Behavioral/Cognitive

Manipulating a "Cocaine Engram" in Mice

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Experience with drugs of abuse (such as cocaine) produces powerful, long-lasting memories that may be important in the development and persistence of drug addiction. The neural mechanisms that mediate how and where these cocaine memories are encoded, consolidated and stored are unknown. Here we used conditioned place preference in mice to examine the precise neural circuits that support the memory of a cocaine-cue association (the "cocaine memory trace" or "cocaine engram"). We found that a small population of neurons (~10%) in the lateral nucleus of amygdala (LA) were recruited at the time of cocaine-conditioning to become part of this cocaine engram. Neurons with increased levels of the transcription factor CREB were preferentially recruited or allocated to the cocaine engram. Ablating or silencing neurons overexpressing CREB (but not a similar number of random LA neurons) before testing disrupted the expression of a previously acquired cocaine memory, suggesting that neurons overexpressing CREB become a critical hub in what is likely a larger cocaine memory engram. Consistent with theories that coordinated postencoding reactivation of neurons within an engram or cell assembly is crucial for memory consolidation (Marr, 1971; Buzsáki, 1989; Wilson and McNaughton, 1994; McClelland et al., 1995; Girardeau et al., 2009; Dupret et al., 2010; Carr et al., 2011), we also found that post-training suppression, or nondiscriminate activation, of CREB overexpressing neurons impaired consolidation of the cocaine memory. These findings reveal mechanisms underlying how and where drug memories are encoded and stored in the brain and may also inform the development of treatments for drug addiction.

Key words: **amygdala; cocaine; conditioning; memory; place preference**

Introduction

Drug addiction is a chronically relapsing brain disease. One obstacle to the treatment of drug abuse (including cocaine abuse) is the high incidence of relapse to drug-taking following months, or even years, of abstinence (Dackis and O'Brien, 2001; Wagner and Anthony, 2002). In currently abstinent, former-cocaine users, mere exposure to environmental cues present at the time of previous cocaine use may evoke powerful memories of the rewarding properties of cocaine, induce drug craving, and precipitate relapse to cocaine-seeking and consumption (Childress et al., 1999). Similarly, recall of a cocaine-related memory may be sufficient to induce relapse to cocaine-seeking and/or cocaine-taking in drug-free rodents with a history of cocaine administration (Shaham et al., 2003; Stefanik et al., 2013). These findings indicate that conditioned cues are important factors in the ongoing cycle of relapse in addiction. Therefore, understanding how the brain associates initially motivationally neutral environmental cues with the rewarding properties of cocaine may inform the development of novel strategies aimed at treating or preventing relapse in humans by inhibiting drug-seeking urges evoked by drug-associated environmental cues.

Despite considerable progress in identifying the neural mechanisms mediating the acute unconditioned rewarding effects of cocaine and other drugs of abuse (Nestler, 2005; Koob and Volkow, 2010; Mameli and Lüscher, 2011), relatively little is known about the molecular, cellular, and circuit mechanisms mediating the persistent conditioned rewarding effects of cocaine. The amygdala, including the lateral amygdala (LA), has been implicated in the process by which an initially neutral cue acquires conditioned rewarding properties by virtue of being paired with a rewarding stimulus, such as food or cocaine (Hiroi and White, 1991; Everitt et al., 2000; Baxter and Murray, 2002; Tye et al., 2008; Morrison and Salzman, 2010). For instance, lesioning (Fuchs et al., 2002) or disrupting synaptic plasticity (Heldt et al., 2014) in the lateral/basolateral amygdala region in rodents, impairs the acquisition of cocaine-conditioned place preference (CPP), in which an initially neutral environmental cue

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is paired with cocaine administration. Moreover, functional magnetic resonance imaging (fMRI) data from humans with a history of cocaine use shows that presentation of cues previously associated with cocaine not only induce craving but increase activation in a number of brain regions, including the amygdala (Chase et al., 2011; Prisciandaro et al., 2014). Together, these findings indicate that the amygdala may play an important role in the formation and expression of cocaine-cue memories that are relevant for addiction. It is of interest to note, however, that electrophysiological recording studies found that only a portion (but not all) neurons in the lateral/basolateral amygdala in either monkeys or rats respond to cues associated with food or cocaine reward (Muramoto et al., 1993; Carelli et al., 2003; Sugase-Miyamoto and Richmond, 2005; Bermudez et al., 2012). Here we investigated which particular neurons in the LA are critical for encoding and storing a memory of the cocaine-cue association in mice. That is, in this series of studies, we searched for the "cocaine memory engram."

Materials and Methods

Mice. Adult (at least 10 weeks of age) wild-type (WT) male and female F1 hybrid mice (C57BL/6NTac × 129S6/SvEvTac) were used for all experiments, except where noted (see Fig. 2*C–E*). For the targeted neuronal ablation experiments (see Fig. 2*C–E*), transgenic mice which express a simian diphtheria toxin receptor (DTR) in a cre-recombinase-inducible fashion were used (iDTR transgenic mice, The Jackson Laboratory, stock no. 007900; Buch et al., 2005; Han et al., 2009). In these mice, the gene encoding a DTR (simian *Hbegf,* heparin-binding epidermal growth factor-like growth factor) is under the control of the ubiquitous ROSA26 locus promoter, but expression of the DTR transgene is dependent on the cre recombinase-mediated removal of a transcriptional STOP cassette. Mice heterozygous for this mutation were maintained on a C57BL/6NTac genetic background. WT littermates were used as controls. Genotypes were determined by PCR analysis of tail DNA samples as previously described (Buch et al., 2005; Han et al., 2009).

Mice were bred at the Hospital for Sick Children and group housed (3–5 mice per cage) on a 12 h light/dark cycle with food and water available *ad libitum*. Behavioral experiments were conducted during the light-phase of the cycle. All procedures were conducted in accordance with the policies of the Hospital for Sick Children Animal Care and Use Committee and conformed to both the Canadian Council on Animal Care and National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

HSV vectors. cDNAs for wild-type full-length CREB fused to N-terminal GFP (kindly provided by Dr Satoshi Kida, Tokyo University of Agriculture, Tokyo, Japan), GFP (subcloned from pEGFP-N1, Clontech Laboratories), cre recombinase (cre, kindly provided by Dr Andras Nagy, Samuel Lunenfeld Research Institute, Toronto, Canada), hM3Dq or hM4Di (kindly provided by Dr Bryan Roth, University of North Carolina) were subcloned into bicistronic HSV vectors containing the constitutive HSV promoter IE4/5 and a CMV promoter. The first named transgene (GFP-CREB or GFP) was driven by the IE 4/5 promoter, while the second (cre, hM3Dq, or hM4Di) was driven by the CMV promoter. For vectors containing CREB alone, GFP-CREB fusion expression was driven by the IE4/5 promoter.

HSV containing these vector amplicons was packaged using a replication-defective helper virus, purified on a sucrose gradient, pelleted, and resuspended in 10% sucrose, as previously described (Carlezon and Neve, 2003; Han et al., 2008). The average titer of the virus stocks was 4.0×10^7 infectious U/ml. Transgene expression using this viral system typically peaks 3 d, and dissipates within 10–14 d, following microinjection (Josselyn et al., 2001; Barrot et al., 2002; Vetere et al., 2011; Cole et al., 2012).

Surgery. Mice were pretreated with atropine sulfate (0.1 mg/kg, i.p.), anesthetized with chloral hydrate (400 mg/kg, i.p.), and placed in a stereotaxic frame. Skin was retracted and holes drilled in the skull bilaterally above the LA (AP = −1.3, ML = ±3.4, V = −5.0 mm from bregma) according to (Paxinos and Franklin, 2001). Viral vector (1.5 μl/side) was microinjected through glass micropipettes connected via polyethelene tubing to a microsyringe (Hamilton) at a rate of 0.1 μl/min. Micropipettes were left in place an additional 10 min following microinjection to ensure diffusion. Micropipettes were slowly retracted, the incision closed and mice treated with analgesic (ketoprophen, 5 mg/kg, s.c.).

Verifying location of vector microinjection and extent of viral infection. Following the final CPP test, mice were transcardially perfused with 0.1 M PBS followed by 4% PFA. Brains were postfixed for 2 h (4°C) and transferred to a 30% sucrose solution. Coronal brain slices (50 μm) across the anterior–posterior extent of LA were collected using a cryostat (Leica CM1850). Every second section was mounted on a gel-coated glass slide and coverslipped with Vectashield fluorescence mounting medium containing DAPI (Vector Laboratories). Consistent with previous reports from several laboratories (Carlezon et al., 1998; Wallace et al., 2004; Brightwell et al., 2005; Airan et al., 2007; Han et al., 2009; Vetere et al., 2011; Cole et al., 2012) microinjection of HSV vectors produced robust localized transgene expression with minimal tissue damage around the site of microinjection. Native GFP-immunofluorescence (which did not differ across vectors) was used to determine placement and extent of the viral infection for each mouse. Based on this, each mouse was classified as a "hit" or "miss" by an examiner unaware of the treatment condition and behavioral results. Mice were defined as hits if robust bilateral GFP expression was observed in LA in at least five consecutive brain sections (across the anterior–posterior plane). All other mice were classified as miss (including those with unilateral, weak or no transgene expression in the LA). Only mice determined to be a bilateral hit were included in subsequent data analysis.

CLARITY. To visualize the placement and extent of viral infection in three dimensions, a set of viral infused brains were processed using the CLARITY technique as described previously (Chung and Deisseroth, 2013; Chung et al., 2013). Briefly, mice were perfused with ice-cold saline followed by a hydrogel solution (consisting of 4% formaldehyde, 4% acrylamide, 0.025% bis-acrylamide, and 0.25% VA044 thermal initiator) and brains were then postfixed in the same solution for 48 h at 4°C. The brains and hydrogel solution were polymerized by increasing the temperature to 37°C. Tissue was cleared for several days using SDS electrophoresis. Once clear, brains were cut on a vibratome (Leica) into 1-mm-thick coronal sections and mounted on glass slides in FocusClear (CelExplorer Labs). Finally, the 1-mm-thick sections were imaged using a Zeiss LSM 710 laser scanning confocal microscope with a 10× 0.3 NA air-objective. Image stacks (150 images with a 6.0 μm step size) were stitched together using Zen imaging software (Zeiss) and 3D reconstructions were assembled using ImageJ and VAA3D.

Stereological counting. To estimate the percentage of total LA neurons infected by the viral vectors, we used unbiased stereology and systematic sampling techniques. Stereological counting was performed using Stereology Investigator software (MBF) on every third section (50 μm thickness, at least 8 sections per mouse) from a subset of mice microinjected with CREB vector ($n = 6$). The entire LA (from −0.70 to −2.5 mm from bregma in the anterior–posterior direction) was traced and the optical fractionator probe was used to randomly place sampling boxes (250 × 250 μm) throughout the LA (West et al., 1991) and the number of $DAPI^+$ and GFP^+ cells per section assessed. Small, bright uniformly DAPI stained nuclei (~5 μm diameter) from putative glial cells were not included in DAPI counts. A counting frame of 5 × 50 μm was used for assessing DAPI and a counting frame of 120 × 120 μm was used for assessing GFP. The total number of $DAPI^+$ cells (putative pyramidal neurons) in the LA was estimated to be 200,000 (sum of left + right LA), consistent with previous reports (Tuunanen and Pitkänen, 2000; von Bohlen und Halbach and Unsicker, 2002). Using this stereological method, we estimate that between 9.84% (±0.89%) of all principal neurons in LA were infected by our viral manipulations.

Drugs. Cocaine HCl (Health Canada) was dissolved in sterile PBS and delivered intraperitoneally at the appropriate dose. Because the behavioral response to cocaine may differ depending on background genetics of mice (Eisener-Dorman et al., 2011), we varied the dose of cocaine in some experiments (see below for precise dosing information). DT was dissolved in sterile PBS and systemically administered (16 μg/kg, i.p.). As

a control, we systemically administered vehicle (PBS). Clozapine-*N*-oxide (CNO, Toronto Research Chemicals) was made in a stock solution of 10 mg/ml in DMSO and then diluted in saline to desired concentration. CNO was injected at a dose of 1 mg/kg, intraperitoneally, 1 h before testing, immediately after training, or 18–48 h after training (depending on experiment).

CPP. CPP was performed using an unbiased, counterbalanced protocol (Josselyn and Beninger, 1993; Prus et al., 2009). The CPP apparatus consisted of two 15 × 20 cm Plexiglas chambers connected by a guillotine door. Each chamber had a unique combination of visual, tactile, and olfactory properties (one side had white walls and a transparent rough floor, whereas the other side had black and white striped walls, a smooth white floor that was wiped with 0.2 ml of 3% acetic acid before each conditioning and test trial). We balanced the two chambers in terms of initial, baseline preference such that during the pretraining (habituation) phase, all groups of mice spent similar time in each chamber. In all experiments conducted herein, we failed to observe a group-wide bias for one chamber before training. The two chambers were separated by a guillotine door (which was open during the pretest habituation and test sessions, but closed during the conditioning sessions). The activity and location of mice was monitored by an overhead CCD camera, connected to a computer running Limelight software (Colbourn Instruments).

The CPP procedure consists of three sequential phases: habituation (pretest), conditioning, and postconditioning test (test). In the habituation phase, drug-free mice were allowed access to the two chambers of the CPP apparatus for 10 min. Time spent in each chamber (defined as the time when mice fully entered the chamber) was calculated for each mouse. To measure this, we used Limelight software to visually divide the CPP apparatus into three zones; chamber1 zone, middle zone, and chamber2 zone (15 × 18 cm; 15 × 4 cm; 15 × 18 cm), thus eliminating the time mice spent in the center of the apparatus from our calculations. Conditioning sessions took place twice daily for 1, 2, or 3 d, with a minimum of 5 h between each session. We chose this method of inducing CPP because of the short time course of transgene expression using these viral vectors. On each conditioning day, mice were confined to one chamber for 15 min immediately following saline (morning) or cocaine (afternoon) administration. The location of the cocaine-paired chamber was randomized and counterbalanced across groups. Eighteen hours following the final training session, CPP memory was assessed in a test session, in which drug-free mice were given free access to the two chambers (as in the pretest). Only the first 5 min of data were used for analysis to exclude possible effects of within-session extinction. We calculated a CPP score for each mouse (time, in seconds, spent in cocaine-paired zone minus time spent in saline-paired zone during the test).

CPP scores were analyzed using one- (*vector*) or two-way (*vector* × *drug*) ANOVAs.

catFISH. We identified neurons specifically activated by exposure to cues previously paired with cocaine using *arc* (activity-regulated cytoskeleton-associated protein) mRNA, as previously described (Han et al., 2007, 2009). Following the completion of training, mice were re-exposed to the chamber previously paired with cocaine for 7 min, and 5 min later, brains were removed. Under basal conditions, neurons express very low levels of *arc* mRNA. Approximately 2 min after the type of stimulation that is associated with synaptic plasticity, *arc* RNA is observed in the nuclei of activated neurons (Guzowski et al., 2001, 2005). However, this robust burst of nuclear *arc* is transient and *arc* RNA is rapidly transported to the cytoplasm, where it can be detected 20–45 min after induction (Guzowski et al., 2001, 2005). Because the time course of the nuclear signal is distinct from the cytoplasmic signal, the subcellular distribution of *arc* mRNA provides a time-stamp of neural activity for a particular neuron, with *arc* mRNA expression in the nucleus being a molecular signature of a neuron that was active ~5 min before. In non-seizure conditions, *arc* mRNA is expressed exclusively in neurons, primarily in principal neurons (Vazdarjanova et al., 2006).

Coronal sections (20 μm) were cut and thaw-mounted on slides, such that each slide contained sections from different treatment conditions (to maximize relevant between-group comparisons within each slide). Digoxigenin (DIG)-labeled antisense and sense riboprobes for arc mRNA were generated using a commercial transcription kit (MaxiScript, Ambion) and RNA labeling mix (Roche Diagnostics) from a plasmid encoding *arc* ORF. *arc* antisense riboprobe was detected with an anti-DIG antibody conjugated with horseradish peroxidase (HRP) FAB fragments (1/300; JacksonImmunoResearch) and visualized with an AlexaFluor 488-conjugated tyramide amplification system (PerkinElmer).

Briefly, sections were fixed with 4% PFA, washed in 2× saline–sodium citrate (SSC) buffer, treated with 0.5% acetic anhydrate/1.5% triethanolamine, and 50% methanol/50% acetone before being incubated in hybridization buffer (with 50% formamide, 5× SSC, 0.1 mg/ml yeast transfer RNA, 0.1% Tween 20, and 50 mg/ml heparin) for 30 min at room temperature. Sections were incubated with *arc* riboprobe (1 ng/μl in hybridization buffer) at 56°C overnight. Twenty-four hours later, sections were treated sequentially with RNaseA (1 μg/ml in 2× SSC at 37°C for 30 min), hydrogen peroxide (1% for 30 min), blocking reagent (Roche Diagnostics) with goat serum (4%, 30 min) and incubated with an anti-DIG HRP-conjugated antibody (described above) in blocking solution for 2 h at room temperature. Tyramide amplification was performed for 30 min at room temperature. Nuclei were visualized with Hoechst 33258 (Sigma-Aldrich) counterstain.

Images were captured using a confocal microscope (Zeiss LSM 710 equipped krypton-argon laser) and acquired using a 40× oil-immersion lens and *z*-sectioned in 1-μm-thick optical sections. Manual cell counts were performed by two observers unaware of the treatment condition. Small, bright uniformly DAPI-stained nuclei (~5 μm diameter) from putative glial cells were not counted. All other whole nuclei cells (putative neurons) in the field-of-view within the LA were counted. Neurons were assigned to one of the following four categories: (1) GFP^+ (infected by vector)/arc^- (not active), (2) arc^+ in the nucleus (recently activated, memory trace/GFP^- (not infected), (3) both GFP^+ and arc^+ in the nucleus, or (4) both GFP^- and arc^-.

Five to 10 slices were analyzed for each mouse and each treatment group contained three to six mice. The portion of neurons positive for *arc* in the nucleus in the infected (GFP^+) or noninfected (GFP^-) population was calculated by slice. The data were analyzed with an ANOVA with between-factor, vector, and a within-factor, neuronal infection status, (infected, noninfected neuron).

Specific methods

*Figure 1*A–C. One day after habituation, WT hybrid mice were trained for cocaine-cue CPP over 1, 2 or 3 consecutive days. In the morning of each training day (between 9:00 and 11:00 A.M.), saline (SAL) was administered directly before mice were placed in one chamber (counterbalanced) and in the afternoon (between 3:00 and 5:00 P.M.), mice either received SAL or COC (15 mg/kg, i.p.) before being placed in the other chamber.

*Figure 1*E,F. One day after habituation, WT hybrid mice were microinjected with CREB or GFP vector. Mice recovered for 1 d before cocaine-cue CPP training (for 2 or 3 d). Saline was administered before chamber placement in morning and COC (15 mg/kg, i.p.) was administered before chamber placement in afternoon.

*Figure 1*G. One day after habituation, WT hybrid mice were trained in cocaine-cue CPP for 3 consecutive days (as above) and the following day, microinjected with CREB or GFP vector. Mice were tested 3 d later (to mimic microinjection-test time course in Fig. 1*F*).

*Figure 1*H. One day after habituation, WT hybrid mice were trained in cocaine-cue CPP over 3 consecutive days. As before, mice were administered SAL in morning and either SAL or COC (1.25, 3, or 15 mg/kg) in afternoon.

*Figure 1*I. One day after habituation, WT hybrid mice were microinjected with CREB or GFP vector. Mice recovered for 1 d before training as above [for 3 d, saline in morning, COC (1.25 mg/kg, i.p.) in afternoon].

*Figure 2*A. One day after habituation, WT hybrid were microinjected with CREB or GFP vector and allowed to recover for 1 d before being trained for 1 d. Mice were administered SAL in morning and COC (30 mg/kg) in afternoon. One day later, drug-free mice were confined in the cocaine-paired chamber for 7 min and brains removed 5 min later.

*Figure 2*C. One day after habituation, iDTR transgenic or WT littermate control mice were microinjected with CREB-cre vector. Two days later, mice were trained for 1 d [SAL in morning and COC (15 mg/kg) in afternoon]. One day after training, DT or PBS was administered once per day for 2 d.

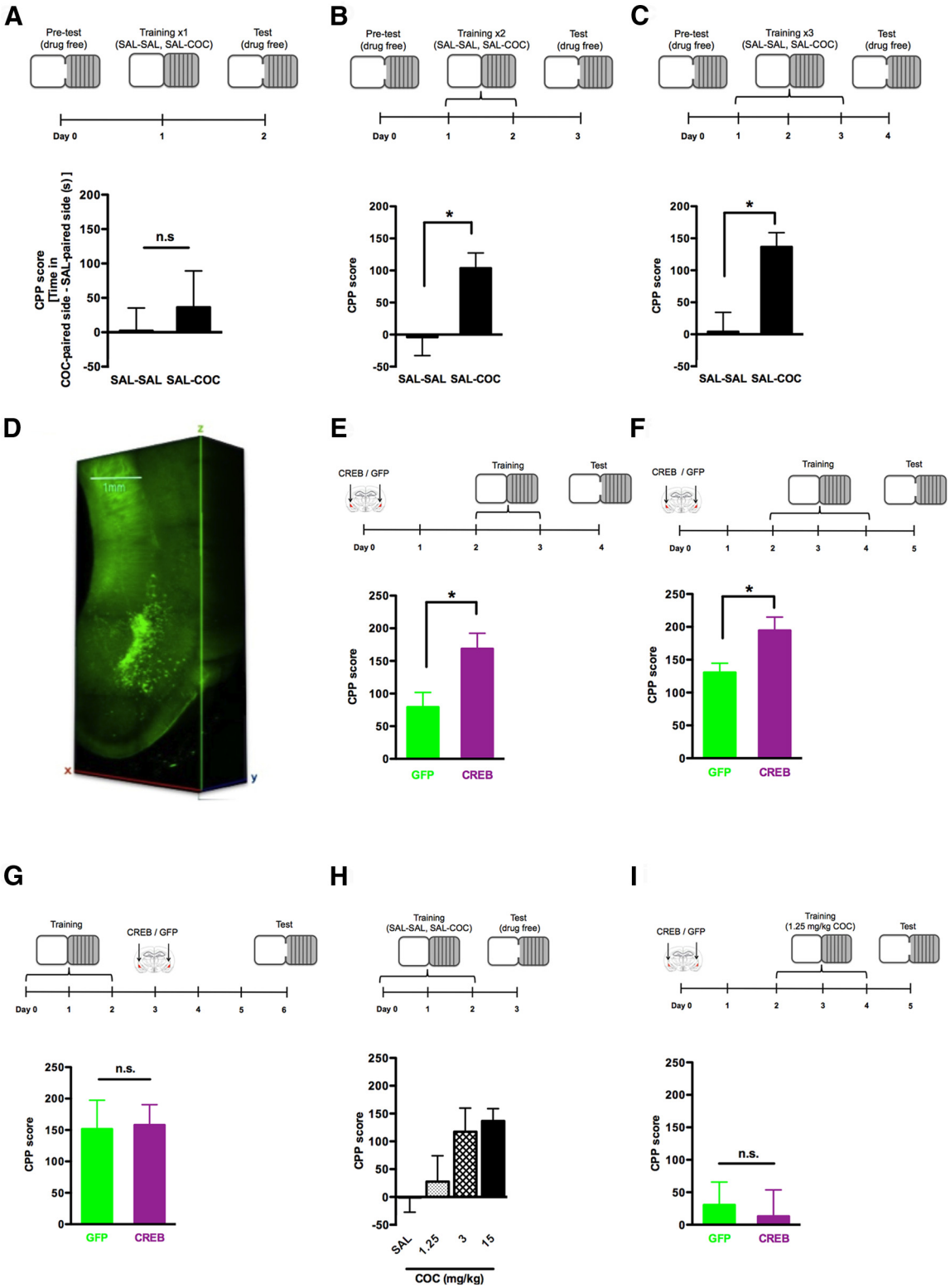

Figure 1. Increasing CREB function in a small population of LA neurons during training enhances cocaine-cue memory formation. ***A–C***, Repeated pairing of cocaine (COC), but not saline (SAL), with initially motivationally neutral cues (chamber) induces a CPP [CPP score = Time (seconds) in COC-paired chamber − time in SAL-paired chamber during the drug-free test]. One pairing: SAL-SAL ($n = 8$), SAL-COC ($n = 8$); two pairings: SAL-SAL ($n = 6$), SAL-COC ($n = 7$); three pairings: SAL-SAL ($n = 8$), SAL-COC ($n = 9$). ***D***, Using CLARITY to clear brain chunk and examine the pattern and extent of viral infection in three dimensions. Microinjecting CREB vector infects a small, random population of neurons that are largely restricted to the LA. 3D rendering of 900-μm-thick coronal brain section containing the LA 5 d postmicroinjection of CREB vector showing distribution of native GFP expression. Scale bar, 50 μm. ***E***, ***F***, Increasing CREB in ~10% of LA principal neurons before training enhances CPP memory formation. Two pairings: CREB vector ($n = 8$), GFP ($n = 8$); three pairings: CREB ($n = 8$), GFP ($n = 12$). ***G***, Microinjecting CREB vector after CPP training has no effect on CPP memory expression. CREB vector ($n = 7$), GFP ($n = 7$). ***H***, Dose–response curve for COC-induced CPP. SAL ($n = 8$), COC 1.25 mg/kg ($n = 8$), 3 mg/kg ($n = 11$), 15 mg/kg ($n = 9$). ***I***, Increasing CREB in LA before training does not induce CPP in mice trained with a subthreshold dose of COC (1.25 mg/kg), suggesting that increasing CREB in LA does not enhance CPP by increasing the unconditioned rewarding properties of COC. CREB vector ($n = 8$), GFP ($n = 8$). In this and all other figures, data presented are mean ± SEM. n.s. denotes not significantly different, * denotes $p < 0.05$. Data presented are mean ± SEM.

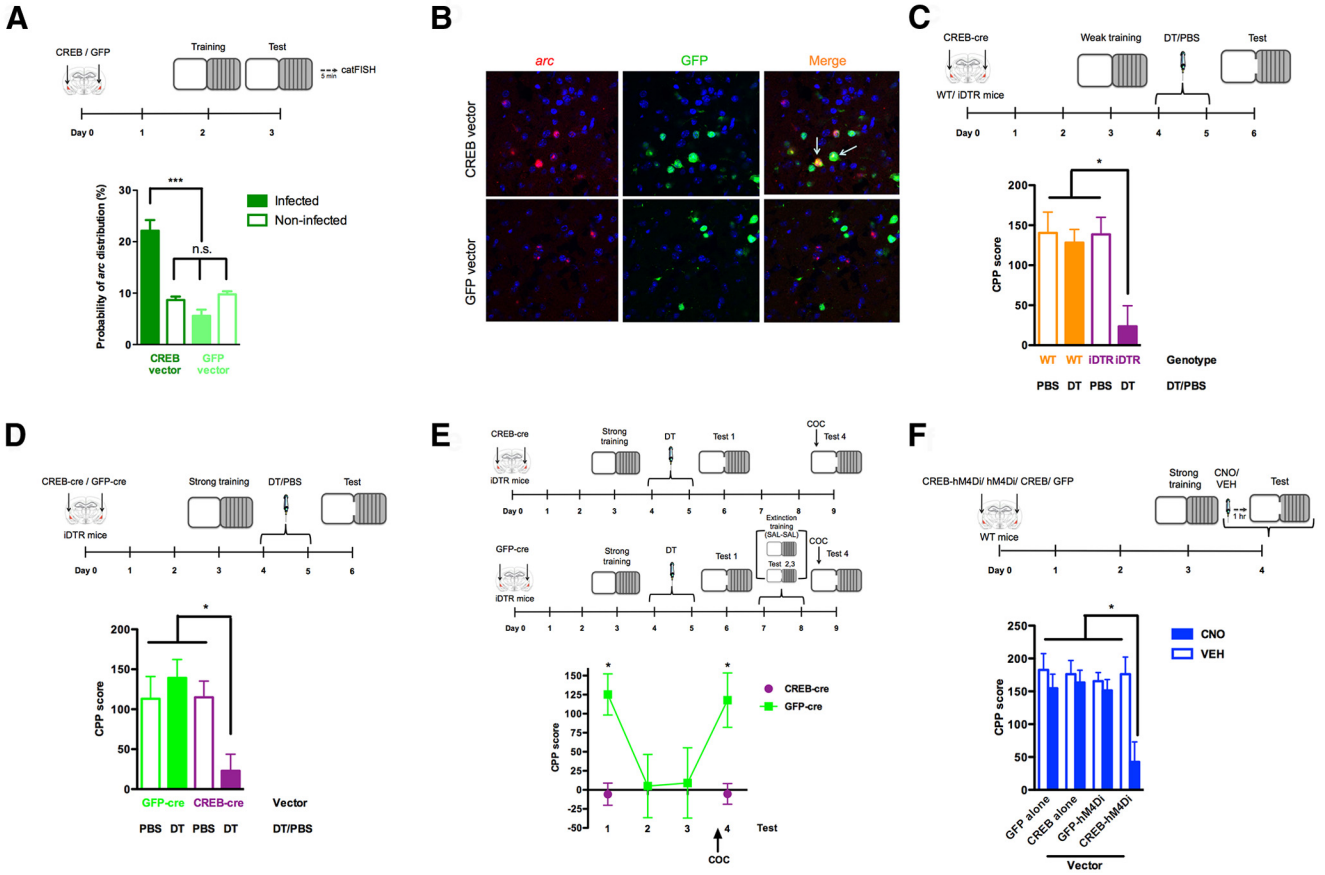

Figure 2. LA neurons with increased CREB function during CPP training become a critical component of a cocaine-cue memory engram. ***A***, ***B***, Neurons overexpressing CREB (but not those expressing GFP only) during training are more likely to be allocated to the cocaine-cue memory trace (arc^+) than their noninfected neighbors. Mice were microinjected with CREB or GFP vector 2 d before training and catFISH for nuclear localized *arc* conducted 5 min after drug-free re-exposure to the chamber previously paired with cocaine. Active neurons (part of memory trace) = neurons with nuclear localized *arc* RNA. ***B***, Example images from results quantified in ***A***. In mice microinjected with CREB vector, *arc* RNA is preferentially localized in GFP^+ (infected) neurons (rather than neighboring uninfected GFP^- neurons), whereas in mice microinjected with GFP vector, *arc* is observed equally in infected and uninfected neurons. CREB vector $n = 31$ slices (5 mice), GFP $n = 21$ slices (3 mice). ***C***, ***D***, Post-training ablation of neurons overexpressing CREB (but not a similar number of random neurons expressing GFP alone) disrupts subsequent expression of cocaine-cue memory. ***C***, iDTR transgenic or WT littermate mice microinjected with CREB-cre vector before training and administered DT (or PBS) after training to ablate CREB-overexpressing neurons. WT mice-PBS ($n = 12$), WT mice-DT ($n = 13$), iDTR mice-PBS ($n = 11$), and iDTR mice-DT ($n = 12$). ***D***, iDTR mice microinjected with CREB-cre or GFP-cre vector before training and administered DT (or PBS) after training. No effect of ablating neurons expressing GFP alone, whereas ablating neurons overexpressing CREB disrupts cocaine-cue memory expression. GFP vector-PBS ($n = 9$), GFP vector-DT ($n = 10$), CREB vector-PBS ($n = 12$), and CREB vector-DT ($n = 11$). ***E***, In contrast to behavioral extinction, COC administration before a memory test fails to reinstate a cocaine-cue memory after ablation of neurons overexpressing CREB. iDTR mice were microinjected with CREB-cre or GFP-cre vector before training and administered DT after training (as above). Ablation of CREB-overexpressing (but not GFP-expressing) neurons disrupted expression of cocaine-cue memory (Test 1, as above). Mice with GFP-cre vector were given extinction training (both chambers paired with SAL) until these mice no longer preferred the chamber originally paired with COC (extinction group). During this time, CREB-cre mice (neuronal ablation group) remained drug-free in homecage. Immediately before a final memory test (Test 4), all mice were administered COC. Cocaine-cue memory was reinstated in the extinction group only (not in the neuron ablation group), suggesting that ablating CREB-overexpressing neurons effectively degrades the cocaine-cue memory trace. GFP-cre + DT ($n = 13$), CREB-cre + DT ($n = 11$). ***F***, Similar to permanent ablation, temporarily silencing neurons overexpressing CREB (using hM4Di DREADD + CNO) before a memory test disrupted cocaine-cue memory expression. GFP vector: CNO ($n = 9$), VEH ($n = 7$); CREB vector: CNO ($n = 8$), VEH ($n = 9$); GFP-hM4Di vector: CNO ($n = 9$), VEH ($n = 8$); CREB-hM4Di vector: CNO ($n = 8$), VEH ($n = 7$). Data presented are mean ± SEM. n.s. denotes note significantly different, * denotes $p < 0.05$, *** denotes $p < 0.001$.

*Figure 2*D. One day after habituation, iDTR transgenic mice were microinjected with CREB-cre or GFP-cre vector and allowed to recover for 2 d. Mice were trained for 1 d [SAL in morning, COC (30 mg/kg) in the afternoon, such that mice with GFP-cre vector would show robust CPP]. One day after training, DT or PBS was administered once per day for 2 d.

*Figure 2*E. One day after habituation, iDTR transgenic mice were microinjected with CREB-cre or GFP-cre vector and allowed to recover for 2 d. Mice were trained for 1 d [SAL in morning and COC (30 mg/kg) in afternoon, as above]. One day after conditioning, DT or PBS was administered (once per day for 2 d). A drug-free test (Test 1) was conducted 24 h later. For the extinction group (iDTR mice with GFP-cre vector), mice received extinction training of SAL-SAL training sessions for 2 d (in which each chamber was paired with SAL administration). Extinction tests (without prior drug injection, Test 2, Test 3) took place at the end of each extinction-training day. During this time, iDTR mice with CREB-cre vector (neuron ablation group) were maintained drug-free in their homecage. Before the final (reinstatement) test (Test 4) all mice received COC (15 mg/kg) immediately before being tested.

*Figure 2*F. One day after habituation, WT hybrid mice were microinjected with CREB, GFP, CREB-hM4Di, or GFP-hM4Di vector. Mice were allowed to recover for 2 d. On day 3, mice were given a single training day [SAL in morning, COC (30 mg/kg) in afternoon]. One day later, mice were tested 1 h following CNO or VEH administration.

*Figure 3*A. One day after habituation, WT hybrid mice were microinjected with CREB-hM4Di vector and allowed to recover for 2 d. One day later, mice were trained [for 1 d, SAL in morning, COC (10 mg/kg) in afternoon]. Immediately after this, CNO or VEH was administered. A drug-free test (no COC or CNO, Test) was conducted 24 h later.

*Figure 3*B. One day after habituation, WT hybrid mice were microinjected with CREB, GFP, CREB-hM4Di or GFP-hM4Di vector. Mice recovered for 2 d. On day 3, mice were given a single training day [SAL in

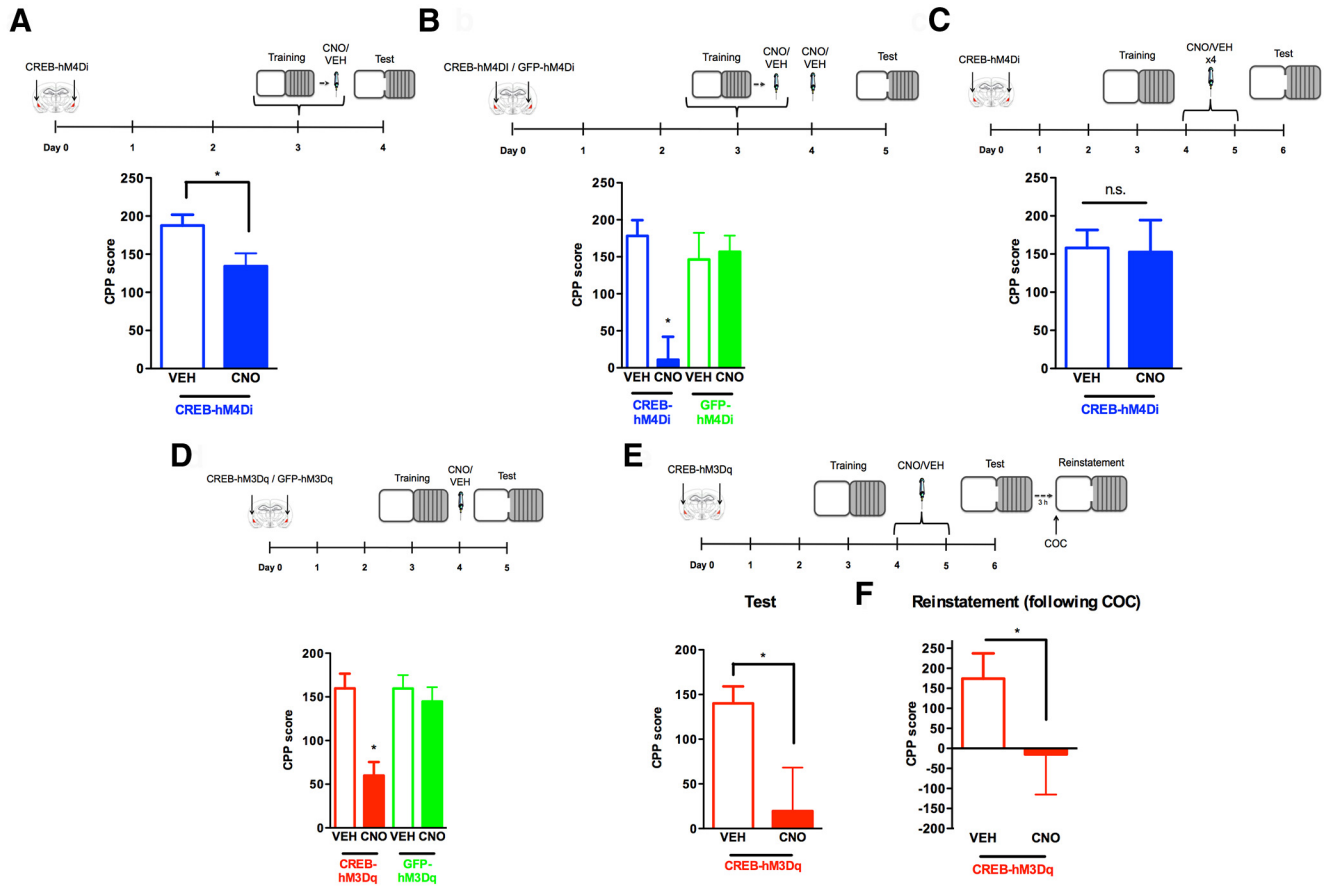

Figure 3. Consolidation of cocaine-cue memory is disrupted by interfering with normal coordinated postencoding activity of LA neurons overexpressing CREB. ***A–C***, Post-training silencing of neurons overexpressing CREB disrupts subsequent cocaine-cue memory expression in a time-dependent manner. ***A***, In mice microinjected with CREB-hM4Di vector, CNO administered immediately after training (to silence CREB-overexpressing neurons) produced a small disruption in subsequent cocaine-cue memory expression (CNO, $n = 7$; VEH, $n = 7$). ***B***, Administering CNO both immediately and 18 h after training in mice with CREB-hM4Di vector robustly impaired subsequent cocaine-cue memory expression. Silencing a similar number of random neurons (GFP-hM4Di vector) had no effect on memory consolidation. CREB-hM4Di vector: CNO ($n = 11$), VEH ($n = 11$); GFP-hM4Di vector: CNO ($n = 8$), VEH ($n = 8$). ***C***, In contrast, memory was intact in mice microinjected with CREB-hM4Di vector if CNO treatment began 18 h after training. CNO ($n = 6$), VEH ($n = 7$). ***D***, ***E***, Nondiscriminately artificially enhancing activity of neurons (using hM3Dq DREADD+CNO) overexpressing CREB (but not a similar number of random neurons expressing GFP) after training disrupts subsequent cocaine-cue memory expression. ***E***, Mice microinjected CREB-hM3Dq vector (GFP-hM3dq used as a control vector). CNO administered 24 h after training disrupts cocaine-cue memory in mice microinjected with CREB-hM3Dq vector only. CREB-hM3Dq vector: CNO ($n = 7$), VEH ($n = 9$); GFP-hM3Dq vector: CNO ($n = 9$), VEH ($n = 9$). ***F***, Memory disruption is pronounced in mice microinjected with CREB-hM3Dq vector administered additional CNO injections (2 injections/d/2 d beginning 18 h after training). Cocaine-cue memory is not reinstated by subsequent administration of COC before a final memory test, suggesting memory trace is degraded. CNO ($n = 6$), VEH ($n = 6$). In this and all other figures, data presented are mean ± SEM. n.s. denotes not significantly different, * denotes $p < 0.05$.

morning, COC (10 mg/kg) in afternoon for mice that were microinjected with CREB or CREB-hM4Di vector and COC (30 mg/kg) for mice with GFP or GFP-hM4Di vector. Different doses of COC were used to equalize the resulting CPPs in VEH-treated mice]. Immediately and 18 h after the last training session, CNO or VEH was administered. A drug-free test (Test) was conducted 24 h later.

*Figure 3*C. One day after habituation, WT hybrid mice were microinjected with CREB-hM4Di vector. Mice recovered for 2 d. On day 3, mice were given a single training day [SAL in morning, COC (10 mg/kg) in afternoon]. Eighteen hours after the last training session, CNO or VEH was administered twice daily for 2 d, with a minimal of 8 h between injections. Drug-free test (Test) was conducted 24 h later.

*Figure 3*D. One day after habituation, WT hybrid mice were microinjected with CREB, GFP, CREB-hM3Dq, or GFP-hM3Dq vector. Mice recovered for 2 d. On day 3, mice were given a single training day [SAL in morning, COC (10 mg/kg) in afternoon for mice microinjected with CREB or CREB-hM4Di vector, whereas a higher dose of COC (30 mg/kg) was administered to mice microinjected with GFP or GFP-hM4Di vector]. Different doses of COC were used to equate the resulting CPPs in mice microinjected with GFP or CREB vector. Eighteen hours after the last training session, CNO or VEH was administered. A drug-free test was conducted 24 h later.

*Figure 3*E,F. One day after habituation, WT hybrid mice were microinjected with CREB-hM3Dq vector. Mice recovered for 2 d. On day 3, mice were given a single training day [SAL in morning, COC (10 mg/kg) in afternoon]. Eighteen hours after last training session, CNO or VEH was administered (2 times per day for 2 d). A drug-free test was conducted 24 h after last injection of CNO or VEH. Figure 3*F*, 3 h after the test session, a reinstatement test was conducted immediately following administration of COC (5 mg/kg).

Results

Assessing cocaine-cue memory in mice using CPP

To examine the neural mechanisms mediating the formation of a cocaine-cue memory in mice, we used CPP. Mice were initially exposed to two distinct, motivationally neutral experimental chambers separated by a removable guillotine door. One day after this habituation session, the guillotine door was closed and one chamber was paired with cocaine administration and the second chamber was paired with saline administration (using an unbiased, counterbalanced design; Josselyn and Beninger, 1993; Prus et al., 2009). Cocaine-cue memory was assessed during a test in which drug-free mice were allowed access to both chambers (with

guillotine door removed) and the amount of time mice spent in both chambers compared. Mice that learned and remembered the cocaine-cue association subsequently spent more time in the chamber previously paired with cocaine administration, thereby exhibiting a CPP (Carlezon et al., 1998; Miller and Marshall, 2005; Airan et al., 2009; Heldt et al., 2014).

We first verified that cocaine reliably induced CPP in adult WT mice in a hybrid genetic background (see Materials and Methods). Mice were trained for 1, 2, or 3 d. On each training day, saline was paired with one chamber (A.M.) and cocaine was paired with the second chamber (P.M.; Fig. 1*A–C*). Control groups received saline administration before placement in both chambers (A.M. and P.M.). To assess cocaine-cue memory, we calculated a CPP score in which we subtracted the amount of time (in seconds) mice spent in the chamber previously paired with saline from time spent in the chamber previously paired with cocaine. Control groups, that received 1, 2, or 3 training days during which saline was paired with both chambers, spent equal time in each chamber during the test (showing no CPP), indicating that mice had no innate preference for either chamber. We observed that two cocaine-cue pairings induced statistically reliable CPP and three cocaine-cue pairings induced robust CPP [Figure 1*A*, 1 pairing; one-way ANOVA (saline, cocaine), $F_{(1,14)} = 0.43$, $p > 0.05$; Figure 1*B*, 2 pairings; $F_{(1,11)} = 7.93$, $p < 0.05$; Figure 1*C*, 3 pairings; $F_{(1,15)} = 13.93$, $p < 0.05$]. The current protocol, therefore, induced a graded CPP, the magnitude of which was related to training intensity.

Increasing CREB function in a random, small population of LA pyramidal/principal neurons enhanced the formation of a cocaine-cue memory

In addition to being implicated in reward-related learning [in which an initially neutral cue is paired with an appetitive unconditioned stimulus, such as food or drugs of abuse; (Hiroi and White, 1991; Everitt et al., 2000; Baxter and Murray, 2002; Tye et al., 2008; Morrison and Salzman, 2010; Heldt et al., 2014), the LA has also been shown to be critical for fear/threat-related learning (LeDoux, 2014), in which an initially neutral cue (typically a tone) is paired with an aversive unconditioned stimulus (typically a shock); Davis, 1992; Fanselow and Gale, 2003; Maren, 2003; Duvarci and Pare, 2014]. Previous results show that the fear/threat memory is sparsely encoded within a small number of neurons in the LA. Specifically, electrophysiological data indicate that although >70% of pyramidal/principal neurons in the rodent LA respond to both tone and shock presentation (suggesting these neurons are "correctly wired" and presumably eligible to become part of the fear/threat memory engram), only a small portion (~10–30%) of these eligible neurons seem to become part of any one fear/threat memory engram (Repa et al., 2001; Rumpel et al., 2005; Reijmers et al., 2007). Similarly, fMRI data suggest that in the human amygdala, fear/threat memories are similarly encoded in a sparse trace (Bach et al., 2011). We (Han et al., 2007, 2009) and others (Zhou et al., 2009; Kim et al., 2014) found that LA neurons compete against one another for recruitment (or allocation) to a fear/threat engram. Furthermore, we found it was possible to bias the outcome of this competition by manipulating CREB function in individual LA neurons. Neurons with relatively higher CREB function were more likely to be included, whereas neurons with relatively lower CREB function were more likely to be excluded, from the fear/threat memory engram. Moreover, increasing CREB in a small population of LA neurons before training was sufficient to enhance fear/threat memory and these neurons were critical for subsequent expression of that memory, suggesting that they became necessary members of the engram (Josselyn et al., 2001; Han et al., 2007, 2009; Zhou et al., 2009; Rexach et al., 2012; Cowansage et al., 2013). Therefore, the formation of a fear/threat memory involves neuronal competition in the LA, the outcome of which is sensitive to relative neuronal CREB function.

We investigated whether similar neural mechanisms underlie the formation of a cocaine-cue memory engram in the LA. To this end, we used replication-defective HSV vectors to increase CREB levels in a small but random population of LA neurons. To subsequently identify infected neurons, we used a virus that also expressed GFP. A virus encoding GFP alone served as a control. Microinjection of HSV overwhelmingly (~99%) infects excitatory, pyramidal/principal neurons in the LA (Cole et al., 2012; Yiu et al., 2014). By titrating the concentration and volume of HSV microinjected into the LA, we infected a small number of seemingly random LA neurons [~10% of LA pyramidal/principal neurons (9.84 ± 0.89%, $n = 6$), as determined by stereological counting].

To better visualize the pattern and extent of neurons infected by intra-LA microinjection of HSV, we took advantage of the recently developed CLARITY (lipid-exchanged, anatomically rigid, imaging/immunostaining compatible, tissue hydrogel) tissue processing technique which permits examination of thick volumes of fixed tissue in three dimensions without the need for sectioning or reconstruction (Chung and Deisseroth, 2013; Chung et al., 2013). Microscopic imaging of brain tissue is normally severely constrained by light scattering caused, in large part, by the fatty lipids that make up the cellular membranes. This results in reduced resolution of images collected from thick brain slices. By replacing the lipids in brain tissue with a clear hydrogel, CLARITY converts an optically opaque brain into a transparent structure. This allows for high cellular resolution images to be collected from relatively thick sections without the need of sectioning. The hydrogel preserves the fine structure of the brain including the localization of proteins and does not reduce the stability of fluorescent signals, such as GFP. We used CLARITY to clear the brains of mice microinjected with our viral vectors and imaged endogenous HSV-GFP signal in 1-mm-thick coronal brain sections. Using this technique, we verified that our vectors infected a small, seemingly random population of neurons in the LA and that the majority of infection was limited to the LA (Fig. 1*D*).

Transgene expression using this type of HSV peaks 2–3 d after microinjection (Josselyn et al., 2001; Barrot et al., 2002; Vetere et al., 2011; Cole et al., 2012). To examine the effects of increasing CREB in a small population of LA neurons during training on formation of a cocaine memory, we microinjected CREB or control vector 2 d before training mice with 2 or 3 cocaine-cue pairings. One day after the final training session, drug-free mice were tested. Increasing CREB in a small, random subpopulation of LA neurons enhanced the CPP produced by either two or three cocaine-cue pairings (Figure 1*E*; $F_{(1,14)} = 7.42$, $p < 0.05$; Figure 1*F*; $F_{(1,18)} = 5.71$, $p < 0.05$). Therefore, similar to a fear/threat memory, enhancing CREB function increased memory for a cocaine-cue association.

To more thoroughly investigate this effect, we conducted a series of control experiments. First, we examined the effects of increasing CREB on the expression of a previously formed cocaine memory, by microinjecting CREB or control vector into the LA 1 d after training. Expression of a previously acquired cocaine-cue memory was not affected by increasing CREB (Figure 1*G*; $F_{(1,12)} = 0.002$, $p > 0.05$), indicating that CREB enhances encoding (rather than expression) of a cocaine-cue memory. Pre-

vious results indicate that similar manipulations of CREB function in the nucleus accumbens alter the (unconditioned) motivational/rewarding properties of cocaine itself (Carlezon et al., 1998). We investigated whether the enhancement of CPP we observed in the present experiments were due to our LA manipulation similarly increasing the unconditioned rewarding properties of cocaine. To test this, we microinjected CREB vector into the LA 2 d before training mice with a subthreshold dose of cocaine (1.25 mg/kg; Carlezon et al., 1998), that is normally not sufficient to support CPP (Figure 1*H*; $F_{(3,32)} = 3.63$, $p < 0.05$; Newman–Keuls *post hoc* tests showed CPP was produced by 3 mg/kg or 15 mg/kg, but not 1.25 mg/kg, of cocaine). If increasing CREB in the LA increased the unconditioned rewarding properties of cocaine, we would predict that this low, subthreshold, dose of cocaine might become sufficient to support CPP. However, we observed no evidence of CPP in mice microinjected with CREB vector in the LA before training with this low dose of cocaine (Figure 1*I*; $F_{(1,14)} = 0.61$, $p > 0.05$). This finding is consistent with the interpretation that increasing CREB in a small population of LA principal neurons does not enhance the rewarding properties of cocaine, but instead enhances memory of the cocaine-cue association.

Neurons overexpressing CREB during training are selectively allocated to the cocaine-cue memory engram

LA neurons are thought to compete against one another for recruitment/allocation (Zhou et al., 2009) to a fear/threat memory engram and neurons with relatively enhanced CREB function are competitively advantaged in this competition (Han et al., 2007, 2009; Zhou et al., 2009). To examine whether CREB-mediated neuronal competition underlies formation of a cocaine-cue memory, we asked whether LA neurons with high levels of CREB are over-represented in the cocaine-cue memory trace.

To visualize neurons that are part of the cocaine-cue memory trace, we took advantage of the unique transcriptional time course of the activity-dependent gene *arc* (Guzowski et al., 1999, 2001). Under basal conditions, neurons typically have low levels of *arc* RNA. Within 5 min of neuronal activity, though, there is a brief rapid burst of *arc* transcription. Within 40 min of this neuronal activity, *arc* RNA is transported from the nucleus to the dendrites. This stereotyped trafficking pattern allows the presence and localization of *arc* RNA to serve as an activity timestamp for individual neurons, with *arc* localized to the nucleus being as a molecular marker of a recently active neuron. Therefore, to visualize neurons that may be part of the cocaine-cue memory engram, we used nuclear *arc* to identify neurons specifically activated by cocaine-associated cues (Guzowski et al., 2001). Mice were microinjected with CREB or GFP vector and trained as above. On the test day, drug-free mice were confined to the chamber previously paired with cocaine. Five minutes later, brains were removed and the overlap of active (*arc* localized to the nucleus, arc^+) and infected (GFP^+) neurons assessed. The overall number of active (arc^+) LA neurons following the test was similar in mice microinjected with CREB and GFP vector, indicating that increasing CREB function did not increase the overall number of neurons expressing *arc* or size of the cocaine-cue memory engram (CREB vector: 8.53 ± 0.63% of LA principal neurons; GFP vector: 8.76 ± 0.52%; $F_{(1,50)} = 0.07$, $p > 0.05$). In contrast, the distribution of arc^+ in infected versus noninfected neurons differed markedly between vector groups. In mice microinjected with CREB vector, infected neurons with high levels of CREB were ~3 times more likely to be arc^+ (part of the memory trace) than their noninfected neighbors. In mice microinjected with GFP vector, infected and noninfected neurons were equally likely to be arc^+ [Figure 2*A*,*B*; significant vector (GFP, CREB) × neuronal infection status (infected, noninfected) interaction; $F_{(1,50)} = 40.53$, $p < 0.001$, *post hoc* Newman–Keuls tests]. Therefore, similar to a conditioned fear/threat memory (Han et al., 2007, 2009; Zhou et al., 2009), LA neurons with increased CREB are preferentially allocated to what is likely a larger cocaine-cue memory engram.

Neurons that overexpress CREB during training are essential for subsequent expression of a cocaine-cue memory

To assess whether these neurons overexpressing CREB at the time of training are necessary for subsequent expression of the resulting cocaine-cue memory, we selectively ablated just this population of neurons after training using a transgenic mouse which takes advantage of DT-induced ablation. We used iDTR mice, in which a functional DTR is expressed only in cells that have undergone Cre recombinase-induced recombination. Subsequent systemic administration of DT induces apoptosis that is limited to those cells which have undergone recombination and express the functional DTR (Buch et al., 2005). To ablate neurons overexpressing CREB after training, we microinjected iDTR transgenic mice with a vector expressing both CREB and Cre recombinase (CREB-cre vector). In these mice, neurons infected by the CREB-cre vector have increased CREB levels and express the DTR, thereby tagging these neurons for subsequent ablation by systemic DT (Han et al., 2009). We microinjected iDTR mice with CREB-cre vector and, before a memory test, systemically administered DT to delete only those neurons overexpressing CREB. If these neurons with high levels of CREB during training were necessary components of what is likely a larger engram of the cocaine-cue association, we would expect that their ablation after training would impair subsequent memory expression. Three control groups were similarly treated, except that neurons overexpressing CREB were not deleted: (1)WT littermate control mice microinjected with CREB-cre vector and administered DT, or (2) PBS or (3) iDTR transgenic mice microinjected with CREB-cre vector but systemically administered PBS rather than DT. During the test, we observed robust CPP in these three control groups. In contrast, ablating neurons overexpressing CREB impaired subsequent cocaine-cue memory expression (iDTR mice microinjected with CREB-cre vector and administered DT after training)[Figure 2*C*; significant *iDTR Genotype* (WT, HET) × Drug (DT, PBS) interaction, $F_{(1,44)} = 5.15$, $p < 0.05$; Newman–Keuls *post hoc* test showed that iDTR transgenic mice+CREB-cre vector + DT group had significantly lower CPP than all control groups, which did not differ from each other].

The disruption of cocaine memory we observed in this experiment, though, could be due to a small lesion of the LA (in that neurons with CREB-cre vector were ablated). To examine this, we conducted a similar experiment, but microinjected iDTR mice with GFP-cre vector (rather than CREB-cre vector) to determine the effects of ablating a small, nonspecific population of LA neurons. To equate the initial strength of the cocaine-cue memory between CREB-cre and GFP-cre vector groups, we trained mice with a higher dose of cocaine (30 mg/kg in single conditioning trial). Indeed, we observed similar CPP scores in iDTR mice microinjected with either CREB-cre or GFP-cre vector, which were administered PBS (rather than DT) before the test. However, in contrast to the effects of ablating neurons overexpressing CREB, we found that ablating a similar number of random neurons expressing GFP failed to impact subsequent expression of the cocaine-cue memory [Figure 2*D*; significant

vector (CREB-cre, GFP-cre) × drug (DT, PBS) interaction; $F_{(1,38)} = 6.63$, $p < 0.05$; Newman–Keuls *post hoc* test showed iDTR mice microinjected with CREB-cre vector, but not GFP-cre vector, administered DT after training had significantly lower CPP than the other groups]. Therefore, ablating neurons overexpressing CREB after training impaired subsequent expression of the cocaine-cue memory, consistent with the interpretation that these specific neurons are necessary for memory expression.

The expression of a cocaine-cue memory may also be decreased by extinction training, during which the conditioned cue is repeatedly presented in the absence of cocaine (Mueller and Stewart, 2000; Shaham et al., 2003; Ciccocioppo et al., 2004; Myers and Carlezon, 2010). Extinction training does not "erase" the original cocaine memory as subsequent cocaine exposure is able to reinstate the cocaine-cue memory (Mueller and Stewart, 2000; Parker and Mcdonald, 2000; Shaham et al., 2003). Therefore, we next asked whether the loss of the cocaine-cue memory we observed after deletion of neurons overexpressing CREB could be similarly reinstated by cocaine exposure. We compared the effects of cocaine re-exposure before a memory test in two groups of mice: (1) extinction group, iDTR mice microinjected with GFP-cre vector and administered DT ("control mice") that received extinction training; and (2) neuron-deletion group, iDTR mice microinjected with CREB-cre vector and administered DT that had neurons overexpressing CREB deleted after training but did not receive extinction training. To extinguish the cocaine-cue memory in the extinction group, we paired both experimental chambers with saline administration (Mueller and Stewart, 2000; Shaham et al., 2003) until mice no longer preferred the chamber initially paired with cocaine [Figure 2*E*; repeated-measures ANOVA for GFP-cre vector (extinction) group comparing Test 1 to Test 3, $F_{(2,38)} = 5.67$, $p < 0.05$, Newman–Keuls *post hoc* test showed mice had significant lower CPP scores during Test 3 than during Test 1]. During this time, mice in the neuron-deletion group remained drug-free in the homecage (and did not receive extinction training). Importantly, both extinction and neuron-deletion groups showed similarly low CPP scores during the last test before cocaine re-exposure [CREB-cre vector (neuron-deletion group) Test 1 versus GFP-cre vector (extinction group) Test 3; $F_{(1,22)} = 0.08$, $p > 0.05$]. Immediately before the final test (Test 4) both the extinction and neuronal-deletion groups received systemic injection of cocaine. As expected based on previous findings, cocaine exposure was sufficient to reinstate the cocaine-cue memory in the extinction group. Strikingly, cocaine exposure did not reinstate cocaine-cue memory in the neuron-deletion group [Figure 2*E*; GFP-cre vector Test 3 vs Test 4: $F_{(1,24)} = 5.00$, $p < 0.05$; Test 4, CREB-cre vs GFP-cre vector: $F_{(1,22)} = 7.48$, $p < 0.05$; CREB-cre vector, Test 1 vs Test 4: $F_{(1,20)} = 0.01$, $p > 0.05$]. Mice in the neuron-deletion group behaved as if they had no prior experience with cocaine. Therefore, in contrast to extinction training (which temporarily suppressed the cocaine-cue memory), post-training ablation of LA neurons overexpressing CREB may permanently degrade the cocaine-cue memory engram.

Consolidation of a cocaine-cue memory is disrupted by post-training silencing of neurons that overexpressed CREB during training

Successful memory consolidation is thought to depend on postencoding reactivation of the activity patterns that were present during the initial learning experience (Marr, 1971; Buzsáki, 1989; Wilson and McNaughton, 1994; McClelland et al., 1995; Girardeau et al., 2009; Dupret et al., 2010; Ego-Stengel and Wilson, 2010; Carr et al., 2011; Dudai, 2012). *In vivo* recording studies show that the frequency of reactivation is highest in the minutes following (Kudrimoti et al., 1999; Tatsuno et al., 2006) but may persist for 18–24 h (Kudrimoti et al., 1999; Karlsson and Frank, 2009) after an experience. Although largely studied in hippocampus and cortex, postencoding reactivation has also been described in other brain regions (Pennartz et al., 2004) and may represent a ubiquitous phenomenon. For instance, Johnson et al. (2009) observed that stimulation of the LA produced polysynaptic field potentials, consistent with the idea that reverberatory activity or reactivation may also occur in LA microcircuits.

We examined whether postencoding reactivation specifically in neurons overexpressing CREB is important for the consolidation of a cocaine-cue memory. To disrupt activity specifically in these neurons, we took advantage of genetically encoded mediators of neural excitability. Although optogenetic approaches are remarkably valuable for controlling neural activity over short time periods (Airan et al., 2009; Gu et al., 2012; Tye and Deisseroth, 2012; Yiu et al., 2014), chemical genetic approaches using DREADDs (designer receptors exclusively activated by designer drug; Armbruster et al., 2007; Nichols and Roth, 2009), which are useful for manipulating neuronal function for more prolonged periods. DREADDS are modified G-protein-coupled receptors which are insensitive to endogenous ligands but activated by a synthetic ligand (CNO, an otherwise pharmacologically inert agent; Armbruster et al., 2007; Nichols and Roth, 2009). Following CNO binding, the Gi/o-coupled hM4Di DREADD activates inwardly rectifying potassium 3 (Kir3) channels to hyperpolarize and transiently silence neurons (Armbruster et al., 2007; Nichols and Roth, 2009). Before we used this strategy to manipulate potential reverberatory activity, we first showed the effects of transiently silencing neurons overexpressing CREB immediately before a memory test.

We microinjected WT hybrid mice with a vector expressing GFP alone, CREB alone, GFP-hM4Di (to transiently silence a random population of neurons) or CREB-hM4Di (to transiently silence neurons overexpressing CREB). Mice were trained as above and systemically administered CNO (1 mg/kg, i.p. or VEH) 1 h before the memory test. Although all three control groups showed robust CPP, mice microinjected with CREB-hM4Di vector and administered CNO before the test (mice in which the neurons overexpressing CREB were silenced just before the test) showed no evidence of CPP [Figure 2*F*; significant vector (GFP, CREB, GFP-hM4Di, CREB-hM4Di) × drug (CNO, VEH) interaction: $F_{(4,54)} = 2.80$, $p < 0.05$, *post hoc* Newman Keuls tests showed only mice microinjected with CREB-hM4Di vector that received CNO showed significantly lower CPP than other groups]. Therefore, temporarily silencing neurons overexpressing CREB (but not a similar number of random LA neurons) disrupted expression of the cocaine-cue memory. These results are consistent with our above findings that post-training ablation of CREB-overexpressing neurons disrupted subsequent memory expression and confirms the effectiveness of this DREADD-mediated silencing approach (Richards et al., 2014).

Having established the utility of this DREADD approach, we next asked whether silencing CREB-overexpressing neurons during the initial post-training period disrupted consolidation of the cocaine-cue memory. We microinjected WT mice with CREB-hM4Di or GFP-hM4Di vector and administered a single injection of CNO (or VEH) immediately after training. Silencing CREB-overexpressing neurons immediately after training (WT mice microinjected with CREB-hM4Di vector systemically administered CNO) produced a mild deficit in subsequent cocaine-cue

memory expression (Figure 3*A*; $F_{(1,12)} = 5.69$, $p < 0.05$). However, cocaine-cue memory expression was completely blocked if we administered CNO both immediately after training and again 18 h later (at which time mice were in the homecage; Figure 3*B*; significant Vector (GFP-hM4Di, CREB-hM4Di) × drug (CNO, VEH) interaction: $F_{(1,34)} = 6.17$, $p < 0.05$, *post hoc* Newman–Keuls tests showed only mice microinjected with CREB-hM4Di vector that received CNO showed significantly lower CPP than other groups]. Interestingly, we observed no memory deficit if CNO administration (2 injections/d for 2 d) began 18 h after training was complete (Figure 3*C*; $F_{(1,11)} = 0.01$, $p > 0.05$). Therefore, disrupting activity in CREB-overexpressing neurons starting immediately after training impaired memory consolidation, an effect that was amplified by repeating disruption 18 h later. However, consolidation was not impaired if the silencing started 18 h after training. These findings help identify the critical temporal windows of consolidation during which ongoing neuronal reactivity may be important for memory consolidation and are consistent with previous estimates of this consolidation time-window. For instance, Chawla et al. (2005) observed high Arc protein in hippocampal CA1 and CA3 regions in the hours following behavioral experience (exploration), as well as the apparent rise of a second wave of activation 8 h later, but levels returned to baseline 24 h later.

Consolidation of a cocaine-cue memory is disrupted by post-training indiscriminate artificial activation of neurons overexpressing CREB

In vivo electrophysiological data suggest that the postencoding reactivation of activity patterns present during the initial learning experience occurs with precise temporal fidelity and that the sequence of neuronal firing is important. Therefore, we next assessed the effects of disrupting the faithfulness of postencoding neuronal reactivation by nondiscriminately enhancing activity in CREB-overexpressing neurons after training. To nondiscriminately increase activity we used the DREADD hM3Dq, which is coupled to Gq receptors. Activation of hM3Dq by CNO produces Gq activation of phospholipase C and intracellular Ca^{2+} release, which depolarizes neurons and increases action potential firing (Armbruster et al., 2007; Nichols and Roth, 2009). In this experiment, we microinjected WT mice with a vector expressing CREB+hM3Dq or GFP+hM3Dq and trained as above. In this way, systemic administration of CNO would increase activity in CREB-overexpressing neurons (CREB-hM3Dq vector) or random neurons (GFP-hM3Dq vector). Interestingly, we observed that a single systemic injection of CNO 18 h after training (while mice were in the homecage) was sufficient to impair subsequent expression of the cocaine-cue memory in mice with CREB-hM3Dq vector. Memory was disrupted in mice microinjected with CREB-hM3Dq vector only; we observed normal CPP in mice microinjected with GFP-hM3Dq vector, indicating that artificially activating a random population of neurons does not impair subsequent memory expression [Figure 3*D*; significant vector (GFP-hM3Dq, CREB-hM3Dq) × drug (CNO, VEH) interaction: $F_{(1,30)} = 6.21$, $p < 0.05$, *post hoc* Newman–Keuls tests showed that mice microinjected with CREB-hM3Dq vector and administered CNO had significantly lower CPP than the other groups]. In contrast to the lack of effect produced by silencing neurons with the hM4Di DREADD beginning 18 h after training (Fig. 3*C*), artificially activating neurons overexpressing CREB and the hM3Dq DREADD with several injections of CNO beginning 18 h after training produced a pronounced memory deficit (Figure 3*E*; $F_{(1,10)} = 5.30$, $p < 0.05$). This cocaine-cue memory was not reinstated by subsequent cocaine administration (Figure 3*F*; $F_{(1,10)} = 6.71$, $p < 0.05$), suggesting that post-training artificial activation of this key component of the memory trace degrades the integrity of the entire memory trace. Artificially increasing firing in this population of LA neurons overexpressing CREB only, independent from intra- or extra-LA components of the memory trace, which are likely at this time window to be inactive, may interfere with normal memory consolidation ("neurons that do not fire together may unwire together").

Discussion

This series of experiments investigated the role of neurons in the LA which become critical for encoding and storing cocaine-cue memories in mice. Our findings indicate that LA neurons compete for allocation to a cocaine-cue memory trace and that neurons overexpressing CREB are competitively advantaged in this competition. Specifically, we used viral vectors to increase CREB levels in a random population of ~10% of LA principal neurons and showed that this small group of neurons become a critical hub of what is likely a larger neuronal cocaine-cue memory network.

Several previous results show that artificially increasing the activity of a similar small population of neurons thought to be in a memory trace impacts the formation and/or expression of that memory. For instance, Liu et al. (2012) reported that optogenetic activation of a small number of dentate gyrus (DG) neurons that were active during context fear/threat training induces freezing (that is, artificially activating these cells served as a sufficient retrieval cue for expression of a conditioned fear/threat memory). Using a similar activity tagging strategy, this group also showed that artificial activation of a small number of DG neurons that happened to be active during novel context exploration could function as a conditioned stimulus in the formation of a new (but false) conditioned fear/threat memory (Ramirez et al., 2013). By contrast, here we manipulated neurons we hypothesized are critical components of a larger memory trace by using CREB overexpression to allocate (or funnel) the memory to a small population of LA neurons before training rather than "tagging" a population of neurons that happened to be active during training.

Using this technique to manipulate neurons which become components of an engram for a cocaine-cue association, we found that post-training ablation or temporary silencing of this population of CREB-overexpressing neurons before a memory test disrupts subsequent expression of a cocaine-cue memory. In contrast to extinction training (which temporarily suppresses cocaine-conditioned memory), our findings suggest that post-training ablation of this population of neurons may functionally erase the cocaine-cue memory, as we observed no reinstatement of the cocaine-cue memory when mice were re-exposed to cocaine. Furthermore, we observed that interfering with the normal post-encoding activity of these neurons (and not a similar number of random LA neurons) disrupts memory consolidation. Either silencing CREB-overexpressing neurons in the hours after training or disrupting the ordered replay/reactivation of these neurons by nondiscriminately activating these neurons impaired cocaine-cue memory consolidation. Our results complement previous findings showing that memory is disrupted by interfering with ordered postencoding reactivation. Although other studies have targeted larger networks during precise intervals of reactivation (hippocampal sharp wave-ripple events; Girardeau et al., 2009; Ego-Stengel and Wilson, 2010; Jadhav et al., 2012), here we show that memory is disrupted by interfering with the activity in a specific population of neurons during temporally

extended consolidation windows. Our findings are consistent with the hypothesis that long-term stabilization of the engram (or cell assembly) requires coordinated reactivation in the hours immediately after an experience.

A similar CREB-mediated neuronal selection process in the LA also underlies the formation of a conditioned fear/threat memory (Han et al., 2007, 2009; Zhou et al., 2009; Kim et al., 2014). Therefore, increasing CREB in a small seemingly random population of LA pyramidal neurons enhanced memory for an association between a cue and footshock (conditioned fear/threat memory) as well for an association between a cue and cocaine (CPP). Together, these findings indicate that the LA plays a general role in assigning biological significance (be it appetitive or aversive) to previously neutral cues. Although CREB is a ubiquitous transcription factor implicated in a range of diverse processes, we recently showed that the neuronal allocation effects of increasing CREB in a small portion of LA neurons were mimicked by increasing excitability and blocked by decreasing intrinsic excitability in CREB-overexpressing neurons (Yiu et al., 2014). These findings indicate that neuronal engram allocation is based on relative neuronal excitability immediately before training. In this series of studies, we artificially manipulated intrinsic excitability and memory allocation by overexpressing CREB. However, endogenous changes in intrinsic excitability are linked to learning in a variety of species [*Hermissenda* (Alkon, 1974) to rodents (Thompson et al., 1996)], suggesting that the current manipulations (CREB overexpression) tap into an underlying fundamental memory process.

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