carryover effect of extinction learning, which may subsequently decrease drug seeking during the subsequent relapse tests after retraining and repunishment (refer to the succeeding texts).

Exp. 2: We tested two groups of rats for cocaine seeking under extinction conditions for 90 minutes in either context A (ABA group; $n = 7$) or context B (ABB group; $n = 7$) or did not test a third group (AB0, no-test group, rats remained in their home cage; $n = 5$). We tested the rats in a single 90-minute session to match the session duration with the approximate time of maximal Fos expression after exposure to environmental cues and contexts previously paired with unconditioned appetitive or aversive stimuli (Cruz *et al.* 2013). At the end of the test session, we deeply anesthetized the rats, perfused them with phosphate-buffered saline (PBS) and 4 percent paraformaldehyde and removed their brains for subsequent Fos immunohistochemistry. We anesthetized and perfused the rats in the no-test group at the same time with the test groups.

Phase 4: self-administration retraining (context A)

Only rats from exp. 1 participated in phases 4–6. We retrained the rats for 6 hours/day for 6 days under a VI-30 schedule of reinforcement as described in the preceding texts in phase 1.

Phase 5: punishment retraining (context B)

We performed punishment retraining as described in phase 2.

Phase 6: relapse tests (contexts A and B)

We tested the rats as described in phase 3.

Fos immunohistochemistry

We based our Fos immunohistochemistry procedure on our previous reports (Bossert *et al.* 2012; Bossert *et al.* 2016). Ninety minutes after exposure to context A or context B, we deeply anesthetized the rats with isoflurane (~80 second) and perfused them transcardially with 100 ml of 0.1 M PBS followed by 400 ml of 4 percent paraformaldehyde in PBS, pH 7.4. We also perfused the ‘no-test’ (AB0) rats (taken from their home cage) at the same time as the tested rats. We removed and post-fixed the brains in 4 percent paraformaldehyde for 2 hours before transferring them to 30 percent sucrose in PBS for 48 hours at 4°C. We subsequently froze the brains in powdered dry ice and stored them at −80°C until sectioning. We cut coronal sections (40 μm) containing the different brain areas using a cryostat (Leica

Microsystems). We divided the sections into five series (200 μm apart), collected them in PBS containing 0.1 percent sodium azide and stored them at 4°C.

We rinsed free-floating sections (3× 10 minutes) in PBS, incubated them for 1 hour in 4 percent bovine serum albumin (BSA) in PBS with 0.4 percent Triton X-100 (PBS-TX) and incubated them overnight at 4°C with rabbit anti-c-Fos primary antibody [Phospho-c-Fos (Ser32), Cell Signaling Tech, RRID: AB_2247211, D82C12 diluted 1:8000] in 4 percent BSA in 0.4 percent PBS-TX. We then rinsed the sections in PBS and incubated them for 2 hours with biotinylated anti-rabbit IgG secondary antibody (BA-1000, Vector Laboratories) diluted 1:600 in 4 percent BSA in 0.4 percent PBS-TX. We rinsed the sections again in PBS and incubated them in avidin–biotin–peroxidase complex (ABC Elite kit, PK-6100, Vector Laboratories) in 0.5 percent PBS-TX for 1 hour. We then rinsed the sections in PBS, developed them in 3,3′-diaminobenzidine, rinsed them in PBS, mounted them onto chrome alum/gelatin-coated slides and air dried them. We dehydrated the slides through a graded series of alcohol concentrations (30, 60, 90, 95, 100, 100 percent ethanol), cleared with Citra Solv (Fisher Scientific) and coverslipped them with Permount (Fisher Scientific).

Imaging and Fos quantification

We digitally captured brightfield images of immunoreactive (IR) cells in the different brain areas using a 10× objective and a Retiga 2000R CCD camera (QImaging) attached to a Zeiss microscope Axio Scope A1. We identified Fos-IR cells by a brown reaction product in the nuclei. For each rat, we quantified cells in both hemispheres of two to three sections and computed a mean of these counts per area. We captured and quantified the following Bregma coordinates: (1) +3.5 to +2.8 mm for dmPFC, vmPFC, OFC and AI; (2) +1.8 to +1.2 mm for NAc shell and core, DMS and DLS; (3) +1.00 to 0.00 mm for LS and MS; (4) +0.1 to −0.4 mm for VP and dorsolateral and ventral BNST); (5) −2.2 to −3.0 mm for BLA and CeA; (6) −2.7 to −3.7 mm for LHb, MHb, PVT and LH; (7) −5.3 to −5.8 mm for VTA and SN; (8) −5.5 to −6.3 mm for vSub; (9) −6.9 to −7.6 mm for DRN; and (10) −7.3 to −8.0 mm for MRN. We analyzed the images using IVision (4.5.0, Biovision Technologies) software. YP, JMB or CC performed the image capture, and JH or CC performed in a blind manner the Fos-IR quantification.

Statistical analysis

Using the statistical program SPSS, we analyzed the data separately for the different phases of training, punishment and relapse testing. For the behavioral data in exp. 1 and 2, we used mixed ANOVAs (see Results for the description of the between-subject and within-subject factors) and followed up on significant main effects and interaction effects ($P < 0.05$) with post-hoc tests (Fisher protected least squares difference). For the Fos data (exp. 2), we used a one-way ANOVA that included the between-subject factor of test context (context A, context B, no test). Because our multi-factorial ANOVAs yielded multiple main and interaction effects, we only report significant effects that are critical for data interpretation. Additionally, for clarity, we primarily indicate post hoc analyses by asterisks in the figures. In Table S1, we provide a complete statistical reporting of the data presented in the paper.

Results

Behavioral data: training, punishment and context-induced relapse (exp. 1 and 2)

Self-administration training and retraining phases (context A)—During the training phase, we trained the rats to self-administer cocaine under a fixed ratio-1 20-second timeout reinforcement schedule during sessions 1–6 and a VI-30 reinforcement schedule during sessions 7–12. In exp. 1 and 2, the rats demonstrated reliable cocaine self-administration, as indicated by significant increases in the number of infusions and active lever presses over the training days (refer to Figs. 1b & 2b and Table S1 for statistical reporting of these data). During retraining (exp. 1), the rats rapidly reacquired cocaine self-administration (Fig. 1e).

Punishment training and retraining phases (context B)—Exp. 1. During both the punishment training and retraining phases, the rats in the paired group decreased both the number of infusions and active lever presses with increasing shock intensity, while the rats in the unpaired group did not (Fig. 1c & f). The repeated-measures ANOVA of the number of infusions, which included the between-subject factor of group (paired, unpaired) and the within-subject factor of session, showed a significant interaction between session and group during both punishment training and retraining ($F_{7,84} = 15.6$, $P < 0.01$ and $F_{7,84} = 16.7$, $P < 0.01$, respectively). We obtained similar statistical results in the analysis of the number of active lever presses during punishment training and retraining (refer to Table S1 for statistical results).

Exp. 2. During the punishment training phase, the rats decreased both the number of infusions and active lever presses with increasing shock intensity over sessions (Fig. 2c). The repeated-measures ANOVA of the number of infusions showed a significant effect of session ($F_{7,126} = 99.9$, $P < 0.01$). The repeated-measures ANOVA of lever presses, which included the within-subject factors of session and lever (active, inactive), showed a significant interaction between the two factors ($F_{7,126} = 26.9$, $P < 0.01$).

Context-induced relapse tests

Exp. 1 (Test 1): We observed selective context-induced relapse to cocaine seeking in context A after suppression of responding in context B in the paired group but not the unpaired group (Fig. 1d). The repeated-measures ANOVA of active lever presses, which included the between-subject factor of group (paired, unpaired) and the within-subject factor of context (A, B), did not show a significant interaction between the two factors ($F_{1,12} = 1.6$, $P = 0.236$). However, the repeated-measures ANOVA for context B, which included the between-subject factor of group (paired, unpaired) and the within-subject factor of lever (active, inactive), showed a significant interaction between the two factors ($F_{1,12} = 6.6$, $P < 0.05$). No significant differences were found between the paired and unpaired groups in context A (group by lever interaction, $P = 0.885$; Fig. 1d).

Exp. 1 (Test 2): We retested the rats for context-induced relapse of cocaine seeking after retraining them in context A and repunishing them in context B. As in test 1, the rats in the paired group, but not the unpaired group, showed context-induced relapse to cocaine seeking

in context A after suppression of responding in context B (Fig. 1g). The repeated-measures ANOVA, which included the between-subject factor of group (paired, unpaired) and the within-subject factor of context (A, B), showed a significant interaction between the two factors ($F_{1,12} = 6.0$, $P < 0.05$). Additionally, like in test 1, we found a significant interaction between group and lever in context B ($F_{1,12} = 11.7$, $P < 0.01$), but not in context A ($P = 0.29$; Fig. 1g).

Exp. 2 (test 1): We observed context-induced relapse of cocaine seeking in context A after punishment-imposed abstinence in context B (Fig. 2d). The repeated-measures ANOVA, which included the between-subject factor of context (A, B) and the within-subject factor of lever (active, inactive), showed a significant interaction between the two factors ($F_{1,12} = 9.0$, $P < 0.05$).

Fos-immunoreactive data (exp. 2)

Frontal cortex (Fig. 2e)—Context-induced relapse in context A after punishment-imposed abstinence in context B was associated with selective activation of dmPFC, vmPFC and AI, but not OFC. One-way ANOVAs showed a significant effect of group [ABA, ABB, AB0 (no test)] for dmPFC ($F_{2,16} = 13.0$, $P < 0.01$), vmPFC ($F_{2,16} = 5.6$, $P < 0.05$) and AI ($F_{2,16} = 13.0$, $P < 0.01$), but not OFC ($P > 0.05$). Post-hoc analyses showed significant differences between ABA versus ABB and AB0 for dmPFC, vmPFC and AI (P values <0.05).

Striatum (Fig. 3a)—Context-induced relapse was associated with selective activation of DMS and DLS but not NAc shell or core. One-way ANOVAs showed a significant effect of group for DMS ($F_{2,16} = 11.8$, $P < 0.01$) and DLS ($F_{2,16} = 3.9$, $P < 0.05$), but not NAc shell or NAc core ($P > 0.05$). Post-hoc analyses showed significant differences between ABA versus ABB and AB0 for DMS and DLS (P values <0.05).

Septum and ventral pallidum (Fig. 3b & c)—Context-induced relapse was not associated with selective activation of LS, MS or VP. One-way ANOVAs showed a significant effect of froup for MS ($F_{2,16} = 3.8$, $P < 0.05$), but not for LS ($F_{2,16} = 3.5$, $P = 0.057$) or VP ($P > 0.09$). Post hoc analysis showed a significant difference between ABA versus AB0 but not ABB for MS ($P < 0.05$).

Bed nucleus of stria terminalis and amygdala (Fig. 4a & b)—Context-induced relapse was associated with selective activation of BLA, but not CeA, dorsal or ventral BNST. One-way ANOVAs showed a significant effect of group for BLA ($F_{2,16} = 15.6$, $P < 0.01$), but not for the other brain regions ($P > 0.1$). Post-hoc analysis showed a significant difference between ABA versus AB0 and ABB for BLA ($P < 0.05$).

Habenula (Fig. 4c)—Context-induced relapse was associated with selective activation of LHb but not MHb. One-way ANOVAs showed a significant effect of group for LHb ($F_{2,16} = 4.9$, $P < 0.05$) but not MHb ($F_{2,16} = 3.5$, $P = 0.056$). Post-hoc analyses showed significant differences between ABA versus ABB and AB0 for LHb (P values <0.05).

Paraventricular nucleus of the thalamus and lateral hypothalamus (Fig. 5a)— Context-induced relapse was associated with selective activation of PVT but not LH. One-way ANOVAs showed a significant effect of group for PVT ($F_{2,16} = 3.7$, $P < 0.05$) but not for LH ($P > 0.1$). Post-hoc analysis showed a significant difference between ABA versus AB0 and ABB for PVT ($P < 0.05$).

Ventral tegmental area and substantia nigra (Fig. 5b)—Context-induced relapse was associated with selective activation of SN but not VTA. One-way ANOVAs showed a significant effect of group for SN ($F_{2,16} = 11.5$, $P < 0.01$) but not for VTA ($P > 0.1$). Post-hoc analysis showed a significant difference between ABA versus AB0 and ABB for SN ($P < 0.05$).

Ventral subiculum and raphe (Fig. 5c & d)—Context-induced relapse was associated with selective activation of vSub and DRN, but not MRN. One-way ANOVA showed a significant effect of group for vSub ($F_{2,16} = 7.9$, $P < 0.01$) and DRN ($F_{2,16} = 3.9$, $P < 0.05$), but not MRN ($P > 0.05$). Post-hoc analyses showed significant differences between ABA versus ABB and AB0 for vSub and DRN (P values <0.05).

Discussion

We report three main findings. First, we found that the phenomenon of context-induced relapse to drug seeking after punishment-imposed abstinence generalized to the psychostimulant drug cocaine. Additionally, context-induced relapse after punishment-imposed abstinence was reliably observed during both the initial relapse test and during a second repeated-measures relapse test performed after retraining and repunishment. Second, we found selective shock-induced suppression of cocaine self-administration and context-induced relapse after punishment-imposed abstinence in rats exposed to paired but not unpaired footshock. These behavioral results replicate results from a recent study of Bouton and Schepers (2015) using food as the operant reinforcer and indicate that (1) punishment contingencies rather than unconditioned shock-induced fear or stress states (Estes 1944) suppress cocaine self-administration in context B and (2) like extinction (Bouton & Swartzentruber 1991; Crombag *et al.* 2008; McNally 2014), the suppressive effect of punishment on drug and non-drug seeking is context-dependent. The third and main finding in our study is that context-induced relapse was associated with selective activation of dmPFC, vmPFC, AI, DMS, DLS, BLA, LHb, PVT, SN, vSub and DRN, but not OFC, NAc, MS, LS, VP, BNST, CeA, MHb, LH, VTA and MRN. However, some of the negative findings (LS, MHb and MRN) may be due to type II error or false negative results, because of the small sample size (n = 5–7/group) and p values that approached statistical significance (P = 0.057–0.067, Table S1).

We discuss these Fos results in the succeeding texts with an emphasis on similarities and differences in brain activation during context-induced relapse after punishment-induced abstinence across drugs (cocaine versus alcohol) and similarities and differences in brain activation during context-induced relapse after punishment versus extinction within a drug (cocaine). We summarize the comparisons between neuronal activation in context A (the

drug self-administration context) versus context B (the punishment or extinction context) in Table 1.

Fos induction during the relapse tests after punishment of cocaine versus alcohol self-administration

The comparison of the Fos expression data in the present study and our previous studies with alcohol (Marchant *et al.* 2014; Marchant *et al.* 2016) indicate some similarities in neuronal activation and notable differences. Context-induced relapse after punishment-imposed abstinence was associated with common (drug-independent) activation of DLS, BLA and vSub. At present, the causal role of these brain regions in relapse after punishment-imposed abstinence of drug seeking is unknown. Based on our recent finding that reversible inactivation of vSub decreases context-induced relapse to alcohol seeking after punishment-imposed abstinence (Marchant *et al.* 2016), we speculate that activation of this brain region during the relapse tests mediates this form of relapse across drug classes.

However, context-induced relapse to cocaine but not alcohol seeking was associated with selective activation of dmPFC, vmPFC, DMS, LHb and PVT, while context-induced relapse to alcohol but not cocaine seeking was associated with selective activation of NAc core and LH. Additionally, inhibition of alcohol but not cocaine seeking in context B was associated with selective activation of LHb.

What might account for the predominantly dissociable pattern of brain activation during context-induced relapse to cocaine versus alcohol seeking after punishment-imposed abstinence? We speculate that this dissociation is likely due to differential encoding of drug–context associations for cocaine versus alcohol during drug self-administration training. This dissociation might be due to differences in pharmacokinetics [slower delivery of oral alcohol to the brain versus very fast brain delivery of intravenous cocaine (Robinson, Brunner & Gonzales 2002; Wise & Kiyatkin 2011)], as well as differences in the discriminative stimulus effects of cocaine versus alcohol (Gatch, Youngblood & Forster 2003; Badiani 2013). Differential encoding of drug–context associations for cocaine versus alcohol during drug self-administration training may also be due to differences in mechanisms underlying cocaine versus alcohol self-administration. For example, 6-hydroxydopamine lesions of the mesolimbic dopamine system decrease cocaine but not alcohol self-administration (Roberts, Corcoran & Fibiger 1977; Rassnick, Stinus & Koob 1993). Conversely, blockade of mu opioid receptors decreases alcohol but not cocaine self-administration (Ettenberg *et al.* 1982; Sanchis-Segura *et al.* 2005; Badiani *et al.* 2011).

Overall, based on the results of the Fos data analyzed in the preceding texts, we propose that the circuits of context-induced relapse to cocaine and alcohol seeking are partially dissociable. However, this conclusion is based on correlational data and should be interpreted with caution, because Fos induction in different brain areas can reflect either the cause or the consequence of relapse to drug seeking and does not necessarily imply that a given brain area plays a causal role in relapse (Bossert *et al.* 2011; Cruz *et al.* 2013).

Fos activation during the renewal (relapse) tests after punishment versus extinction for cocaine seeking

To our knowledge, only one study was published on Fos induction during context-induced reinstatement of cocaine seeking after extinction. As in our study, Hamlin, Clemens and McNally (2008) reported that context-induced reinstatement of cocaine seeking after extinction is associated with Fos induction in vmPFC and BLA, suggesting similarities in mechanisms of context-induced relapse, independent of the method used to achieve abstinence in context B (extinction or punishment). However, context-induced relapse to cocaine seeking after punishment but not extinction was associated with selective Fos induction in dmPFC, dorsal striatum (both DMS and DLS), PVT and SN, while the opposite pattern was observed for LH. Thus, it appears that there are both similarities and differences in brain activation during context-induced relapse to cocaine seeking after punishment versus extinction.

Regarding similarities, a question for future research is whether the common activation of BLA during both context-induced relapse after extinction (Hamlin *et al.* 2008) and context-induced relapse after punishment of cocaine seeking (present study) reflects a common function of the BLA in these two forms of relapse. BLA inactivation decreases context-induced relapse to cocaine seeking after extinction, indicating that the BLA is critical for this form of relapse (Fuchs *et al.* 2005). Our Fos data suggest that the BLA is also critical for context-induced relapse after punishment-imposed abstinence. However, BLA activity encodes aversive conditioning (Morrison & Salzman 2010), and BLA lesions prevent punishment-induced suppression of cocaine and food seeking (Pelloux *et al.* 2013; Jean-Richard-Dit-Bressel & McNally 2015). Thus, future studies are needed to determine whether BLA lesions or reversible inactivation will inhibit or potentiate context-induced relapse after punishment-imposed abstinence.

The differences described in the preceding texts in neuronal activation after context-induced relapse after extinction versus punishment may reflect differences in circuits controlling relapse after punishment versus extinction, as previously shown for drug priming-induced reinstatement of drug seeking (Panlilio, Thorndike & Schindler 2005; Marchant *et al.* 2013). However, it cannot be ruled out that other procedural differences between our study and Hamlin *et al.* (2008) study may account for differences in brain activation. These procedural differences include the duration of the training session (extended access for 6 hours/day versus limited access for 2 hours/day) or the feeding conditions (free feeding versus restricted feeding for 1 hour/day).

Concluding remarks

The goal of our Fos mapping study was to begin characterizing brain areas potentially involved in context-induced relapse to cocaine seeking after punishment abstinence. We found that this relapse was associated with selective activation of dmPFC, vmPFC, AI, DMS, DLS, BLA, LHb, PVT, SN, vSub, and DRN. The analysis of our Fos mapping results within the context of previous Fos mapping studies on context-induced relapse of alcohol seeking and context-induced relapse to cocaine seeking after extinction suggests some similarities and also notable differences. This analysis suggests that the circuits controlling

context-induced relapse after punishment across drug classes and context-induced relapse after punishment versus extinction within a drug class are likely partially dissociable. A question for future research is whether the brain areas selectively activated during the context-induced relapse to cocaine seeking after punishment-imposed abstinence play a causal role in this relapse. Future studies using classical neuropharmacological methods and novel optogenetic and chemogenetic methods can answer this question.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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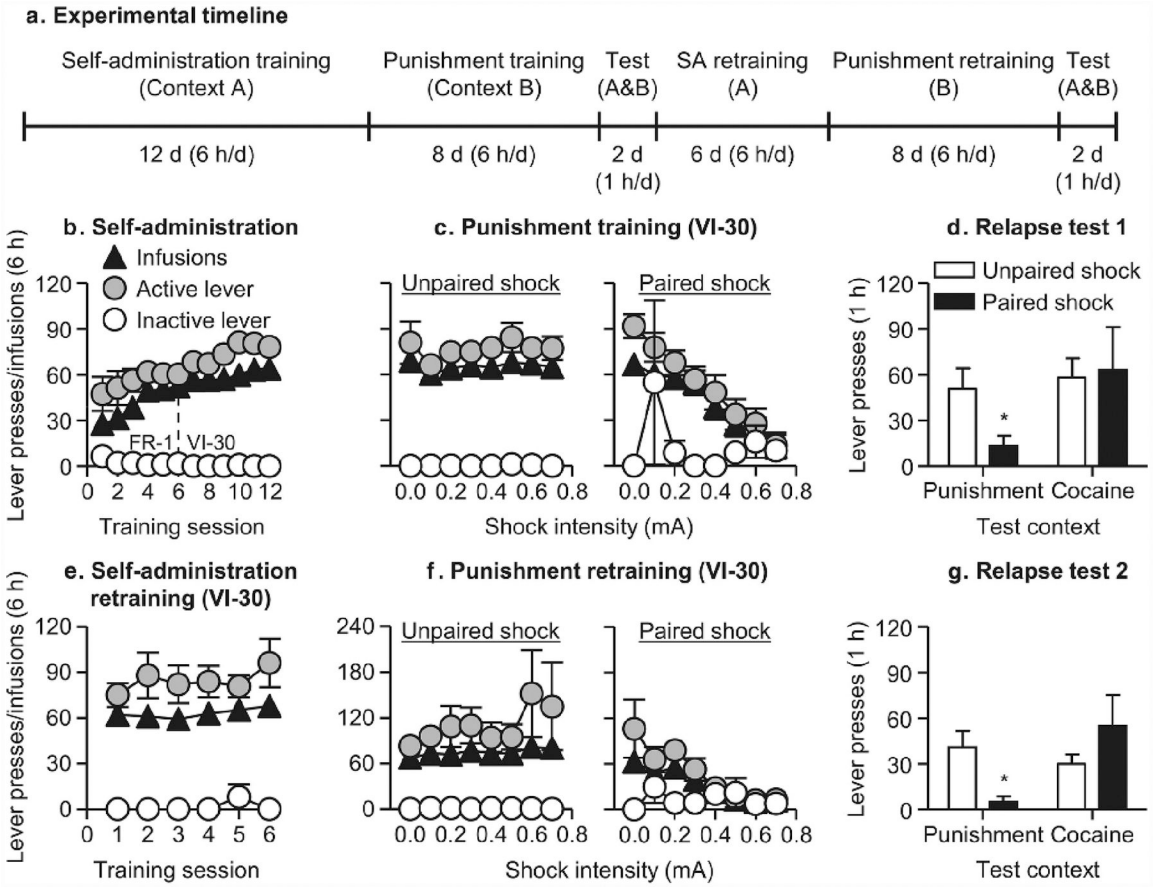

Figure 1.

Effects of paired versus unpaired footshock on cocaine self-administration and context-induced relapse to cocaine seeking. (a) Timeline of exp. 1. (b) Self-administration training: number of cocaine infusions (0.75 mg/kg/infusion) and lever presses during self-administration training (12 days) in context A ($n = 14$). The reinforcement schedules were fixed ratio-1 20-second timeout (days 1–6) and variable interval 30 seconds (VI-30) (days 7–12). (c) Punishment training: number of cocaine infusions and lever presses (VI-30) for the unpaired (left panel; $n = 6$) and paired (right panel; $n = 8$) shock groups in context B with shock intensity increasing from 0.0 to 0.7 mA by 0.1 mA each day (8 days). (d) Relapse test 1: number of lever presses on the previously active lever during the 1-hour extinction sessions in context B (punishment) and context A (cocaine). Lever presses led to contingent presentations of the compound tone–light cue previously paired with cocaine infusions during training, but not cocaine. (e) Self-administration retraining: number of cocaine infusions and lever presses during the retraining phase in context A under a VI-30 reinforcement schedule. (f) Punishment retraining: number of cocaine infusions and lever presses (VI-30) for the unpaired (left panel) and paired (right panel) shock groups in context B. (g) Relapse test 2: number of lever presses on the previously active lever during the 1-hour extinction sessions in contexts A and B. All data are mean ± SEM. *Different from the unpaired group, $P < 0.05$

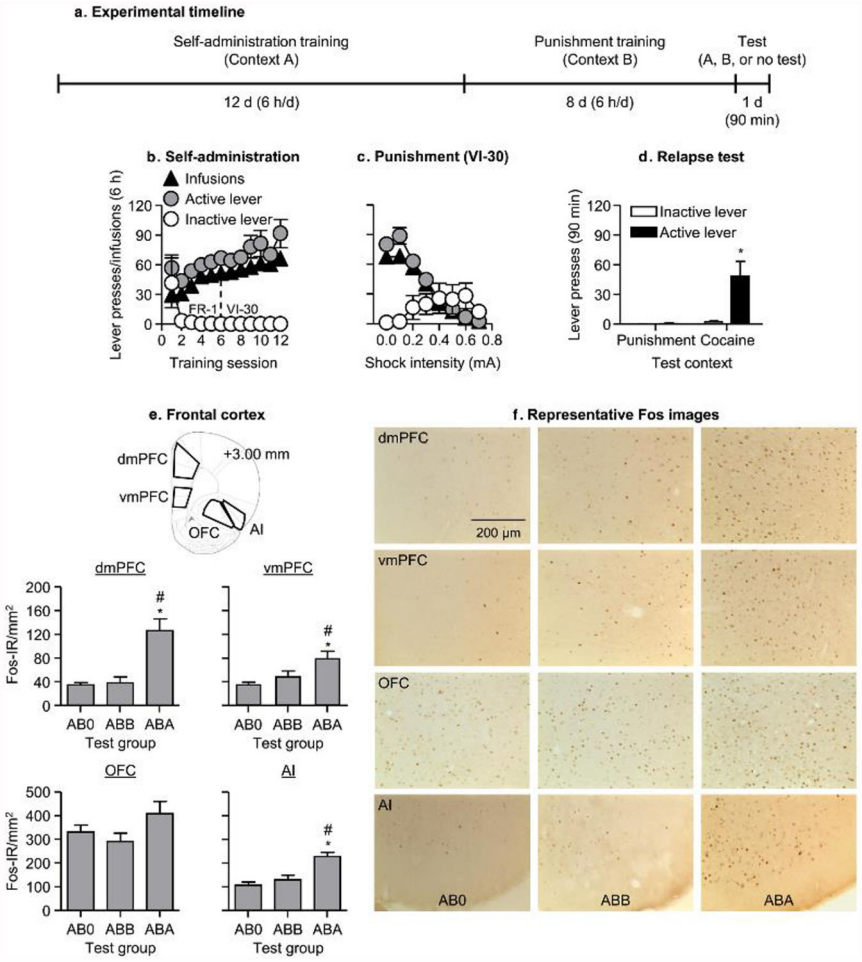

Figure 2.

Fos expression after context-induced relapse to cocaine seeking: frontal cortex. (a) Timeline of exp. 2. (b) Self-administration training: number of cocaine infusions (0.75 mg/kg/infusion) and lever presses during self-administration training (12 days) in context A (n = 19). The reinforcement schedules were fixed ratio-1 20-second timeout (days 1–6) and variable interval 30 seconds (days 7–12). (c) Punishment training: number of cocaine infusions and lever presses (variable interval 30 seconds) in context B with shock intensity increasing from 0.0 to 0.7 mA by 0.1 mA each day (8 days). (d) Relapse test: number of lever presses on the previously active lever and on the inactive lever during the 90-minute extinction session in context B (punishment) and context A (cocaine). Lever presses led to contingent presentations of the compound tone–light cue previously paired with cocaine infusions during training, but not cocaine. (e) Frontal cortex: number of Fos-immunoreactive nuclei per square millimeter in dorsomedial prefrontal cortex (dmPFC), ventromedial prefrontal cortex (vmPFC), orbitofrontal cortex (OFC) and anterior insula (AI) for rats not tested (AB0) or tested in the punishment context B (ABB) or cocaine context A (ABA). (f) Representative Fos images: representative photomicrographs of dmPFC, vmPFC, OFC and AI (scale bar = 200 µm). All data are mean ± SEM. #Different from AB0; *different from ABB, $P < 0.05$, n = 5–7 per group

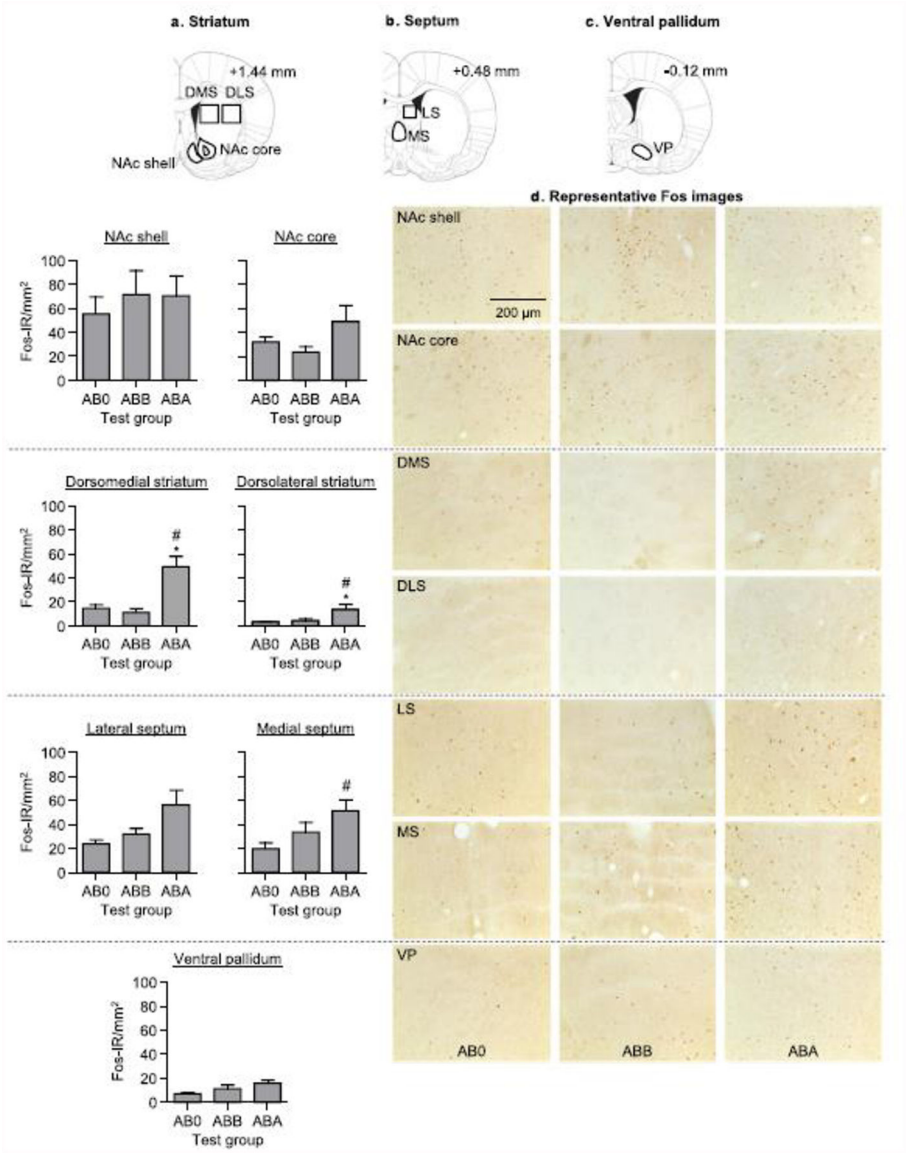

Figure 3.

Fos expression after context-induced relapse to cocaine seeking: striatum, septum and ventral pallidum. Number of Fos-immunoreactive nuclei per square millimeter in (a) striatum: nucleus accumbens shell (NAc shell), nucleus accumbens core (NAc core), dorsomedial striatum (DMS) and dorsolateral striatum (DLS); (b) septum: lateral (LS) and medial (MS) septum; and (c) ventral pallidum (VP) for rats in the AB0, ABB and ABA groups. (d) Representative Fos images: representative photomicrographs of NAc shell, NAc core, DMS, DLS, LS, MS and VP for the AB0, ABB and ABA groups (scale bar = 200 µm). All data are mean ± SEM. #Different from AB0; *different from ABB, $P < 0.05$, n = 5–7 per group.

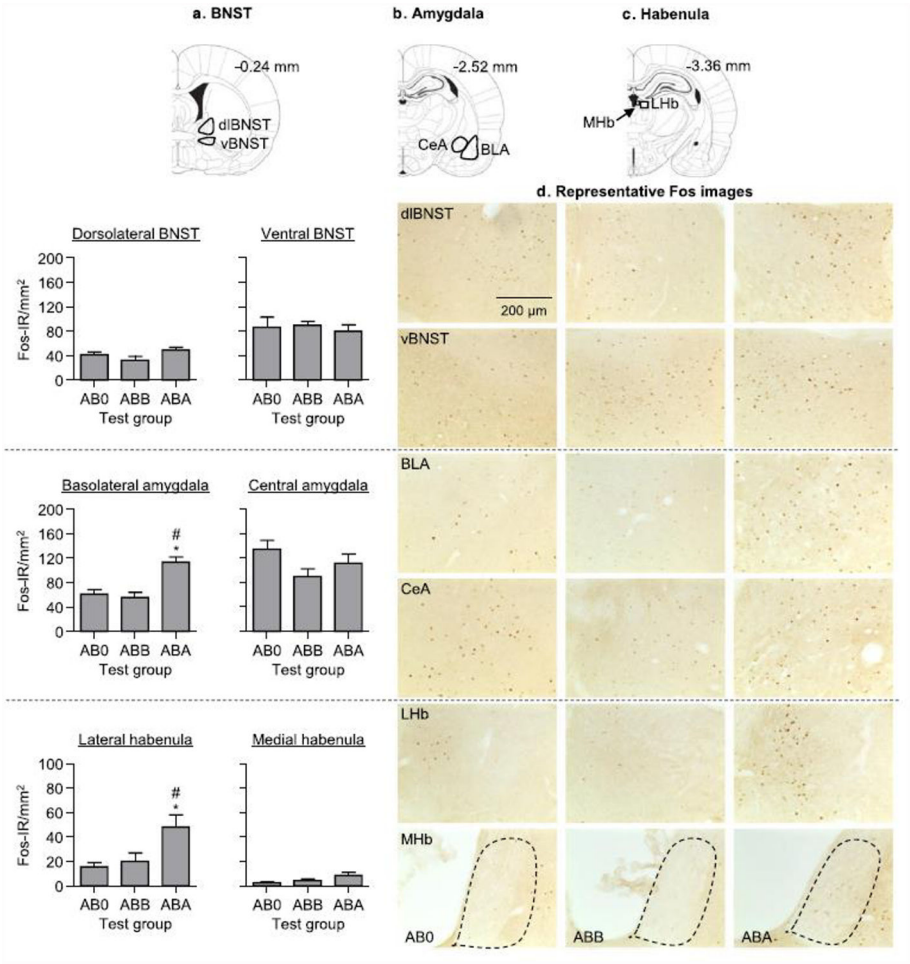

Figure 4.

Fos expression after relapse to cocaine seeking: BNST, amygdala and habenula. Number of Fos-immunoreactive nuclei per square millimeter in (a) BNST: dorsolateral and ventral bed nucleus of the stria terminalis (dlBNST, vBNST); (b) amygdala: basolateral and central amygdala (BLA, CeA); and (c) habenula: lateral and medial habenula (LHb, MHb) for rats in the AB0, ABB and ABA groups. (d) Representative Fos images: representative photomicrographs of dlBNST, vBNST, BLA, CeA, LHb and MHb for AB0, ABB and ABA groups (scale bar = 200 µm). All data are mean ± SEM. #Different from AB0; *different from ABB, $P < 0.05$, n = 5–7 per group

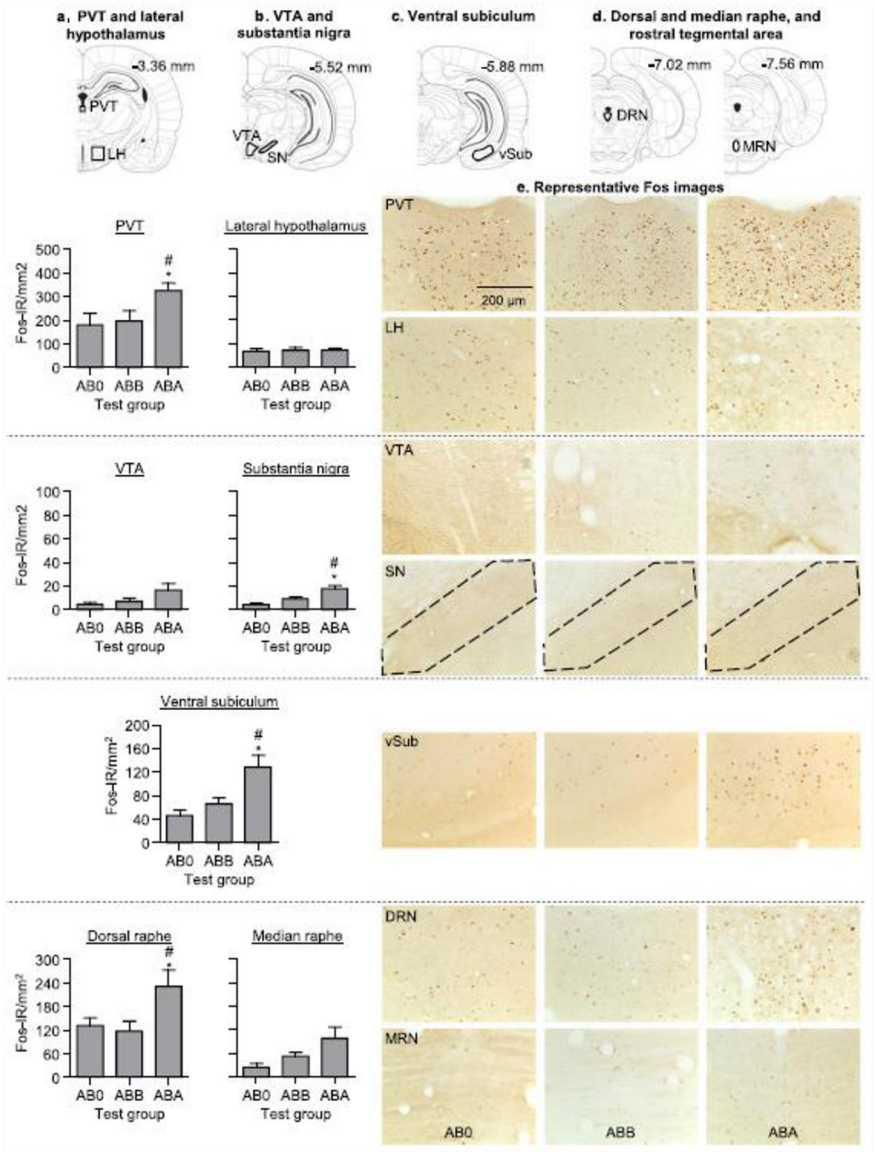

Figure 5.

Fos expression after relapse to cocaine seeking: paraventricular nucleus of the thalamus, lateral hypothalamus, ventral tegmental area, substantia nigra, ventral subiculum and raphe nuclei. Number of Fos-immunoreactive nuclei per square millimeter in (a) paraventricular nucleus of the thalamus (PVT) and lateral hypothalamus (LH); (b) ventral tegmental area (VTA) and substantia nigra (SN); (c) ventral subiculum (vSub); and (d) raphe nuclei: dorsal and median raphe nuclei (DRN, MRN) for rats in the AB0, ABB and ABA groups. (e) Representative Fos images: representative photomicrographs of PVT, LH, VTA, SN, vSub, DRN and MRN for AB0, ABB and ABA groups (scale bar = 200 µm). All data are mean ± SEM. #different from AB0; *different from ABB, $P < 0.05$, $n = 5–7$ per group

Table 1.

Comparison of Fos induction in different brain areas during the relapse tests in contexts A and B between the present study and previous studies on context-induced relapse to alcohol seeking after punishment-imposed abstinence (Marchant et al. 2014; Marchant et al. 2016) or context-induced reinstatement of cocaine seeking after extinction (Hamlin et al. 2008).

Brain region	Punishment: cocaine versus alcohol		Cocaine: punishment versus extinction	
	Cocaine	Alcohol	Punishment	Extinction
dmPFC	A > B	A = B	A > B	A = B
vmPFC	A > B	A = B	**A > B**	**A > B**
DMS	A > B	A = B	A > B	A = B
DLS	**A > B**	**A > B**	A > B	A = B
NAc core	A = B	A > B	A = B	A = B
NAc shell	A = B	A = B	A = B	A = B
vBNST	A = B	A = B	A = B	A = B
dBNST	A = B	A = B	A = B	A = B
LS	A = B	A = B	A = B	
BLA	**A > B**	**A > B**	**A > B**	**A > B**
CeA	A = B		A = B	A = B
LHb	A > B	A < B	A > B	
MHb	A = B	A = B	A = B	
PVT	A > B	A = B[a]	A > B	A = B
LH	A = B	A > B	A = B	A > B
VTA	A = B		A = B	A = B
SN	A > B		A > B	A = B
vSub	**A > B**	**A > B**	A > B	