

Maturation of silent synapses in amygdala-accumbens projection contributes to incubation of cocaine craving

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In rat models of drug relapse and craving, cue-induced cocaine seeking progressively increases after withdrawal from the drug. This 'incubation of cocaine craving' is partially mediated by time-dependent adaptations at glutamatergic synapses in nucleus accumbens (NAc). However, the circuit-level adaptations mediating this plasticity remain elusive. We studied silent synapses, often regarded as immature synapses that express stable NMDA receptors with AMPA receptors being either absent or labile, in the projection from the basolateral amygdala to the NAc in incubation of cocaine craving. Silent synapses were detected in this projection during early withdrawal from cocaine. As the withdrawal period progressed, these silent synapses became unsilenced, a process that involved synaptic insertion of calcium-permeable AMPA receptors (CP-AMPA). *In vivo* optogenetic stimulation-induced downregulation of CP-AMPA at amygdala-to-NAc synapses, which re-silenced some of the previously silent synapses after prolonged withdrawal, decreased incubation of cocaine craving. Our findings indicate that silent synapse-based reorganization of the amygdala-to-NAc projection is critical for persistent cocaine craving and relapse after withdrawal.

Relapse to drug addiction can occur after prolonged abstinence¹ and is often precipitated by exposure to drug-associated cues that provoke drug craving². In rat models of drug relapse, cue-induced drug craving progressively increases after withdrawal from cocaine and other abused drugs^{3,4}, a phenomenon known as incubation of drug craving⁵. Incubation of cocaine craving is partially mediated by delayed time-dependent drug-induced accumulation of GluA2-lacking CP-AMPA in NAc^{6,7}, a brain region that is critical for relapse rat models^{8,9}. The molecular triggering events and the specific glutamatergic projections to NAc that are involved in this form of long-lasting cocaine-induced synaptic plasticity are unknown^{3,7}.

We and others have reported that, in the NAc, non-contingent exposure to cocaine generates silent excitatory synapses, potentially immature synapses that express stable NMDA receptors (NMDARs) with AMPARs that are either absent or highly labile^{10–12}. Silent synapses are abundant during early developmental stages and subsequently mature into fully functional synapses by recruiting AMPARs^{13,14}. The functional relevance of cocaine-induced silent synapses in animal models of drug reward and relapse is unknown. We recently hypothesized that the generation and subsequent unsilencing and maturation of silent synapses are a critical component of cocaine-induced brain adaptations that mediate cocaine reward and relapse¹⁵. Here, we studied the role of cocaine-induced silent synapses in the basolateral amygdala (BLA)-to-NAc glutamatergic projection in the incubation

of cocaine craving. We focused on this projection because of its critical role in cue-induced drug seeking^{8,16} and appetitive conditioned responses^{17,18}.

RESULTS

Recording of BLA-to-NAc shell excitatory synapses

We examined excitatory synapses in the BLA-to-NAc shell projection¹⁹. To determine the BLA subregion that projects to the NAc shell, we anesthetized a group of rats ($n = 5$) and stereotaxically injected the retrograde tracer fluorogold (0.1 ng per μ l per side) into the NAc shell, the region we intended to record from (Fig. 1a). A week later, we obtained BLA slices with fluorogold-positive neurons enriched throughout the NAc shell (Fig. 1b). We then injected a ChR2::YFP-expressing adeno-associated vector (AAV2) into the BLA of the rats ($n = 5$) and observed ChR2-expressing neurons and neural processes in the BLA 3 weeks later (Fig. 1c–g). Whole-cell current-clamp recordings from these ChR2-expressing BLA neurons showed that optical stimulation ($\lambda = 473$ nm, 0.5 ms) induced action potentials (Fig. 1h). We found extensive neural fibers expressing ChR2, which presumably originated from ChR2-expressing neurons in the BLA (detected by their YFP signals; Fig. 1i). Whole-cell voltage-clamp (-70 mV) recording from NAc shell neurons showed that optical stimulation ($\lambda = 473$ nm \times 0.5 ms) of these ChR2-expressing fibers induced postsynaptic currents that were inhibited by the AMPAR

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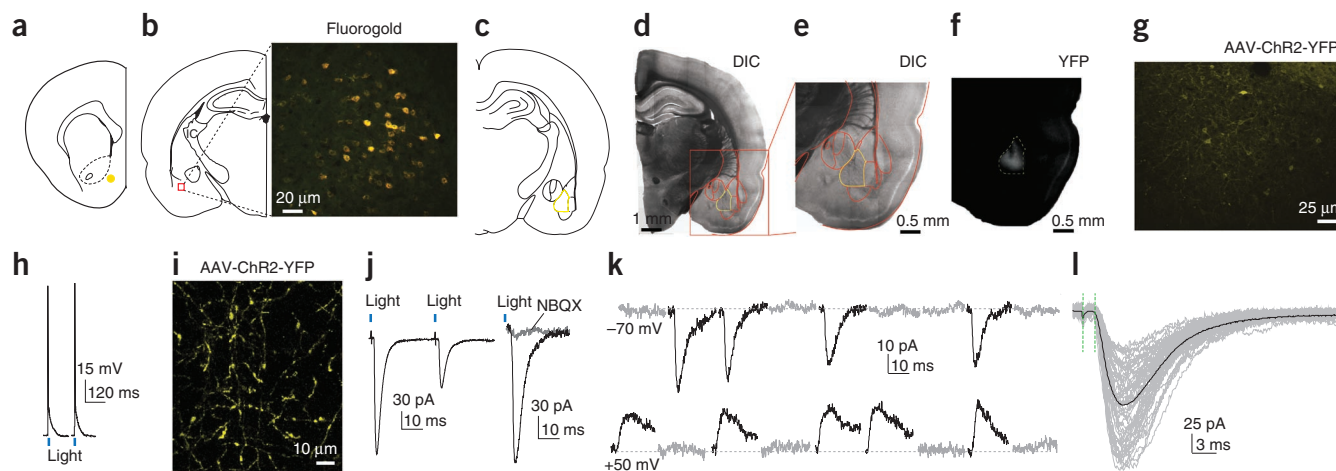


Figure 1 Recording of BLA-to-NAc excitatory synapses. (a) Diagram showing the position in the NAc shell at which retrograde tracer fluorogold was stereotaxically injected. (b) Diagram and image of the BLA. Injection of fluorogold resulted in labeling of BLA neurons. (c) Diagram of the BLA (circled in yellow), where the ChR2-expressing AAV2 was injected. (d) Differential interference contrast image showing a brain slice with intra-BLA viral injection. Yellow outline indicates the BLA. (e) Magnification of the image shown in d (red square). (f) YFP image showing fluorescence (viral expression) in the BLA. (g) Fluorescence image showing that intra-BLA injection of ChR2-YFP-expressing virus resulted in expression of ChR2-YFP in neurons and neuronal fibers in the BLA. (h) Optical stimulation elicited action potentials in ChR2-expressing BLA neurons. Example traces are shown. (i) Example image of the NAc shell in a rat injected in the BLA with ChR2/YFP AAV2. Extensive ChR2-expressing fibers are present. (j) NBQX perfusion eliminated optically elicited synaptic currents in NAc neurons. Example traces are shown. (k) Example EPSCs elicited at BLA-to-NAc synapses by optical stimulations in the minimal stimulation assay. Failed and successful synaptic responses were readily discernible. (l) Optically evoked individual (gray) and averaged EPSCs (black) from an example NAc neuron showing a short latency of BLA-to-NAc synaptic transmission. The dashed green lines indicate that the latency was measured between the stimulation artifact and initiation of EPSCs. Examples in j and k are taken from saline-experienced control rats (1 day after the end of the training phase).

antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX, 5 μ M, paired *t* test, $t_3 = 23.3$, $P = 0.0002$; Fig. 1j), suggesting that synaptic currents generated from the BLA-to-NAc projection are predominantly glutamatergic.

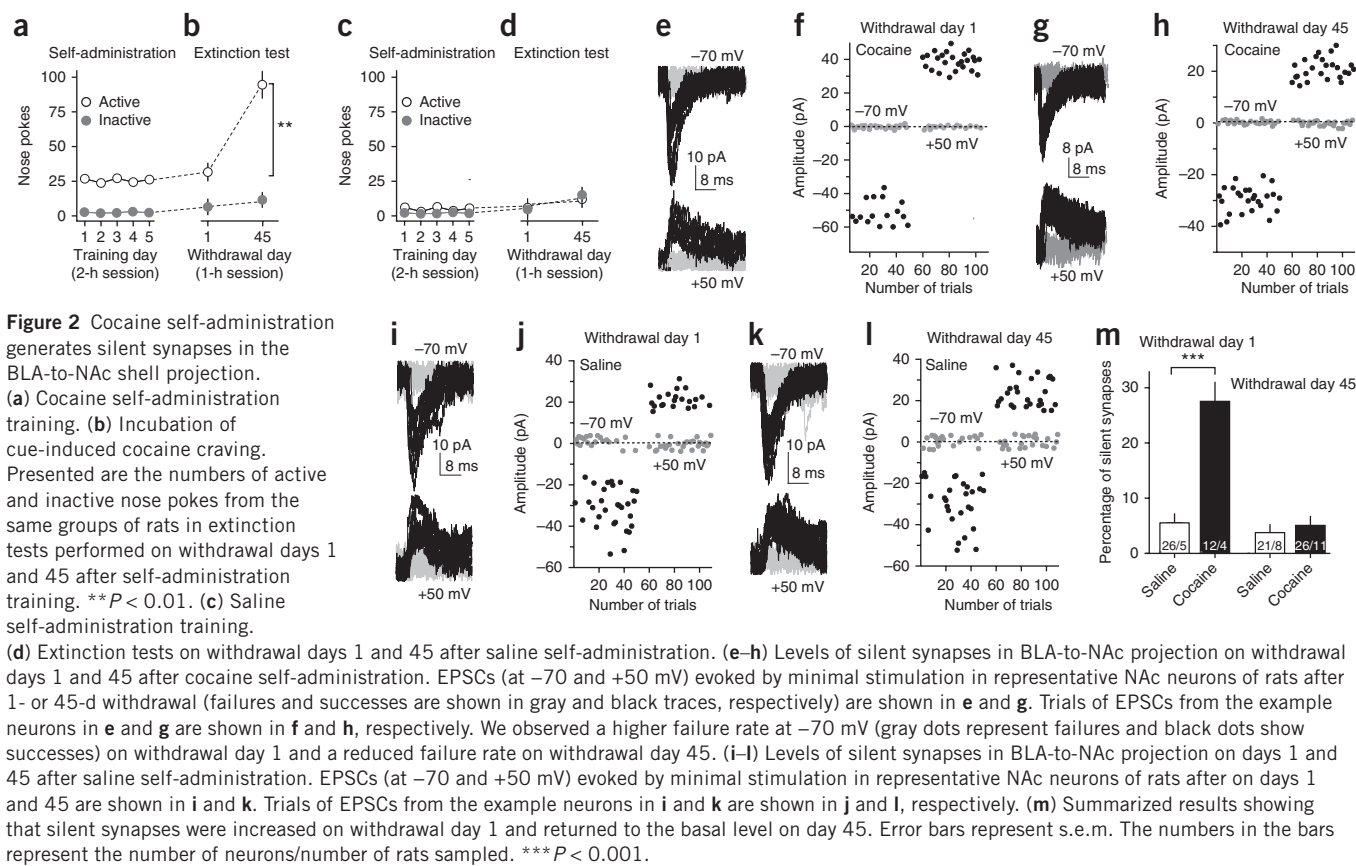
We next verified that that we could perform the minimal stimulation assay^{10,11,13,20} to estimate the level of silent synapses in the BLA-to-NAc projection. We adjusted the parameters of optical stimulation (duration, intensity of stimulation and position of the laser path) such that successes and failures of excitatory postsynaptic currents (EPSCs) could be recorded and clearly separated at -70 and $+50$ mV (Fig. 1k). Optically evoked EPSCs exhibited short delays between the light pulse and the onset of synaptic response (0.85 ± 0.16 ms, $n = 156$ neurons in 85 rats), indicative of monosynaptic transmission (Fig. 1l). Secondary synaptic responses with >2 -ms delays were not observed. Thus, we were able to estimate the level of silent synapses selectively in the BLA-to-NAc excitatory monosynaptic projection. Although BLA-targeted viral injections may potentially infect the neighboring central nucleus (CeA), it is unlikely that the CeA contributed to our recorded synaptic responses. CeA neurons are largely GABAergic neurons, and GABA receptor-mediated currents were negligible in light-evoked responses in NAc slices ($\sim 7\%$ inhibition by 100 μ M picrotoxin, $n = 22$ cells from 4 rats). In addition, CeA neurons do not directly project to NAc¹⁹.

Cocaine self-administration generates silent synapses

To examine incubation of cue-induced cocaine craving, we trained rats to nose poke for intravenous infusions of cocaine for 6 d (one overnight session followed by 2 h d^{-1} for 5 d); each infusion was paired with a light cue. As previously demonstrated^{21–23}, this training procedure led to reliable cocaine self-administration over the 5-d training period (Fig. 2a). We then used a within-subjects repeated-measure procedure that has been used in studies of incubation of craving for cocaine and other drugs^{24,25} to test rats for cue-induced cocaine seeking in extinction sessions performed after 1 withdrawal day or 42–47 d

(referred to herein as day 45). During testing, rats were re-exposed to the drug self-administration chambers (the drug environment), and nose pokes (the operational measure of drug seeking and craving) led to contingent presentations of the light cue, but not cocaine²⁶. We found that cue-induced cocaine seeking after 45 withdrawal days was higher than after 1 d (incubation of cocaine craving; withdrawal day (1, 45) \times nose poke operandum (active, inactive) interaction, $F_{1,24} = 25.3$, $P < 0.0001$; Fig. 2b). In contrast, no significant time-dependent changes in responding to the light cue during testing were observed in drug-naive rats that previously nose poked for saline injections during the training phase (incubation of cocaine craving, withdrawal day (1, 45) \times nose poke operandum (active, inactive) interaction, $F_{1,19} = 1.22$, $P = 0.2827$; Fig. 2c,d). These data are consistent with several reports on incubation of cocaine craving in which a limited-access (2-h daily sessions) cocaine self-administration training procedure was used^{26–28}. In most studies of mechanisms of incubation of cocaine craving^{3,7,29}, investigators used an extended-access (6-h daily sessions) training procedure, which leads to stronger incubation after withdrawal than the limited-access training procedure²⁶.

Using this self-administration procedure, we trained different groups of rats (Supplementary Fig. 1) and measured silent synapses in the BLA-to-NAc (shell subregion) glutamatergic projection after 1 or 45 withdrawal days. We performed the minimal stimulation assay of silent synapses^{10,11,13,20} using optical stimulation in NAc slices from rats receiving BLA injection of ChR2-expressing AAV2 to assess successful and failed EPSCs at BLA-to-NAc shell synapses. We observed that the level (percentage of all synapses) of silent synapses in the BLA-to-NAc shell projection was increased on withdrawal day 1, but not on day 45 (withdrawal day \times training condition (cocaine, saline) interaction, $F_{1,81} = 25.6$, $P < 0.0001$; Fig. 2e–m). Thus, cocaine self-administration leads to the formation of silent synapses in the BLA-to-NAc shell projection; these silent synapses were detected during early (day 1), but not late (day 45), withdrawal.



Unsilencing of cocaine-generated silent synapses

During brain development, silent synapses are either pruned away or mature into fully functional synapses by recruiting AMPARs, resulting in very low levels of silent synapses in adulthood^{14,30}. Although both processes may occur simultaneously after withdrawal from cocaine, the second possibility is supported by evidence of elevated cell-surface and synaptic levels of CP-AMPA in the NAc after prolonged withdrawal from cocaine^{6,31–34}. We therefore tested whether cocaine-generated silent synapses were unsilenced during the withdrawal period by recruiting these receptors. CP-AMPA conduct minimal current at depolarized membrane potentials³⁵. Thus, synaptic insertion of these receptors can be detected by increased rectification of EPSCs at positive membrane potentials³⁵. In rats trained with the same cocaine self-administration regimen (Supplementary Fig. 1), we found increased rectification of AMPAR EPSCs at BLA-to-NAc synapses after 45 withdrawal days ($t_{27} = 3.5$, $P = 0.002$; Fig. 3a–d). Furthermore, AMPAR EPSCs at these synapses became sensitive to Naspam (200 μ M), a selective antagonist of CP-AMPA³⁵ ($t_7 = 3.8$, $P = 0.007$; Fig. 3e–i). Our data, obtained from rats trained to self-administer cocaine under limited-access 2-h daily sessions, differ from that of a recent report³⁴ in which accumulation of CP-AMPA in the NAc after 45 withdrawal days was observed in rats trained to self-administer cocaine for 6 h d^{-1} , but not 2 h d^{-1} . These differences might be a result of the fact that we initiated the training phase with an overnight extended-access cocaine self-administration. Another difference between ours and previous studies is that we assessed accumulation of CP-AMPA specifically in the BLA-to-NAc projection, increasing our ability to detect small changes in CP-AMPA levels, assuming that CP-AMPA do not accumulate in all NAc synapses after our limited-access cocaine self-administration training procedure.

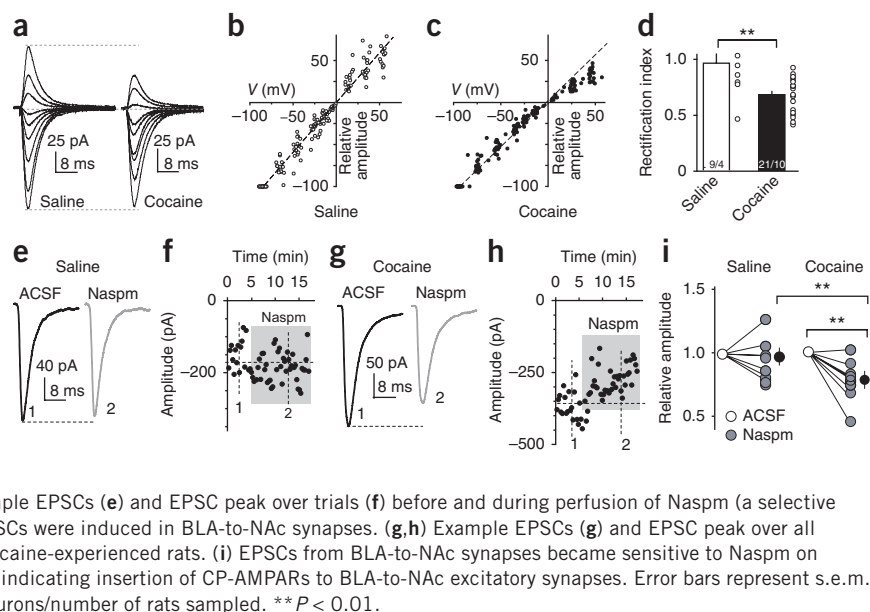
Furthermore, the rats that we used were younger than those previously used in studies of the role of CP-AMPA in incubation of cocaine craving^{3,7} and therefore may be more susceptible to withdrawal-induced CP-AMPA accumulation.

These results support the possibility that the disappearance of silent synapses after prolonged withdrawal from cocaine is mediated by a maturation or unsilencing process that at least partially involves recruitment of CP-AMPA. We tested this possibility by assessing both silent synapse formation and CP-AMPA accumulation on withdrawal day 10 using cocaine and saline training and extinction test conditions identical to those described above. At the behavioral level, cue-induced cocaine seeking in the extinction tests was higher after 10 withdrawal days than after 1 d (withdrawal day (1, 10) \times nose poke operandum interaction, $F_{1,37} = 10.9$, $P < 0.0001$; Fig. 4a). At this intermediate withdrawal day, the level of silent synapses was decreased toward basal levels (saline, $3.9 \pm 2.9\%$; cocaine, $10.1 \pm 3.3\%$; $t_{19} = 1.4$, $P = 0.18$; Fig. 4b–d). In addition, sensitivity to Naspam, indicative of CP-AMPA accumulation, began to emerge at the BLA-to-NAc projection in cocaine-trained rats on withdrawal day 10. Planned-comparison contrasts demonstrated a significant effect of Naspam in cocaine-trained rats on withdrawal day 10 ($F_{1,20} = 16.1$, $P = 0.04$), but not in saline-trained rats ($P > 0.99$; Fig. 4e–i). This is a more rapid appearance of CP-AMPA than has been observed previously⁷, possibly as a result of our selective assessment of the BLA-to-NAc projection or the initial overnight training session that we used, but was not used in previous studies.

Under our experimental conditions, the time courses for the incubation of cocaine craving, disappearance of silent synapses and accumulation of CP-AMPA exhibited parallel changes during the withdrawal period (Fig. 4j), suggesting that the disappearance of

Figure 3 Insertion of CP-AMPA receptors in BLA-to-NAC synapses after 45 withdrawal days.

(a) Example EPSCs elicited at -70 to $+50$ mV (with 10 -mV increment) from BLA-to-NAC synapses; data were collected on withdrawal day 45 in rats previously trained to self-administer saline or cocaine. Whole-cell voltage-clamp recordings were made in the presence of the GABA_A receptor-selective antagonist picrotoxin (100 μ M) and the NMDA receptor-selective antagonist *D*-aminophosphonovaleric acid (AP5, 50 μ M). (b,c) *I*-V curves of EPSCs from BLA-to-NAC synapses; the influence of reversal potentials of EPSCs was factored in for each recorded neuron (Online Methods). (d) On withdrawal day 45, an increased rectification of EPSCs was detected at BLA-to-NAC synapses from cocaine-experienced, but not saline-experienced, rats. Scattered dots indicate the values of individual cells. $**P < 0.01$.



silent synapses is mediated in part by an unsilencing process that involves CP-AMPA synaptic insertion. If this speculation is correct, selective inhibition of CP-AMPA receptors after prolonged withdrawal

(day 45) should at least partially re-silence the previously silent synapses. Accordingly, we assessed silent synapses in the BLA-to-NAC projection (Supplementary Fig. 1) on withdrawal day 45.

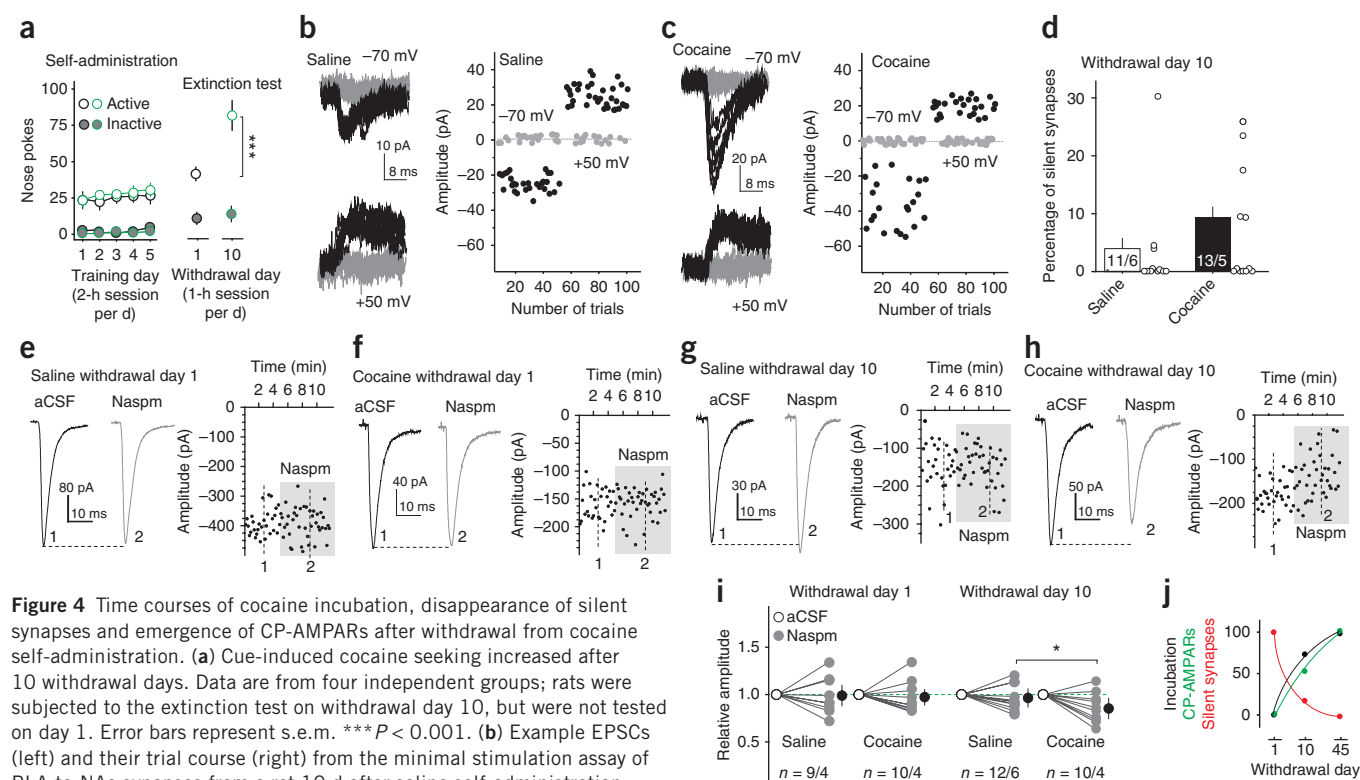


Figure 4 Time courses of cocaine incubation, disappearance of silent synapses and emergence of CP-AMPA receptors after withdrawal from cocaine self-administration. (a) Cue-induced cocaine seeking increased after 10 withdrawal days. Data are from four independent groups; rats were subjected to the extinction test on withdrawal day 10, but were not tested on day 1. Error bars represent s.e.m. $***P < 0.001$. (b) Example EPSCs (left) and their trial course (right) from the minimal stimulation assay of BLA-to-NAC synapses from a rat 10 d after saline self-administration. (c) Example EPSCs (left) and their trial course (right) from the minimal stimulation assay of BLA-to-NAC synapses from a rat 10 d after withdrawal from cocaine self-administration. (d) The level of silent synapses in the BLA-to-NAC projection decreased toward the basal (saline control) level after withdrawal from cocaine. Error bars represent s.e.m. (e,f) Example EPSCs from BLA-to-NAC synapses (left) and their trial course (right) before and during perfusion of Naspm from rats 1 d after withdrawal from saline (e) or cocaine (f) self-administration. aCSF, artificial cerebrospinal fluid. (g,h) Example EPSCs from BLA-to-NAC synapses (left) and their trial course (right) before and during perfusion of Naspm from rats 10 d after saline (g) or cocaine (h) self-administration. (i) Summary showing that the sensitivity of EPSCs from BLA-to-NAC synapses to Naspm exhibited a small, but significant, increase after 10 d of withdrawal from cocaine. (j) Time courses (arbitrarily spaced) of incubation, disappearance of silent synapses and insertion of CP-AMPA receptors after withdrawal from cocaine. Data are normalized by setting the withdrawal scores to 0% and withdrawal day 45 scores to 100%. *n* values are presented as the number of neurons/number of rats sampled. Error bars represent s.e.m. $*P < 0.05$.

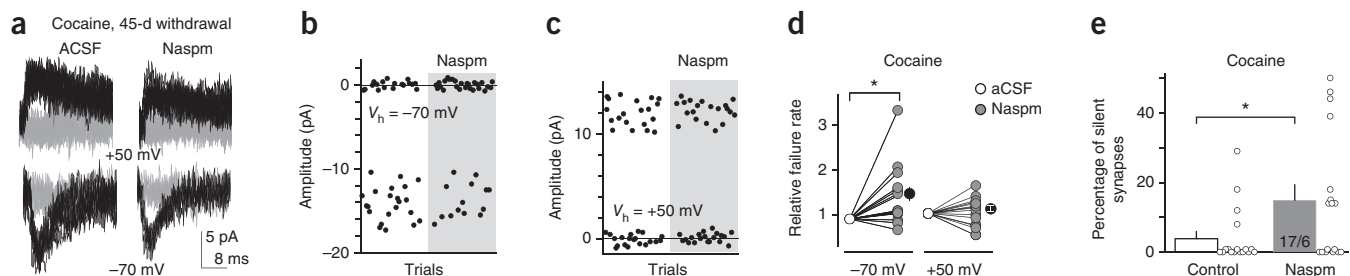


Figure 5 Blockade of CP-AMPA receptors re-silences silent synapses in BLA-to-NAc shell projection on withdrawal day 45. EPSCs were elicited by minimal stimulations (recorded at -70 and $+50$ mV) from BLA-to-NAc synapses 45 d after withdrawal from cocaine self-administration. (**a–c**) Example (**b**) and trials (**b,c**) of EPSCs from a cocaine-experienced rat before and during perfusion of Naspm. (**d**) Blockade of CP-AMPA receptors increased the failure rates of EPSCs at -70 mV, but not at $+50$ mV. (**e**) Blockade of CP-AMPA receptors on withdrawal day 45 caused a re-emergence of silent synapses in the BLA-to-NAc shell projection, suggesting that a large portion of cocaine-generated silent synapses was un-silenced by recruiting CP-AMPA receptors. Error bars represent s.e.m. The numbers in the bars represent the number of neurons/number of rats sampled. $*P < 0.05$.

In cocaine-experienced rats, inhibiting CP-AMPA receptors with Naspm significantly increased the failure rate of minimal stimulation-induced synaptic responses at -70 mV ($t_{14} = 2.72$, $P = 0.01$), but not at $+50$ mV ($t_{15} = 0.29$, $P = 0.78$), in the BLA-to-NAc projection (**Fig. 5d**). In saline-experienced rats, perfusion of Naspm did not affect the failure rate at either -70 ($t_8 = 1.0$, $P = 0.33$) or $+50$ mV ($t_8 = 1.7$, $P = 0.13$). Consequently, inhibition of CP-AMPA receptors with Naspm resulted in the reemergence of silent synapses in cocaine-experienced rats ($t_{16} = 2.4$, $P = 0.028$; **Fig. 5e**), but not in saline-experienced rats ($t_{17} = 0.14$, $P = 0.89$; **Supplementary Fig. 2**). Note that perfusion of Naspm did not result in full recovery of cocaine-generated silent synapses to the level observed on withdrawal day 1, suggesting that other mechanisms are involved in silent synapse maturation (see Discussion). Nonetheless, these results suggest that a substantial portion of cocaine-generated silent synapses in the BLA-to-NAc projection are un-silenced after withdrawal from cocaine by recruiting CP-AMPA receptors.

LTD internalizes CP-AMPA receptors at BLA-to-NAc synapses

Our results suggest a silent synapse-based reorganization of the BLA-to-NAc projection during the development of incubation of

cocaine craving; silent synapses are generated during cocaine self-administration and subsequent recruitment of CP-AMPA receptors un-silences these synapses. To further test this hypothesis, we attempted to optogenetically re-silence cocaine-generated silent synapses in the BLA-to-NAc projection by preferentially internalizing CP-AMPA receptors using an *in vivo* long-term depression (LTD)-based manipulation on withdrawal day 45. If our hypothesis is correct, then this manipulation should switch the newly matured/un-silenced silent synapses that contain CP-AMPA receptors from the ‘incubated’ state (withdrawal day 45) to their ‘pre-incubated’ silent synapse state (withdrawal day 1), which would then result in reduced cue-induced cocaine seeking after prolonged withdrawal from the drug.

The LTD protocol was developed on the basis of previous protocols that induce internalization of AMPARs from excitatory synapses^{36,37}. We modified the protocol such that the induction stimulation could be used in our optogenetic setup to selectively target the BLA-to-NAc synapses, did not substantially affect typical AMPARs in saline-experienced rats and preferentially induced internalization of CP-AMPA receptors in cocaine-experienced rats. Because inhibiting or removing CP-AMPA receptors appears to be

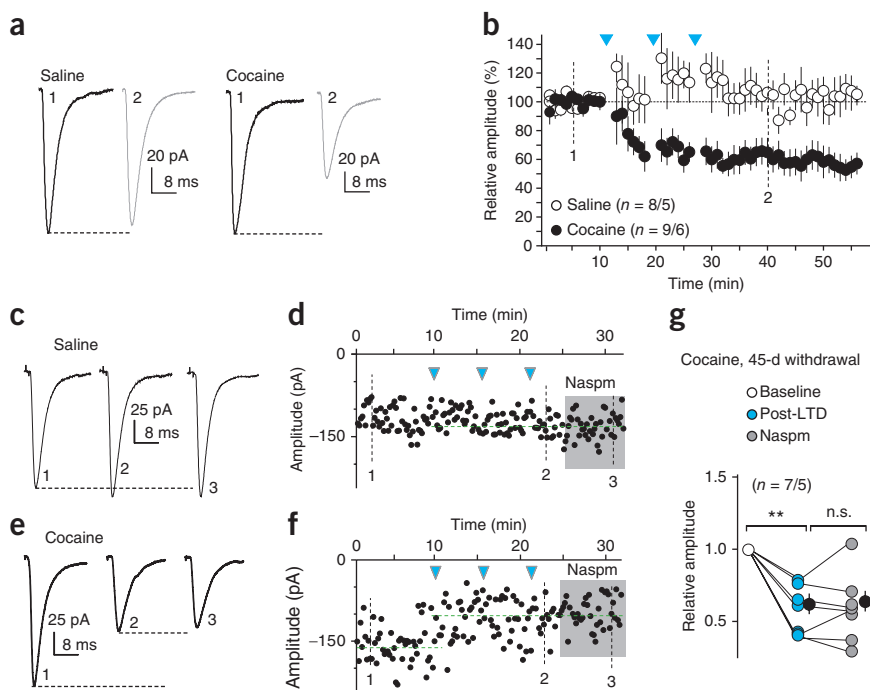


Figure 6 LTD induction at BLA-to-NAc synapses selectively internalizes CP-AMPA receptors on withdrawal day 45. (**a,b**) Example traces (**a**) and summarized results (**b**) showing that the LTD induction protocol induced a persistent reduction of the peak amplitudes of EPSCs at BLA-to-NAc shell synapses from cocaine-experienced, but not saline-experienced, rats. Arrowheads indicate the time points of application of LTD protocols. Error bars represent s.e.m. (**c–f**) Example EPSCs and the time course showing that BLA-to-NAc shell excitatory synapses, although highly sensitive to Naspm after withdrawal from cocaine (**Fig. 3e–i**), became Naspm insensitive after LTD induction (**e–g**). In saline-experienced rats, EPSCs in this projection were Naspm-insensitive before (**Fig. 3e–i**) or after LTD induction (**c,d**). (**g**) At BLA-to-NAc synapses, LTD induction induced a persistent decrease in the peak amplitude of EPSCs and EPSCs lost their sensitivity to Naspm after LTD, suggesting a selective reduction (internalization) of CP-AMPA receptors from these synapses. Error bars represent s.e.m. n values are presented as the number of neurons/number of rats sampled. $**P < 0.01$. n.s., not significant ($P = 0.69$).

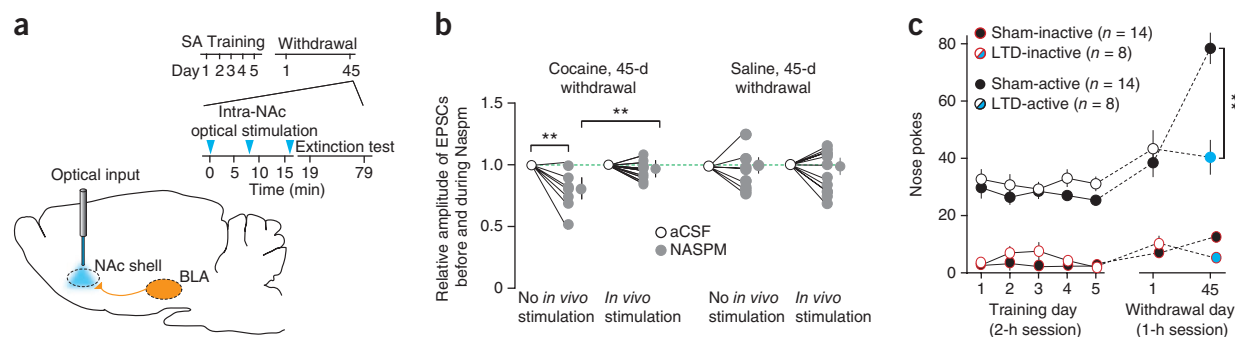


Figure 7 Reversing the maturation of silent synapses in the BLA-to-NAc projection reverses incubation of cocaine craving. **(a)** Diagrams showing the timeline of behavioral experiments and the LTD induction protocol in BLA-to-NAc synapses before the test for cue-induced cocaine seeking on withdrawal day 45. **(b)** After 45 d of withdrawal from cocaine, EPSCs at BLA-to-NAc synapses were significantly inhibited by perfusion of NaspM. After *in vivo* LTD induction, EPSCs from these synapses became resistant to NaspM. EPSCs were insensitive to NaspM before or after *in vivo* LTD in saline-experienced rats. Error bars represent s.e.m. $**P < 0.01$. **(c)** *In vivo* LTD induction prevented incubated cue-induced cocaine seeking in the extinction test. Error bars represent s.e.m. n indicates the number of rats. $**P < 0.01$.

sufficient to re-silence a large portion of cocaine-generated silent synapses after prolonged withdrawal from the drug (Fig. 5), an LTD induction protocol that meets the above criteria should selectively re-silence the matured or un-silenced synapses in the BLA-to-NAc projection.

We first verified the efficacy of this LTD protocol in brain slices. We virally expressed ChR2 in the BLA of rats trained to self-administer cocaine or saline (Supplementary Fig. 1) and recorded EPSCs from BLA-to-NAc synapses on withdrawal day 45. The LTD protocol induced long-lasting depression of EPSCs in cocaine-trained, but not saline-trained, rats (training condition \times LTD protocol interaction, $F_{57,855} = 3.2$, $P < 0.0001$; Fig. 6a,b). Furthermore, although EPSCs from cocaine-trained rats exhibited increased sensitivity to NaspM (Fig. 3e-i and Supplementary Fig. 2), this effect was abolished after LTD induction ($t_6 = 0.4$, $P = 0.7$; Fig. 6c-g). These results suggest that the AMPARs internalized by LTD induction were primarily CP-AMPA, which were located at matured silent synapses.

Although the mechanisms of the preferential sensitivity of CP-AMPA to this protocol are unknown, CP-AMPA inserted during the withdrawal period may be loosely tethered to the postsynaptic density and are therefore more susceptible to regulation^{31,33}. Supporting this possibility, CP-AMPA that accumulate in the NAc after prolonged withdrawal from cocaine are preferentially removed from synapses by mGluR1 stimulation³³. Given that optical stimulation of BLA-to-NAc projection predominantly activated excitatory monosynaptic events (Fig. 1j), the LTD protocol could be delivered to the ChR2-expressing BLA-to-NAc projection *in vivo* via NAc optical fibers with minimal effect on inhibitory synapses or indirect inputs.

Reversal of incubation of cocaine craving by LTD

In the final experiment, we used the LTD protocol described above *in vivo* to reverse the maturation of cocaine-generated silent synapses in the BLA-to-NAc projection on withdrawal day 45 and determined the effect of this manipulation on enhanced (incubated) cue-induced cocaine seeking. We delivered the LTD induction protocol 19 min before the extinction test for cue-induced cocaine seeking on day 45 via the NAc optical fibers, which preferentially targeted BLA-to-NAc synapses (Fig. 7a). In a subset of rats, we first verified the *in vivo* efficacy of this LTD protocol in preferentially internalizing CP-AMPA in the BLA-to-NAc projection by preparing NAc slices immediately after *in vivo* LTD induction on day 45. In these slices, EPSCs from

BLA-to-NAc synapses lost the NaspM sensitivity (one-tailed t test, $t_{10} = 1.8$, $P = 0.045$; Fig. 7b) that would otherwise (without LTD induction) be present. Thus, the optical LTD protocol was effective *in vivo*, as well as *in vitro*, at preferentially internalizing CP-AMPA at BLA-to-NAc synapses.

We then applied this LTD protocol immediately before the day 45 extinction test. LTD induction reduced cue-induced cocaine seeking to a level similar to that observed on day 1 (LTD induction \times nose poke operandum interaction, $F_{1,20} = 11.1$, $P = 0.003$; Fig. 7c and Supplementary Fig. 3). Thus, internalization of CP-AMPA, which likely re-silenced the previously silent synapses in the BLA-to-NAc projection, reversed incubation of cue-induced cocaine craving on withdrawal day 45 to the pre-incubated day 1 levels.

DISCUSSION

Studies using pharmacological inactivation or site-specific lesions have implicated the glutamatergic projection from BLA to NAc in cue-induced cocaine seeking in rat models^{8,16}. Biochemical, electrophysiological and pharmacological evidence indicates that time-dependent delayed accumulation of CP-AMPA in NAc is critical for the incubation of cocaine craving⁷. Our results provide a logical link between these two sets of data by demonstrating that silent synapses are generated in the BLA-to-NAc projection during cocaine self-administration, and that some of these silent synapses are unsilenced after withdrawal from cocaine by recruiting CP-AMPA, which are important for the incubation of cocaine craving.

Cocaine exposure and synaptogenesis

Previously, we proposed that cocaine-induced generation of silent synapses shares mechanisms with synaptogenesis¹⁵. Three features are particularly notable in this regard: synaptogenesis involves formation of new synaptic structures, reflected in postsynaptic morphology as an increase in the number of dendritic spines³⁸, synaptogenesis and circuitry development are often initiated or modulated by postsynaptic activation of CREB³⁹, and one of the pro-synaptogenesis functions of CREB is mediated by upregulation of GluN2B-containing NMDARs^{10,40}. Similarly, cocaine exposure increases the number of dendritic spines in the NAc^{41,42} and activates CREB⁴³. In addition, cocaine-induced CREB activation in NAc is involved in both silent synapse formation and cocaine-induced increases in dendritic spines¹⁰. Finally, cocaine-induced generation of silent synapses is controlled by synaptic insertion of GluN2B-containing NMDARs^{10,11}.

During brain development, generation and subsequent maturation of unsilencing of silent synapses is critical for synaptic stabilization and circuitry formation¹⁴. Our results suggest that, in the developed brain, generation of silent synapses can be resumed in particular neural circuits after cocaine exposure to reorganize and reshape synapses, neural circuits and future behaviors¹⁵. If the silent synapse-mediated circuitry reorganization is achieved by synaptogenesis as we hypothesize, this process may not only substantially and persistently change the pattern of information flow in this projection, but also create a new path of information flow via newly formed synapses and circuits that did not exist before cocaine exposure¹⁵.

Our previous results suggest that cocaine-induced generation of silent synapses occurs in heterogeneous populations of NAc neurons via the same GluN2B-based molecular mechanisms¹¹. However, different mechanisms are likely involved for certain types of NAc neurons. A recent study¹² showed that, in mice, prior non-contingent cocaine exposure induces higher levels of silent synapses in ~2–3% of Fos-positive NAc neurons that were acutely activated by cocaine. Furthermore, generation of silent synapses in these neurons was not accompanied by increased GluN2-containing NMDARs and was potentially achieved by internalization of AMPARs from existing synapses, rather than synaptogenesis-related mechanisms¹². Thus, although cocaine-induced generation of silent synapses is likely to be a common phenomenon in different NAc neuronal populations, the cellular processes underlying silent synapse formation may differ.

Maturation of silent synapses and craving incubation

Our data suggest that, after silent synapses are generated during cocaine self-administration, some of these synapses mature by recruiting CP-AMPA receptors after prolonged withdrawal from cocaine. The main evidence for this notion is that BLA-to-NAc synapses that were highly sensitive to Nasp after 45 withdrawal days became Nasp-insensitive after LTD induction (Figs. 3 and 6). Furthermore, at this time point, pharmacological inhibition of CP-AMPA receptors resulted in the re-emergence of silent synapses (Fig. 5). However, Nasp treatment only recovered ~60% of the initial level of silent synapses (Fig. 5d), suggesting that some silent synapses may have been pruned away after withdrawal from cocaine, as is the case with some silent synapses during development³⁸. Another possibility is that some silent synapses matured by recruiting Nasp-insensitive, GluA2-containing AMPARs.

We employed a LTD-based *in vivo* manipulation, rather than NAc application of Nasp, to manipulate CP-AMPA receptors and silent synapses in a projection-specific manner. However, our results cannot rule out two other scenarios. First, the LTD protocol might also affect synapses that were previously non-silent. This could happen if some previously non-silent synapses recruited CP-AMPA receptors after prolonged withdrawal from cocaine and became susceptible to the LTD protocol. Second, the CP-AMPA receptor-containing synapses silenced after prolonged withdrawal from cocaine might not be the same silent synapses that were generated during early withdrawal. This could happen if some previously non-silent synapses lost all of their typical AMPARs and recruited new CP-AMPA receptors after prolonged withdrawal from cocaine. Nevertheless, the observations that the LTD manipulation removed CP-AMPA receptors from BLA-to-NAc synapses after 45 withdrawal days (Fig. 6) and that inhibiting CP-AMPA receptors caused a re-emergence of silent synapses at this time point (Fig. 5) suggest that the most parsimonious interpretation of our data is that the LTD manipulation re-silenced the unsilenced synapses.

Finally, we confirmed the role of NAc CP-AMPA receptors in incubation of cue-induced cocaine craving and our results suggest a previously

unknown role for silent synapses in this incubation. A question for future research is whether these particular cocaine-induced neuroadaptations are also involved in relapse provoked by re-exposure to the drug itself, as assessed by reinstatement of cocaine seeking induced by a drug-priming injection⁴⁴ or resumption (re-acquisition) of drug self-administration under different reinforcement schedules⁴⁵. We suspect that the mechanisms that we have identified are selective to incubation of cue-induced drug craving. This is because there is either little, or mixed, evidence for time-dependent changes in cocaine priming-induced reinstatement or resumption of cocaine self-administration after withdrawal from cocaine^{3,4,26,46}.

Concluding remarks

Previously, we and others found that silent synapses were generated in the NAc after non-contingent cocaine exposure^{10–12}. Here, we observed that, after cocaine self-administration, silent synapses were generated in a particular glutamatergic projection and were necessary for the development of incubation of cocaine craving. These results raise two questions for future research. First, do the generation and subsequent maturation of silent synapses occur in other glutamatergic projections to NAc⁴⁷ and do they mediate other aspects of cocaine relapse^{9,44}? Second, does silent synapse-mediated circuitry reorganization occur in other brain regions that have been implicated in incubation of cocaine craving³? Some studies have observed differences in cocaine-induced plasticity between the NAc core and shell⁴⁸. However, the NAc core and shell undergo similar AMPAR plasticity after prolonged withdrawal from cocaine; both regions exhibit accumulation of CP-AMPA receptors^{6,32,49}. Furthermore, CP-AMPA receptors in both NAc subregions are necessary for the expression of incubation of cocaine craving⁶. These results warrant future studies examining withdrawal period-associated generation and maturation of silent synapses in NAc core. However, in regards to our results, removal of CP-AMPA receptors from NAc core synapses after prolonged withdrawal from cocaine represents a promising pharmacotherapeutic approach to attenuate incubation of cocaine craving^{33,50}. Future studies will be needed to delineate the role of silent synapse-based re-organization of neural circuits involving the NAc in drug craving and the potential for exploiting this understanding to devise new therapies for cocaine relapse.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.R.L., Y.M., Y.H.H., S.R.S., M.E.W., E.J.N., Y.S., O.M.S. and Y.D. contributed to the design of the experiments and the analyses, and the writing of the manuscript. B.R.L., Y.M., X.W., M.O., M.I., P.A.N., N.M.G., T.E.B., A.S., C.G. and M.K.L. conducted the experiments and performed the analyses.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. Male Sprague-Dawley rats (Harlan and Charles River), postnatal day 28–30 at the beginning of the experiments, were used. Rats were single housed under a 12-h light/dark cycle (on at 700, off at 1900). Temperature ($22 \pm 1^\circ\text{C}$) and humidity ($60 \pm 5\%$) were controlled. Behavioral experiments were performed during the day time. Rat usage was in accordance with protocols approved by the Institutional Animal Care and Use Committees at Washington State University and University of Pittsburgh.

Viral vectors. Recombinant adeno-associated vectors (rAAV2) expressing venus-tagged ChR2 H134R were pseudotyped with AAV1/2 capsid proteins. HEK293T cells were co-transfected with the plasmids pF6 (adenoviral helper plasmid), pRVI (cap and rep genes for AAV serotype 2), pH21 (cap gene for AAV serotype 1 and rep gene for serotype 2) and the rAAV plasmid using linear polyethylenimine assisted transfection²³. The helper plasmids were kindly provided by M. Schwarz (Max Planck Institute)⁵¹. Cultures grown in DMEM (Biochrom) with 10% substituted fetal bovine serum (vol/vol, Biochrom, #S0115) were harvested from 15- × 15-cm dishes after 48 h. rAAV was isolated and purified as described⁵¹. Briefly, HEK293T cells were lysed with sodium desoxycholate and repeated freeze-thaw cycles in the presence of Benzonase-Nuclease HC (Novagen). From the supernatant, rAAVs were isolated by iodixanol gradient centrifugation from the 40% and 54% interphase. rAAVs were then desalted by ultrafiltration, filtered through 0.2- μm Millex-GV filter units (Millipore), and stored at 4°C in 500 μl of phosphate-buffered saline (PBS) with 10 mM MgCl_2 and 25 mM KCl. The *ChR2* gene was packaged with a *YFP* gene into a recombinant, replication-defective form of the adenovirus (AAV).

Fluorogold and ChR2-YFP imaging procedures. For retrograde tracing, rats were anesthetized with a ketamine/xylazine mixture (50 and 5 mg per kg of body weight, intraperitoneal) and placed in a stereotaxic apparatus (Stoelting). Fluorogold solution (1% in saline, wt/vol) was injected iontophoretically (+5 μA , 7 s on/7 s off, 10 min) into the NAc shell with a glass micropipette (tip diameter = 20 μm). The coordinates used to target the NAc shell were +1.8 (anterior-posterior), 0.9 (medial-lateral) and 6.5 (dorsal-ventral from brain surface). After a 2-week survival period, rats were perfused transcardially with 0.1 M PBS followed by 4% formaldehyde (wt/vol) in PBS. Brains were removed carefully and given an additional 48-h postfix in 4% formaldehyde, and then transferred to 30% (wt/vol) sucrose in PBS for 48 h before sectioning. Coronal sections (50 μm) were cut with a Leica RM 2000R freezing microtome. Sections were washed in PBS and mounted on gelatin-coated slides and then coverslipped with DPX mounting medium. Expression of fluorogold in BLA was examined using a Zeiss Axioplane2 imaging microscope and images were taken at 10 \times original magnification using infinity digital camera. For imaging of ChR2-YFP, rats were injected with AAV-ChR2-YFP into the BLA (see below). After approximately 3 weeks, rats were transcardially perfused with 0.1 M PBS followed by 4% formaldehyde in PBS. Brains were removed carefully and given an additional 48-h postfix in 4% formaldehyde, and then transferred to 30% sucrose in PBS for 48 h before sectioning. Coronal sections (50 μm) were cut with a Leica RM 2000R freezing microtome. Sections were washed in PBS and mounted on gelatin-coated slides and then coverslipped with DPX and Prolong Antifade (Invitrogen) mounting medium. Expression of ChR2-YFP in the BLA and NAc shell was examined using a Zeiss LSM5210 Meta confocal microscope and images were taken at 10 \times and 40 \times original magnification.

Behavioral studies. Cocaine HCl (provided by National Institute on Drug Abuse Drug Supply Program) was dissolved in 0.9% NaCl saline (wt/vol). Ketamine and xylazine were mixed for anesthesia (purchased from Washington State University College of Veterinary Medicine or Drug Enforcement Agency–designated vendor at University of Pittsburgh).

A 28 gauge injection needle was used to bilaterally inject 1 μl ($0.2 \mu\text{l min}^{-1}$) of the AAV2-ChR2-YFP solution via Hamilton syringe into the BLA (AP, -2.50 ; ML, ± 4.80 ; DV, -8.50) using a Thermo Orion M365 pump. Injection needles were left in place for 5 min following injection. ChR2-YFP was given 1–2 weeks before any experimental manipulation to ensure maximal infection; thus, electrophysiological analyses were conducted ~ 3 weeks (1-d withdrawal) or ~ 8 weeks (45-d withdrawal) post infection.

Self-administration surgery has been described previously^{10,21}. Briefly, a silastic catheter was inserted into the right auricle through the external jugular vein and the distal end was led subcutaneously to the back between the scapulas. Catheters are constructed from silastic tubing (~ 5 cm, inner diameter = 0.020 inches, outer diameter = 0.037 inches) connected to a Quick Connect Harness (SAI Infusion). Rats were allowed to recover for 5–14 d. During recovery, the catheter was flushed daily with 0.1 ml of heparin (10 U ml^{-1}) and gentamicin antibiotic (5 mg ml^{-1}) in sterile saline to help protect against infection and catheter occlusion.

Experiments were conducted in operant-conditioning chambers enclosed within sound-attenuating cabinets (Med Associates). Each chamber contains an active and inactive nose poke, a food dispenser, the conditioned stimulus (CS) light in each nose poke and a house light. No food or water was provided in the chambers during the training sessions.

Cocaine self-administration training began 5–14 d after surgery. On day 1, rats were placed in the self-administration chamber for an overnight training session on a fixed ratio (FR) 1 reinforcement schedule. Nose-poking in the active hole resulted in a cocaine infusion (0.75 mg per kg in 0.10 ml over 6 s) and illumination of a CS light inside the nose poke hole. The CS light remained on for 6 s, whereas the house light was illuminated for 20 s, during which active nose pokes were counted but resulted in no cocaine infusions. After the 20 s, the house light was turned off, and the next nose poke in the active hole resulted in a cocaine infusion. Nose pokes in the inactive hole had no reinforcement consequences, but were recorded. Rats that received at least 40 cocaine infusions in the overnight session were allowed to self-administer cocaine for 2 h for 5 consecutive days ~ 24 h after the overnight training on an FR1 reinforcement schedule. The same or similar cocaine self-administration procedures were used in our previous studies^{21–23}. Rats ($n = 5$) that did not meet the overnight number of infusions criterion ($n = 5$) were removed from the study.

We assessed incubation of cue-induced cocaine craving in extinction tests (1 h) conducted after 1, 10 or 45 d of withdrawal from cocaine self-administration. During the test sessions, active nose pokes resulted in contingent delivery of the CS light cue, but not cocaine. For behavioral assays without electrophysiology, we used within-subject assessment of incubation of cocaine craving^{24,26}; the same rats were tested for cocaine seeking on withdrawal days 1 and 45, or on days 1 and 10. For electrophysiology experiments, we used between-subject assessments; different groups of rats were killed on either withdrawal day 1, 10 or 45 without the cocaine seeking extinction tests.

Optogenetic procedures. For *in vivo* optical control of BLA projections, two 105- μm core optic fibers were modified for attachment to an internal cannula creating the optical neural interface (ONI). When the ONI was secured *in vivo* to the guide cannula, the stripped fiber extended 1.0 mm past the tip of the cannula. This experimental setup was based on a previously verified setup⁵² with slight modifications. The ONI was secured *in vivo* to the cannula head-mount only during stimulation.

For *in vivo* stimulation, the ONI was attached with a fiber-optical adaptor to a 473-nm blue laser diode (IkeCool), and light pulses were generated through a stimulator (A-M Systems). Optic fiber light intensity was measured using a light sensor (Thor Labs, S130A) and light intensity was adjusted to ~ 10 mW. Prior to attaching the ONI to head-mount guide cannula, the rat was briefly sedated with isoflurane to allow smooth insertion and to prevent damage to the optic fibers. Once the rat was awake, an LTD protocol was administered (5-Hz blue light pulses were administered for 3 min and repeated three times with 5-min intervals; pulse duration, 1.0 ms) in their home cage. Once this 19-min LTD protocol was concluded, the rat was placed in a testing chamber. Control rats were also briefly sedated with isoflurane and a sham optic fiber was attached to the head-mount guide cannula. Following awakening, the rats remained in their home cage for 19 min and were subsequently placed into a testing chamber.

For *in vitro* stimulation, all evoked responses were delivered using an IkeCool laser at 473 nm, ~ 10 mW, through the microscope's 40 \times objective. The duration (0.01–1 ms) of the light pulse was decreased until an optimal evoked response was achieved.

Electrophysiological studies. Before decapitation, the rats were anesthetized with isoflurane and subsequently transcardially perfused with 4°C cutting solution (135 mM *N*-methyl-D-glucamine, 1 mM KCl, 1.2 mM KH_2PO_4 , 0.5 mM CaCl_2 , 1.5 mM MgCl_2 , 20 mM choline- HCO_3 , 11 mM glucose, pH adjusted to

7.4 with HCl, and saturated with 95% O₂/5% CO₂). The rat was decapitated, and then the brain was removed and glued to a block before being sliced using a Leica VT1200s vibratome in 4 °C cutting solution. We cut 300- μ m-thick coronal slices were cut such that the preparation contained the signature anatomical landmarks (for example, the anterior commissure) that clearly delineate the NAc subregions. After allowing 1–2 h for recovery, one slice was transferred from a holding chamber to a submerged recording chamber where it was continuously perfused with oxygenated aCSF maintained at 30 \pm 1 °C.

AP5 was used at a concentration of 50 μ M to inhibit NMDAR-mediated responses. Picrotoxin (100 μ M) was used to inhibit GABA_AR-mediated responses. Spermine (100 μ M) was added freshly to the internal solution to restore the endogenous polyamine that blocks GluA2-lacking AMPARs. NBQX (5 μ M) was used to inhibit AMPAR-mediated responses. 1-Naphthylthylacetyl spermine trihydrochloride (Naspm) (200 μ M) was used to selectively inhibit GluA2-lacking AMPARs. AP5, NBQX and Naspm were purchased from R&D Systems, and all other chemicals were purchased from Sigma-Aldrich.

Standard whole-cell current- or voltage-clamp recording were used with a MultiClamp 700B amplifier (Molecular Device). During recordings, slices were superfused with aCSF that was heated to 30 \pm 1 °C by passing the solution through a feedback-controlled in-line heater (Warner Instruments) before entering the chamber. Recordings were made under visual guidance (40 \times , differential interference contrast optics) with electrodes (3–5 M Ω). Expression of Chr2-YFP in neurons or processes was verified using an Olympus BX51WI fluorescent/DIC microscope before recordings. The intracellular and extracellular solutions were described previously^{10,11}. For all recordings, series resistance was 8–14 Ω M and was left uncompensated. Series resistance was monitored continuously during all recordings, and a change beyond 15% resulted in exclusion of the cell from data analyses. Synaptic currents were recorded with a MultiClamp 700B amplifier (Molecular Devices), filtered at 3 kHz, amplified five times, and then digitized at 20 kHz with a Digidata 1440A analog-to-digital converter (Molecular Devices).

Neurons in the NAc shell were randomly selected for silent synapse recording. Minimal stimulation experiments were performed as previously reported^{11,13,20}. After obtaining a small (<50 pA) EPSC at –70 mV, the duration of the light pulse was reduced in small increments to the point that failures versus successes of synaptically evoked events (EPSCs) could be clearly distinguished visually. Pulse duration and frequency were then kept constant for the rest of the experiment. The amplitude of both AMPAR and NMDAR EPSCs resulting from single vesicle release is relatively large (for example, ~15 pA for AMPAR mEPSCs)¹¹, which facilitates the judgment of success versus failures of EPSCs; thus, they were defined visually. For each cell, 50–100 traces were recorded at –70 mV, and 50–100 traces were recorded at +50 mV. Recordings were then repeated at –70 mV and +50 mV for another round or two. Each cell was recorded >2 rounds. Only cells with relatively constant failure rates (changes <10%) between rounds were included for calculation of the percentage of silent synapses. Percent silent synapses were calculated using the equation $1 - \ln(F_{-70})/\ln(F_{+50})$, in which F_{-70} was the failure rate at –70 mV and F_{+50} was the failure rate at +50 mV. The major difference between minimal stimulations delivered by optical fibers and electrical electrode is that electrical stimulation can be confined to one or a few afferents around the well-defined stimulation site (that is, the tip of the stimulation electrode), whereas minimal optical stimulation will preferentially influence the afferents with the highest expression of Chr2 despite not necessarily being located together. This difference may not be a major concern for several reasons. First, the amplitudes of successful synaptic responses elicited by minimal optical stimulations are similar to those of mEPSCs (see example traces throughout the manuscript), suggesting that one or very few afferents are stimulated each time. Thus, the premise that only a very small number of synapses are activated each time is met, even without knowing the actual activation spot. Second, given that only fibers with the highest expression of Chr2 are activated with short-duration stimulation, we assume that this same set of fibers is repeatedly activated throughout the trial, similar to minimal electrical stimulation in which the same set of fibers that is most sensitive to electrical stimulation is assumed to be activated repeatedly. Third, when performing the minimal optical stimulation assay, the stimulation intensity and duration was adjusted such that failure rate was around 0.5 (range = 0.2–0.8, median = 0.5). Successes and failures were readily separable, with the pattern similar to that observed in previous studies detecting silent synapses^{10,11,13,20,53} using minimal electrical stimulation. Thus, even with potentially

different properties of optical stimulation, minimal optical stimulation can still detect silent synapses, at least semiquantitatively.

To examine AMPAR subunit composition, an *I-V* curve was plotted. Evoked BLA-to-NAc AMPAR-mediated EPSCs were measured at the membrane potentials of –70, –50, –30, –10, 0, 10, 30 and 50 mV. The rectification index was calculated by comparing the peak amplitude at +50 mV to –70 mV after offsetting the reversal potential⁵⁴.

Light-evoked LTD was induced at BLA-to-NAc synapses in cocaine- or saline-treated rats. After 10 min of stable baseline recording, an induction protocol was used that consisted of three trains of stimuli at 5 Hz (pulse duration, 0.5 ms; train duration, 3 min; 5 min apart), while holding the cells at –70 mV.

Experiment 1. Incubation of cocaine craving and measurement of silent synapses in the BLA-NAc projection on withdrawal day 1, 10, and day 45 (Figs. 2 and 4). We first verified a short incubation procedure. Briefly, we trained the rats with one overnight session on an FR1 reinforcement schedule with either saline (0.1 ml per infusion) or cocaine HCl (0.75 mg per kg, 0.1 ml per infusion). If the rats had at least 40 cocaine infusions during the overnight session, they began five consecutive 2-h training days under the same reinforcement schedule on the second following day. After completing the 5-d training with at least 10 infusions per day, the rats were placed back in the home cages for withdrawal. On withdrawal day 1 and/or 45, rats were placed in the operant chambers for a 1-h extinction test, during which nose pokes in the active holes resulted in presentation of light cues but not cocaine infusion. The number of nose pokes in the active holes was used as a measure of cocaine seeking. For these experiments, saline self-administering rats were used as control. In a different group of rats, we assessed extinction responding after 10 withdrawal days (Fig. 4). Animal use: withdrawal day 1: saline 17 rats, cocaine 20 rats; withdrawal day 10: saline 8 rats, cocaine 9 rats; withdrawal day 45: saline 9 rats, cocaine 11 rats.

We next examined cocaine-induced generation of silent synapses in rats that were treated with the same incubation-inducing cocaine regimen as described above. For this study, rats were bilaterally injected with AAV2-Chr2-YFP into the BLA and then implanted with a silastic catheter into their right jugular vein. After approximately 2 weeks of recovery, self-administration training was conducted as described above. After completing the 5-d training, the rats were used for electrophysiological analysis of silent synapses after 1, 10 or 45 d of withdrawal in their home cages (saline self-administration: day 1, 26 cells from 5 rats; day 10, 11 cells from 8 rats; day 45, 21 cells from 6 rats; cocaine self-administration: day 1, 12 cells from 3 rats; day 10, 13 cells from 5 rats; day 45, 26 cells from 11 rats). To estimate the number of silent synapses, minimal stimulation experiments were performed. Optogenetic stimulation was used to obtain a small EPSC at BLA-to-NAc synapses while holding the cells at –70 mV. Then, the duration of the light pulse was reduced in small increments to a point at which failures versus successes could be clearly defined. The recordings at the two membrane potentials were then repeated 1–2 rounds to ensure the stability. Responses immediately after (within 2.5 min) the change of holding potentials were not included for analysis. Percent silent synapses were calculated using the equation $1 - \ln(F_{-70})/\ln(F_{+50})$, in which F_{-70} was the failure rate at –70 mV and F_{+50} was the failure rate at +50 mV¹¹.

Experiment 2. AMPAR subunit composition in the BLA-to-NAc projection on withdrawal day 1, 10 and 45 (Figs. 3 and 4). The self-administration procedures were identical to the ones used in Experiment 1. Rats were killed for electrophysiological analyses after 1, 10, or 45 d of withdrawal (saline self-administration: day 1, 9 cells from 4 rats; day 10, 12 cells from 6 rats; day 45, 8 cells from 8 rats; cocaine self-administration: day 1, 10 cells from 4 rats; day 10, 11 cells from 6 rats; day 45, 21 cells from 10 rats). Picrotoxin and AP5 were added to the bath to isolated AMPAR-mediated responses and spermine was added to the internal solution to restore the endogenous polyamines that contribute to the rectification at depolarized potentials, which are indicative of GluA2-lacking AMPARs. Light pulses, <1 ms in duration, were used to evoke BLA-to-NAc EPSCs for generation of the *I-V* curve. The rectification index was calculated by comparing the peak amplitude at +50 mV to –70 mV after correcting for the reversal potential. Additional verification of the CP-AMPA component was achieved by locally applying Naspm. Light-evoked AMPAR-mediated EPSCs were obtained from saline (8 cells from 7 rats) or cocaine self-administration (8 cells from 4 rats) rats while locally perfusing aCSF through a large (inner diameter = 100–150 μ m)

bore pipette. Once a stable baseline response was achieved, the solution was switched to one that contained Nasp. Following stabilization and diffusion of solutions, data were collected for at least 5 min.

Experiment 3. Reemergence of silent synapse after blockade of CP-AMPA receptors on withdrawal day 45 (Fig. 5). The self-administration procedures were identical to the ones used in Experiment 1. Rats were killed for electrophysiological analysis after long-term withdrawal (42–47 days) (saline self-administration, 18 cells from 5 rats; cocaine self-administration, 17 cells from 6 rats). Minimum stimulation assay (described in Experiment 1) was performed first in the absence and then in the presence of Nasp. A large bore pipette, as described in Experiment 2, was used to locally supply either bath solution (control) or bath solution with the addition of Nasp.

Experiment 4. LTD at BLA-to-NAc synapses (Fig. 6a,b). The self-administration procedures were identical to the ones used in Experiment 1. Rats were killed for electrophysiological analysis after long-term withdrawal (42–47 d) (saline self-administration, 8 cells from 5 rats; cocaine self-administration, 9 cells from 6 rats). Picrotoxin was added to the bath in some conditions to verify the synaptic properties. Light pulses, <1 ms in duration, were used to evoke BLA-to-NAc EPSCs while holding the cell at -70 mV. Once a stable baseline was obtained for ~ 10 min, a light-evoked LTD-induction protocol was administered: 0.5–1-ms flashes, 5 Hz for 3 min, three times with 5-min intervals. Following this induction, recordings were continued for at least 30 min.

Experiment 5. Removal of the Nasp-sensitive component of BLA-to-NAc EPSCs by LTD on withdrawal day 45 (Fig. 6c–g). This set of recordings examined the CP-AMPA component in rats with a previous history of saline self-administration (10 cells from 7 rats) or cocaine self-administration (8 cells from 5 rats) before and after LTD induction on withdrawal day 45. We measured light-evoked AMPAR-mediated EPSCs while locally perfusing aCSF containing picrotoxin through a large bore pipette. Once a stable response was achieved, we applied a light-evoked LTD protocol (5 Hz, three times, 5-min intervals). After completion of the LTD induction, the local perfusion solution was switched to one that contained Nasp. Following stabilization, data were collected for at least 10 min.

Experiment 6. *In vivo* LTD attenuates cue-induced cocaine seeking on withdrawal day 45 (Fig. 7). The self-administration procedures were identical to those used in Experiment 1. Two groups of rats ($n = 8$ –14 per group) were tested for extinction responding (1-h session) on withdrawal days 1 and 45. Prior to the final test, on withdrawal day 35, rats were anesthetized with sodium pentobarbital (40 mg per kg) and placed in a stereotaxic apparatus. A bilateral 26 gauge guide cannula was inserted 1.0 mm above the NAc shell and secured with cranial screws and dental cement. On withdrawal day 45, the rats were divided into two groups: one group had a sham optic fiber inserted into the guide cannula; the other group received a true optic fiber ONI. The ONI was adjusted to a power level ~ 10 mW

at the optic fiber tip and the rat was briefly anesthetized to allow smooth insertion of optic fiber. Sham controls received the same brief anesthesia and installation of optical fiber. Once the rats had recovered and were freely moving, we initiated the LTD protocol with an IkeCool laser connected to a pulse generator (5 Hz for 3 min, three times, 5-min intervals) in the rats' home cage. The sham control group received an identical treatment except that the optic fiber was not functional. Once the LTD stimulation train was completed, the rats were placed in the operant chamber for a 1-h extinction test.

Data acquisition and analysis. In all electrophysiology experiments, the data were coded before analysis. Data were then de-coded for the final results. All results are shown as mean \pm s.e.m. Each experiment was replicated in at least four rats (1–3 cells were recorded from each rat) for electrophysiological analysis and eight rats for behavioral tests. No data points were excluded unless specified in the experimental procedure. For Experiments 1–6, a total of 257 rats were used, among which 5 rats were excluded before data collection because of catheter leakage or clogging, 12 rats were excluded before data collection because of surgery-associated infection or substantial (>15%) loss of body weight, 9 rats were excluded during data collection because of misplacement of cannula (found during preparation of brain slices), and 5 rats were excluded because they did not meet the cocaine self-administration training criterion. Data from the repeated runs for the same experiment were pooled together for statistical analysis. Technical replicates were used for some of the key experiments, such as insertion of CP-AMPA receptors, in which biophysical (rectification of AMPAR EPSCs) and pharmacological (sensitivity to Nasp) approaches were both employed. Sample size for each experiment was determined on the basis of either our previous experience with similar experiments or those that have been routinely used in similar studies published in this journal. Normal distribution was assumed for all statistics. This is based on our previous work related to silent synapses, in which a typical normal distribution was observed¹⁰. Variance was estimated for most major results and no significant difference was found between control and manipulation groups. Statistical significance was assessed using *t* tests (when two groups are compared) or one or two-way ANOVAs (when multiple groups or repeated measured were involved), followed by Bonferroni post-tests.

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Erratum: Maturation of silent synapses in amygdala-accumbens projection contributes to incubation of cocaine craving

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In the version of this article initially published online, the Nasp_m mean data point (black circle) under cocaine in Figure 3i was displaced upward, appearing at a vertical-axis value of ~1.0. The error has been corrected for the print, PDF and HTML versions of this article.

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