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SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Group 1: Reinstatement of cocaine-seeking behaviour: brain activation patterns in C-LA rats

Self-administration. Rats that were designated for C-LA self-administration were surgically implanted with jugular catheters. Surgeries were performed under general anesthesia (isoflurane, 5% for induction, 1-3% for maintenance), and intravenous catheters were aseptically inserted in the right jugular vein as previously described [1]. The vein was pierced with a 21-gauge needle, and the tubing was inserted and secured inside the vein by tying the vein with suture thread. The catheter was made of Micro-Renathane tubing (0.037-inch diameter; Braintree Scientific, Braintree, MA, USA) that was attached to a guide cannula (Plastics One, Roanoke, VA, USA), secured with dental acrylic cement, and anchored with a mesh (2 cm square). The catheter port was placed on a small incision on the back and closed with a small plastic cap and metal cover cap to keep the inside part of the catheter clean and protected. All incisions were closed using veterinary tissue adhesive. After 7-10 days of recovery, the rats began cocaine self-administration training in daily 6 h sessions. Each session was initiated by the extension of two retractable levers into the operant chamber (29 cm × 24 cm × 19.5 cm; Med Associates, St. Albans, VT, USA). Responses on the active lever were reinforced on a fixed-ratio 1 (FR1) schedule with intravenous (IV) cocaine (National Institute on Drug Abuse, Bethesda, MD, USA; 0.25 mg/0.1 ml/infusion), dissolved in 0.9% sodium chloride (Hospira, Lake Forest, IL, USA) and infused over 4 s. Each reinforced response was followed by a 20 s

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timeout (TO20 s) period that was signaled by the illumination of a cue light above the active lever. Responses on the inactive lever were recorded but had no scheduled consequences.

Cannulation. Fourteen days after beginning self-administration training, the rats were implanted with a guide cannula (23-gauge, 15 mm, Plastics One, Roanoke, VA, USA) that was aimed at the pPVT (anterior/posterior, -3.3 mm; medial/lateral, ±2.2 mm from bregma; dorsal/ventral, -3.2 mm from dura, 20° angle; [2]) and positioned 2 mm above the target injection point. After 7 days of recovery, the animals resumed self-administration training for an additional 7 days.

Extinction. Immediately following the completion of 21 daily self-administration sessions, the rats underwent extinction training for 14-20 days (until the extinction criteria of ≤ 10 responses over three consecutive sessions was reached) [3]. All of the extinction sessions lasted 2 h and began with extension of the levers into the operant chambers, with the same schedule of self-administration sessions but without reward (cocaine) delivery.

Intra-pPVT microinjection and reinstatement tests. On the last day of extinction training, each rat received a sham injection for habituation to the microinjection. Twenty-four hours later, they received an intra-pPVT microinjection of 0.5 μg OrxA [3] or vehicle (Matzeu et al., 2016). The microinjections in the pPVT were performed using a microinfusion pump (Harvard 22 Syringe Pump, Holliston, MA, USA) and injectors that extended 2.00 mm beyond the guide cannula. The injections were performed at a flow rate of 0.5 μl/min over 1 min. The injectors were left in place for an additional minute to allow for diffusion away from the injector tip. Following the injections, the rats were returned to their home cages for 2 min. Half of the rats were then placed in the operant chambers under extinction conditions for 2 h and tested for the reinstatement of cocaine-seeking behavior. The other half of the rats remained in their home cage for 2 h. This latter subgroup was used to control for possible operant chamber effects (in addition to the

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pPVT injection) on brain activation.

Immunohistochemistry. Immediately after the 2 h reinstatement session (or after 2 h in the home cage), the rats were deeply anesthetized by CO_2 and transcardially perfused with cold 4% paraformaldehyde in 0.1 mM sodium tetraborate, pH 9.5. Brains were removed, postfixed in 4% paraformaldehyde overnight, and stored in 30% (w/v) sucrose, 0.1% (w/v) sodium azide, and potassium phosphate-buffered saline (KPBS) solution. The brains were sectioned coronally (40 µm) on a cryostat at -20ºC. The sections were then processed for Fos immunodetection. Briefly, coronal sections were blocked for 90 min using 5% normal donkey serum, 0.1% bovine serum albumin (BSA), and 0.3% Triton-X in PBS, followed by incubation for 24 h at room temperature with anti-Fos antibody (1:1000, rabbit monoclonal antibody, Cell Signaling, Danvers, MA, USA). The tissue sections were then incubated with ImmPress reagent with secondary antibody for 90 min (anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA). Fos immunostaining was visualized using DAB-Ni (Vectors Laboratories, Burlingame, CA, USA). Fos-positive (Fos^+) neurons were counted within sections that incorporated the pPVT (4 sections/rat; typical range: -3.14 mm to -3.80 mm from bregma), BNST (5 sections/rat; typical range: -0.26 mm to -0.92 mm from bregma), CeA (5 sections/rat; typical range: -1.88 mm to -2.80 mm from bregma), prelimbic cortex (PrL) and infralimbic cortex (IL; 4 sections/rat; typical range: 3.70 mm to 2.20 mm from bregma), nucleus accumbens core (NACc) and NAC shell (NACsh; 5 sections/rat; typical range: 2.20 mm to 1.00 mm from bregma), and basolateral amygdala (BLA; 5 sections/rat; typical range: -1.88 mm to -2.80 mm from bregma). Fos^+ neurons were acquired with a bright-field microscope (Keyence, BZ-X700, Itasca, IL, USA) and counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Injection tracks were verified in sections that incorporated the pPVT using a bright-field microscope (**Fig. 1D**), and off-target cannulations

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were excluded from the study. For immunohistochemistry, one age-matched naive group of rats (n = 6) was prepared. All of the histochemical data were compared with data from age-matched experimentally naive rats that were handled daily for 5 min and never exposed to the operant chambers.

Group 2: Reinstatement of cocaine-seeking behaviour: inactivation of the BNST and CeA in C-LA rats

Self-administration. The rats underwent the same self-administration training as described for Group 1.

Cannulation. Fourteen days after beginning self-administration training, the rats were implanted with a guide cannula that was aimed at the pPVT and bilaterally implanted with cannulas that were aimed at the BNST (anterior/posterior, -0.30 mm; medial/lateral, ±2.00 mm from bregma; dorsal/ventral, -4.80 mm from dura) [2] or CeA (anterior/posterior, -2.20 mm; medial/lateral, ±4.20 mm from bregma; dorsal/ventral, -5.50 mm from dura) [2]. The BNST and CeA guide cannulas were positioned 2.00 mm above the target injection site. After 7 days of recovery, the animals resumed self-administration training for an additional 7 days.

Extinction. All of the extinction sessions lasted 2 h using the same schedule of self-administration but without reward (cocaine) delivery.

Microinjections in the CeA and BNST and reinstatement tests. On the last day of extinction training, each rat received sham injections for habituation to the microinjection procedure. Twenty-four hours later, they received an intra-pPVT microinjection of 0.5 μg OrxA [3]. To transiently inactivate the CeA or BNST, intra-CeA or BNST injections of the γ-aminobutyric acid-

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A ($GABA_A$) and $GABA_B$ receptor agonists muscimol and baclofen (M/B) or vehicle were performed. Muscimol and baclofen were prepared at concentrations of 0.6 and 0.06 mM, respectively. All the injections were performed simultaneously at a flow rate of 0.5 µl/min over 1 min. The injectors were left in place for an additional minute to allow for diffusion away from the injector tip. Following the injections, the rats were returned to their home cages for 2 min and then placed in the operant chambers under extinction conditions for the reinstatement test. After the test, the rats were euthanized by CO_2 inhalation, and their brains were collected and snap frozen. The brains were sectioned coronally (40 µm) on a cryostat at -20ºC, fixed, and stained with DAB-Ni. Injection tracks were verified in sections that incorporated the pPVT, CeA, and BNST using a bright-field microscope (**Fig. 3D-F**). Only rats with cannula placements that were located in the appropriate brain region were included in the data analysis.

Group 3: Reinstatement of reward-seeking behaviour and CeA activation pattern: short access to cocaine versus sweetened condensed milk

Cocaine short access

Self-administration. Following recovery from jugular surgery, the rats began IV cocaine self-administration training in daily C-SA (2 h) sessions (instead of daily 6 h sessions) under the same schedule of reinforcement as described for Group 1 and Group 2 (i.e., FR1, TO20 s).

Cannulation. Fourteen days after beginning self-administration training, the rats were implanted with a guide cannula that was aimed at the pPVT (see Group 1 and Group 2).

Extinction. All of the extinction sessions lasted 2 h using the same schedule of self-administration sessions but without reward (cocaine) delivery.

Intra-pPVT microinjection and reinstatement test. The sham injections and microinjections in the

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pPVT were performed as described for Group 1. Following the injections, the rats were returned to their home cages for 2 min and then placed in the operant chambers under extinction conditions for 2 h and tested for the reinstatement of cocaine-seeking behavior.

Immunohistochemistry. Brain processing for immunohistochemistry and cannula placement verification were performed as for Group 1. Fos^+ neurons were counted within sections that incorporated the CeA and pPVT. Injection tracks were verified (**Fig. 4C**), and rats with off-target cannulations were excluded from the data analysis.

Sweetened condensed milk

Self-administration, extinction. To avoid satiety that can be induced by the excessive ingestion of sweetened condensed milk (SCM), SCM self-administration was established in daily 30 min sessions on an FR1 TO20 s schedule of reinforcement. Sessions were initiated by the extension of both levers into the operant chamber, and responses on the right active lever resulted in the delivery of SCM (2:1 in tap water, 0.1 ml) into a drinking receptacle. Responses on the left inactive lever were recorded but had no scheduled consequences. The concentration of SCM that was used in the present study came from a classic study of SCM reinforcement [4] that has been shown to maintain breakpoints (an index of reinforcing efficacy) under a progressive-ratio schedule of reinforcement that is comparable to those that are measured with cocaine at the present dose [4-6]. Under this condition (schedule of reinforcement and concentration of SCM), we have extensively reported that reliable and comparable behavior occurs for cocaine and SCM (e.g., [7-9]).

Cannulation. Fourteen days after beginning self-administration training, the rats were implanted with a guide cannula that was aimed at the pPVT as described for Group 1.

Extinction. All of the extinction sessions lasted 2 h using the same schedule of self-

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administration sessions but without reward (SCM) delivery.

Intra-pPVT microinjections and reinstatement test. The sham injection and microinjection in the pPVT were performed as described for Group 1. Following the injections, the rats were returned to their home cages for 2 min and then placed in the operant chambers under extinction conditions for 2 h and tested for the reinstatement of SCM-seeking behavior.

Immunohistochemistry. Brain processing for immunohistochemistry and cannula placement verification were performed as described for the C-SA group. Injection tracks were verified (**Fig. 5C**), and rats with off-target cannulations were excluded from the study.

Group 4: Reinstatement of cocaine-seeking behaviour in C-LA rats: effect of CRF1 receptor blockade in the CeA

Self-administration and extinction. Cocaine self-administration and extinction training were conducted similarly to Group 1 and Group 2.

Cannulation. Cannulas were implanted in the pPVT and CeA as described for Group 2.

Intracerebral microinjections and reinstatement test. Once the rats' behavior was extinguished, they received sham injections (see Groups 1-3). Twenty-four hours later, the rats were injected with 0.5 μg OrxA in the pPVT and bilaterally injected in the CeA with the CRF_1 receptor antagonist CP154526 (0, 0.3, or 0.6 μg) [10]. The injections were performed simultaneously at a flow rate of 0.5 μl/min over 1 min. The injectors were left in place for an additional minute to allow for diffusion away from the injector tip. Following the injections, the rats were returned to their home cages for 2 min and then placed in the operant chambers under extinction conditions for the reinstatement test. After the reinstatement test, the rats were euthanized by CO_2 inhalation, and their brains were harvested and snap frozen. The brains were sectioned

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coronally (40 µm) on a cryostat at -20ºC, fixed, and stained with DAB-Ni. Injection tracks were verified in sections that incorporated the pPVT and CeA using a bright-field microscope (**Fig. 6C**). Off-target cannulations were excluded from the study.

RESULTS

Group 1: Reinstatement of cocaine-seeking behaviour: brain activation patterns in C-LA rats

Fos immunoreactivity

Prelimbic cortex (**Fig. S1A-G**). A significant increase in Fos^+ neurons was observed in the PrL following OrxA injections in both the operant chamber and home cage conditions compared with naive rats and rats that received vehicle (one-way ANOVA: $F_{4,24} = 16.88$, $p < 0.001$; Sidak *post hoc* test: $p < 0.05$, *vs*. naive or respective vehicle), with stronger activation when rats were placed in the operant chambers following the OrxA injection (Sidak *post hoc* test: $p < 0.05$; **Fig. S1G**) that could have been induced by the rats' operant response (i.e., lever press).

Infralimbic cortex (**Fig. S1H-N**). An increase in Fos^+ neurons was observed in the IL following OrxA injections in rats that were returned to the operant chamber or their home cage (one-way ANOVA: $F_{4,24} = 6.23$, $p < 0.01$; Sidak *post hoc* test: $p < 0.05$, *vs*. naive or respective vehicle; **Fig. S1N**).

Nucleus accumbens core (**Fig. S1O-U**). Fos expression remained unchanged following vehicle or OrxA injection in the pPVT (one-way ANOVA: $F_{4,24} = 1.700$, $p = 0.1828$; **Fig. S1U**).

Nucleus accumbens shell (**Fig. S1V-Bi**). The number of Fos^+ neurons in the NACsh did not change in any of the conditions tested (one-way ANOVA: $F_{4,24} = 1.107$, $p = 0.3761$; **Fig. S1Bi**).

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Basolateral amygdala (**Fig. S1Ci-li**). An increase in Fos^+ neurons was observed in the BLA following OrxA injections when rats were placed in the operant chambers or returned to their home cage compared with naive rats and rats that received vehicle (one-way ANOVA: $F_{4,24} = 16.59$, $p < 0.001$; Sidak *post hoc* test: $p < 0.05$, *vs*. naive), with stronger activation in rats that were placed in the operant chambers following the OrxA injection (Sidak *post hoc* test: $p < 0.01$; **Fig. SIi**). Similar to the PrL, this higher BLA activation was likely attributable to the animals' operant responses during reinstatement.

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Figure Legends

Figure S1. Activation patterns in the PrL, IL, NACc, NACsh, and BLA following intra-pPVT injection of OrxA to induce the reinstatement of cocaine-seeking behavior. **(A**) Schematic illustration of the rostrocaudal level where Fos^+ neurons were counted in the PrL. Typical photomicrographs illustrate Fos activation in the PrL in naive rats (**B**), in rats that were injected with vehicle (**C**) or OrxA (**D**) and exposed to the operant chamber, and in rats that were injected with vehicle (**E**) or OrxA (**F**) and exposed to the home cage. (**G**) Number of Fos^+ neurons in the PrL. $^{**}p < 0.01$, $^{***}p < 0.001$, *vs*. naive; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, *vs*. respective vehicle; $^{+}p < 0.05$, $^{+++}p < 0.001$, *vs*. Orx operant chamber (Sidak *post hoc* test). (**H**) Schematic illustration of the rostrocaudal level where IL Fos^+ neurons were counted. Typical photomicrographs illustrate Fos activation in the IL in naive rats (**I**), in rats that were injected with vehicle (**J**) or OrxA (**K**) and exposed to the operant chamber, and in rats that were injected with vehicle (**L**) or OrxA (**M**) and exposed to the home cage. (**N**) Number of Fos^+ neurons in the IL. $^{*}p < 0.05$, $^{**}p < 0.01$, *vs*. naive; $^{\#}p < 0.05$, *vs*. respective vehicle; $^{+}p < 0.05$, *vs*. Orx operant chamber (Sidak *post hoc* test). (**O**) Schematic illustration of the rostrocaudal level where NACc Fos^+ neurons were counted. Typical photomicrographs illustrate Fos activation in the NACc in naive rats (**P**), in rats that were injected with vehicle (**Q**) or OrxA (**R**) and exposed to the operant chamber, and in rats that were injected with vehicle (**S**) or OrxA (**T**) and exposed to the home cage. (**U**) The number of Fos^+ neurons was not different following OrxA or vehicle injections. (**V**) Schematic illustration of the rostrocaudal level where NACsh Fos^+ neurons were counted. Typical photomicrographs illustrate Fos activation in the NACsh in naive rats (**W**), in rats that were injected with vehicle (**X**)

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or OrxA (**Y**) and exposed to the operant chamber, and in rats that were injected with vehicle (**Z**) or OrxA (**Ai**) and exposed to the home cage. (**Bi**) The number of Fos^+ neurons was not different following OrxA or vehicle injections. (**Ci**) Schematic illustration of the rostrocaudal level where BLA Fos^+ neurons were counted. Typical photomicrographs that illustrate Fos activation in the BLA in naive rats (**Di**), in rats that were injected with vehicle (**Ei**) or OrxA (**Fi**) and exposed to the operant chamber, and in rats that were injected with vehicle (**Gi**) or OrxA (**Hi**) and exposed to the home cage. (**Ii**) Number of Fos^+ neurons in the BLA. $^*p < 0.05$, $^{***}p < 0.001$, *vs*. naive; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, *vs*. respective vehicle; $^{++}p < 0.01$, $^{+++}p < 0.001$, *vs*. Orx operant chamber (Sidak *post hoc* test). The data are expressed as mean ± SEM.

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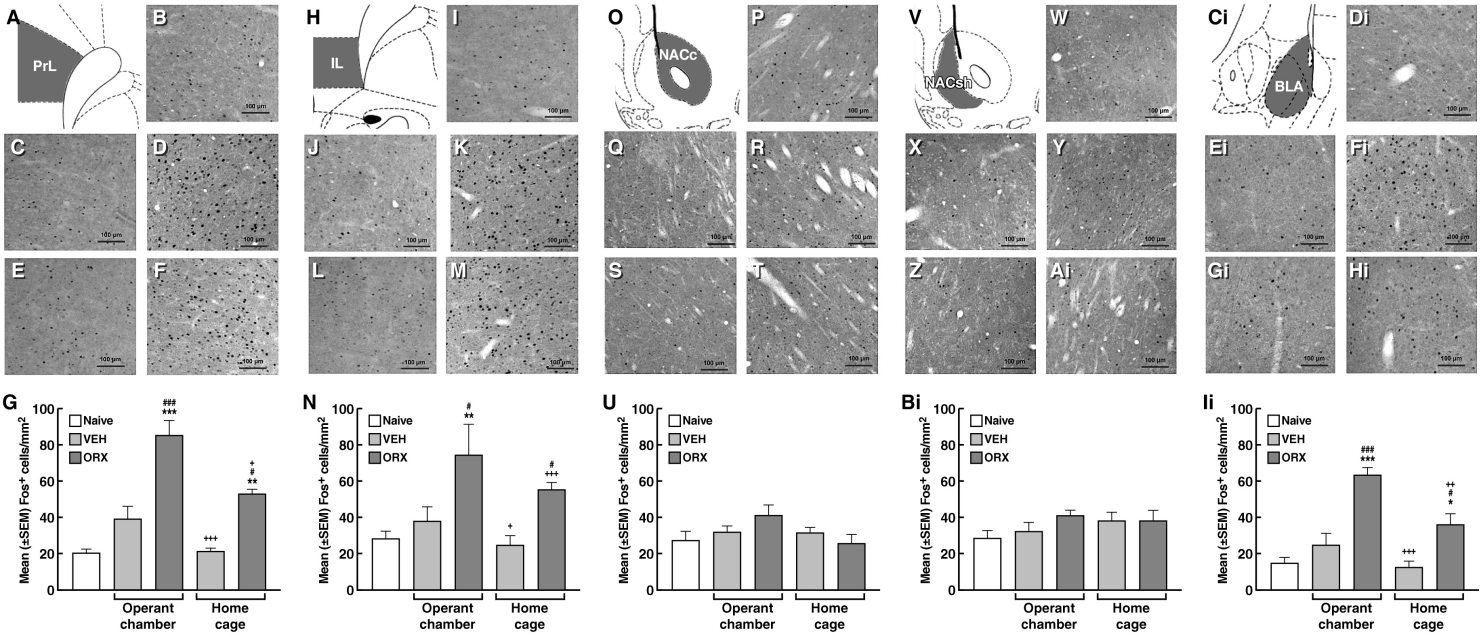

Supplemental Figure 1