Silent synapses in selectively activated nucleus accumbens neurons following cocaine sensitization

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Cocaine-induced alterations in synaptic glutamate function in nucleus accumbens are thought to mediate drug-related behaviors such as psychomotor sensitization. However, previous studies have examined global alterations in randomly selected accumbens neurons regardless of their activation state during cocaine-induced behavior. We recently found that a minority of strongly activated Fos-expressing accumbens neurons are necessary for cocaine-induced psychomotor sensitization, whereas the majority of accumbens neurons are less directly involved. We assessed synaptic alterations in these strongly activated accumbens neurons in Fos-GFP mice, which express a fusion protein of Fos and GFP in strongly activated neurons, and compared these alterations with those in surrounding non-activated neurons. Cocaine sensitization produced higher levels of ‘silent synapses’, which contained functional NMDA receptors and nonfunctional AMPA receptors only in GFP-positive neurons, 6–11 d after sensitization. Thus, distinct synaptic alterations are induced in the most strongly activated accumbens neurons that mediate psychomotor sensitization.

Cocaine produces short- and long-lasting neuroadaptations at excitatory glutamatergic synapses in the ventral tegmental area and nucleus accumbens1–4, two brain areas that mediate drug reward5,6. These neuroadaptations include alterations of synaptic strength, as assessed by AMPA receptor (AMPAR)/NMDA receptor (NMDAR) ratios7–12, AMPAR subunit composition7,12–14, and changes in the expression of synaptic plasticity7,10–17, which are hypothesized to underlie long-lasting alterations in drug-associated behaviors1–4.

It is important to note that all of these alterations were assessed in randomly selected neurons in ex vivo brain slices regardless of their previous activation state during behavior, with the implicit assumption that most or all of these neurons undergo similar alterations and have similar roles in cocaine-induced behaviors. However, we recently found that cocaine-induced locomotor sensitization in a novel environment is mediated primarily by a distinct minority of sparsely distributed accumbens neurons that are more strongly activated than the surrounding majority of neurons, as indicated by Fos expression15. Cocaine sensitization in a novel environment is context specific, with cocaine inducing sensitized locomotion only in the environment paired with repeated cocaine injections during sensitization, and not in a non–drug-paired environment15. Cocaine administration to these sensitized rats correspondingly induces the activation marker Fos in only 2–3% of accumbens neurons that were selected by stimuli in the drug-paired environment15. Selective inhibition of these neurons activated in the drug-paired environment disrupted sensitized cocaine-induced locomotion, whereas inhibition of other neurons activated in a non–drug-paired environment had no effect15.

Thus, these selectively activated neurons undergo different alterations and have different roles in behavior than the majority of less-activated neurons following cocaine sensitization in a novel environment.

We hypothesize that repeated strong activation of these selectively activated neurons during sensitization induces unique synaptic alterations relative to the surrounding majority of neurons that are not as strongly activated. We used Fos-GFP transgenic mice, which have a transgene containing a Fos promoter that drives expression of a Fos-GFP fusion protein in strongly activated neurons19,20. We compared glutamatergic synaptic properties of the strongly activated neurons that expressed GFP with those of the surrounding majority of less-activated accumbens neurons that did not express GFP following cocaine-induced locomotion in previously cocaine-sensitized and naive mice.

RESULTS

Cocaine-induced locomotor activity and GFP expression

Previously, we found that cocaine sensitization in a novel environment produces enhanced or sensitized cocaine-induced activation of nucleus accumbens neurons as measured by Fos immunohistochemistry, which corresponds with sensitized cocaine-induced locomotion18,21,22. Here, cocaine-induced locomotion and neuronal activation in accumbens could also be sensitized in Fos-GFP mice. Mice in the repeated cocaine group received five injections, once per day, of 15 mg per kg of body weight of cocaine, whereas the acute cocaine group received 5 injections, once per day, of saline. The mice in both groups were given test injections of 20 mg per kg

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cocaine 6–11 d after the repeated injections. Prior repeated cocaine injections enhanced cocaine-induced locomotion on test day relative to cocaine-induced locomotion for the acute cocaine group (repeated, 449 ± 38 m, n = 7 mice; acute, 254 ± 36 m, n = 7 mice; t_{12} = 3.71, P < 0.01).

Neuronal activation was assessed in the same mice by counting GFP-expressing (GFP+) neurons in the nucleus accumbens shell (Fig. 1). Cocaine test injections induced significantly higher levels of GFP expression in the repeated cocaine group than in the acute cocaine group (repeated, 136 ± 10 cells per mm²; n = 7 mice; acute, 97 ± 14 cells per mm²; n = 7 mice; t_{12} = 2.29, P < 0.05). In the accumbens shell region, 136 of 167 GFP+ neurons (81.4%, n = 3 mice) also expressed Fos (Fig. 1). Lack of coexpression of Fos in a minority of GFP+ neurons could be a result of different time courses for induction and degradation of the two proteins after neuronal activation, or different antibody sensitivities and thresholds chosen for each protein when determining whether a nucleus was immunoreactive or not.

We assessed the phenotype of GFP+ neurons using DARPP-32 as a marker of GABAergic medium spiny projection neurons (Fig. 1). In the repeated cocaine group (n = 6 mice), 92 of 95 GFP+ neurons (96.8%) were DARPP-32+ whereas only three GFP+ neurons were DARPP-32–. In the acute cocaine group (n = 5 mice), 53 of 55 GFP+ neurons (96.4%) were DARPP-32+, whereas only two GFP+ neurons were DARPP-32–. Thus, the vast majority of cocaine-activated GFP+ neurons in both groups were medium spiny neurons. The high degree of co-labeling in GFP+ neurons for both DARPP-32 and Fos ensured that whole-cell recordings from the majority of GFP+ neurons that we used were from strongly activated medium spiny neurons.

Figure 2 Synaptic strength in activated nucleus accumbens neurons.
(a) Representative area of medial accumbens shell in which whole-cell recordings were performed (dashed line rectangle) and GFP+ nuclei were counted. A GFP+ neuron in the accumbens shell is indicated by a white arrow in the GFP panel. The same neuron was filled with Alexa 568 fluorophore included in the patch pipette and indicated with a white arrow in the Alexa 568 panel. (b) AMPAR/NMDAR ratios were significantly lower in GFP+ versus GFP– neurons following cocaine test injections in the repeated cocaine group (GFP+ neurons, n = 16 cells from 16 mice; GFP– neurons, n = 17 cells from 12 mice; *P < 0.01), but not in the acute cocaine group (GFP+ neurons, n = 8 cells from 6 mice; GFP– neurons, n = 12 cells from 7 mice). AMPAR/NMDAR ratios were significantly higher in GFP+ neurons in the repeated cocaine group than in GFP– neurons in the acute cocaine group (**P < 0.05). (c) AMPAR/NMDAR ratios obtained from randomly selected accumbens neurons from mice challenged with saline on test day, 6–11 d after repeated cocaine or repeated saline injections. AMPAR/NMDAR ratios were significantly higher in the repeated cocaine group than in the repeated saline group (repeated cocaine, n = 10 cells from 4 mice; repeated saline, n = 12 cells from 4 mice; naive, n = 7 cells from 2 mice; *P < 0.05). Data are expressed as mean ± s.e.m.
Reductions in AMPAR/NMDAR ratios may be a result of higher proportions of GluR2-lacking AMPARs, as the loss of this subunit would increase inward rectification. We measured rectification of glutamatergic excitatory postsynaptic currents (EPSCs) by dividing the EPSC amplitude at –80 mV by the amplitude at +40 mV (ref. 25). If more GluR2-lacking AMPARs were expressed following cocaine sensitization, then EPSCs should show greater inward rectification at depolarized membrane potentials. However, we did not observe a significant interaction of GFP and repeated cocaine exposure on AMPAR EPSC rectification (F1,27 = 0.15, P = 0.70; Fig. 3a). Also, 1-naphthyl acetyl-spermine (NASPM), a selective blocker of GluR2-lacking AMPARs, did not alter the amplitude of evoked EPSCs between GFP+ and GFP− neurons in both repeated cocaine mice (F32,416 = 1.26, P = 0.157) and acute cocaine mice (F32,288 = 0.82, P = 0.752; Fig. 3b). Thus, reduced AMPAR/NMDAR ratios in GFP− neurons following repeated cocaine were not a result of substantial changes in AMPAR subunit composition and single cocaine injections did not result in incorporation of GluR2-lacking AMPARs in GFP− neurons in acute cocaine mice.

To further assess whether smaller AMPAR/NMDAR ratios resulted from decreased AMPAR function, we determined the effects of a smaller subset of glutamate synapses on GFP+ neurons in acute cocaine mice. In repeated cocaine mice, bath-applied AMPA generated inward currents were observed between GFP+ and GFP− neurons in the acute cocaine group (F34,442 = 0.47, P = 0.995). Thus, lower AMPAR/NMDAR ratios in GFP+ neurons, compared with GFP− neurons, in repeated cocaine mice was at least partly a result of decreased AMPAR function.

We recorded spontaneous AMPAR-mediated EPSCs (sEPSCs) in GFP+ and GFP− neurons (Fig. 5). These sEPSCs likely represent action potential–independent quantal release events, as tetrodotoxin did not affect sEPSC frequency or amplitude in GFP+ or GFP− neurons from repeated cocaine mice (frequency, F1,22 = 0.05, P = 0.824; amplitude, F1,22 = 0.04, P = 0.834; GFP+, n = 6 cells, 5 mice; GFP−, n = 7 cells, 3 mice; data not shown). sEPSC amplitudes did not significantly differ between GFP+ and GFP− neurons in either the repeated or acute cocaine groups (F1,88 = 0.21, P = 0.645; Fig. 5a). However, for sEPSC frequency, we observed a significant interaction of GFP and repeated cocaine exposure (F1,88 = 13.1, P < 0.01). Post hoc tests revealed significantly lower sEPSC frequency in GFP+ neurons than in GFP− neurons from the repeated cocaine group (P < 0.05). In contrast, sEPSC frequency was significantly greater in GFP+ neurons than in GFP− neurons from the acute cocaine group (P < 0.05). In addition, post hoc tests revealed significantly higher sEPSC frequency in the GFP− neurons of the repeated cocaine group compared with GFP− neurons from the acute cocaine group (P < 0.05; Fig. 5b).

Altered sEPSC frequency in the absence of amplitude changes suggest presynaptic alterations in glutamate release. However, measurement of paired-pulse facilitation of evoked EPSCs, which are thought to reveal changes in the probability of neurotransmitter release (p0), did not differ between GFP+ and GFP− neurons from repeated and acute cocaine groups (repeated, F6,90 = 0.16, P = 0.986; GFP+, n = 8 cells from 6 mice; GFP−, n = 9 cells from 7 mice; acute, F6,78 = 0.67, P = 0.677; GFP+, n = 7 cells from 5 mice; GFP−, n = 8 cells from 4 mice; Supplementary Fig. 1). This lack of difference argues against presynaptic changes in p0. Thus, our data indicate a reduction in the number of functional AMPARs in the absence of a change of AMPAR properties in neurons from the repeated cocaine group.

Silent synapses
What might explain the lower AMPAR/NMDAR ratios, lower AMPA-induced inward currents with bath application, lower sEPSC frequency, and lack of changes in mean sEPSC amplitude or paired-pulse facilitation in GFP+ neurons from cocaine-sensitized mice? One possibility is that a larger subset of glutamate synapses on GFP+ neurons are silent, in that they contain functional NMDARs, but not inward currents.
AMPARs. Silent synapses\textsuperscript{26–32} can be detected by measuring evoked EPSCs at –80 mV, where AMPARs, but not NMDARs, are activated, and at +40 mV, where NMDARs contribute strongly to EPSCs. We set the minimal stimulus intensity to evoke EPSCs by adjusting stimulator output to elicit AMPAR-mediated currents in approximately 30–70% of trials at –80 mV. Once set, stimulus intensity was unchanged during the assessment of synaptic failures at +40 mV. There was a significant interaction of GF and repeated cocaine exposure for the difference in the failure rates between the –80- and +40-mV holding potentials ($F_{1,51} = 12.1$, $P < 0.01$). Post hoc tests revealed that this difference was significantly different in GFP+ neurons (at –80 mV, 46.9 ± 3.2%; at +40 mV, 19.8 ± 4.2%; $P < 0.001$), but not significantly different in GFP– neurons (at –80 mV, 33.6 ± 3.8%; at +40 mV, 28.8 ± 4.4%; $P > 0.05$) (Fig. 6a–c). In contrast, failure rates in neurons from acute cocaine mice did not differ between –80- and +40-mV holding potentials in GFP+ neurons (at –80 mV, 51.4 ± 7.5%; at +40 mV, 54.8 ± 7.7%, $P > 0.05$) or in GFP– neurons (at –80 mV, 48.0 ± 7.7%; at +40 mV, 45.1 ± 6.4%, $P > 0.05$).

These failure rates obtained at –80 mV and +40 mV were used to estimate the proportion of silent synapses using the equation $1 – \ln(\text{failure rate at } –80 \text{ mV})/\ln(\text{failure rate at } +40 \text{ mV})$ (refs. 27, 32). We observed a significant interaction of GFP and repeated cocaine exposure ($F_{1,51} = 12.9$, $P < 0.01$). Post hoc tests revealed a significantly greater proportion of silent synapses in GFP+ neurons than in GFP– neurons ($P < 0.001$; Fig. 6a–c), whereas the proportion of silent synapses was not different between GFP+ and GFP– neurons in the acute group ($P > 0.05$).
Silent synapses were also assessed using the coefficient of variation (CV) for AMPAR EPSCs at –80 mV and NMDAR EPSCs at +40 mV. When silent synapse levels were increased, the CV of AMPAR EPSCs was higher compared with when silent synapses were absent, resulting in a lower CV-NMDAR/CV-AMPAR ratio. We observed a significant interaction of GFP and repeated cocaine exposure (F_{1,34} = 4.79, P < 0.05). Post hoc tests revealed that, in the repeated cocaine group, the CV-NMDAR/CV-AMPAR ratio was significantly lower (P < 0.05) in GFP+ neurons (CV-AMPAR = 0.40 ± 0.03, CV-NMDAR = 0.27 ± 0.02, ratio = 0.70 ± 0.05) than in GFP- neurons (CV-AMPAR = 0.28 ± 0.03, CV-NMDAR = 0.25 ± 0.02, ratio = 0.91 ± 0.07) (Fig. 6d–f). In the acute cocaine group, the CV-NMDAR/CV-AMPAR ratio did not differ (P > 0.05) in GFP+ neurons (CV-AMPAR = 0.39 ± 0.03, CV-NMDAR = 0.38 ± 0.02, ratio = 0.97 ± 0.05) compared to GFP- neurons (CV-AMPAR = 0.33 ± 0.04, CV-NMDAR = 0.29 ± 0.03, ratio = 0.89 ± 0.07). Taken together, the results of the minimal stimulation and CV analyses indicate that cocaine sensitization was associated with a greater proportion of silent synapses in GFP+ neurons than in GFP- neurons.

A previous study identified significantly elevated levels of silent synapses in medium spiny neurons 1–2 d, but not 7–14 d, after 3–5 d of repeated cocaine injections in rats. For comparison, we used the minimal stimulation assay to measure silent synapses from randomly selected neurons in our mice 1–2 d and 6–11 d following repeated cocaine or repeated saline injections. Although there was no significant interaction of repeated cocaine and withdrawal duration for the difference in the failure rates between –80- and +40-mV holding potentials (F_{1,34} = 2.9, P > 0.05). Post hoc tests revealed significantly different failure rates for neurons in the 1–2 d withdrawal group for the repeated cocaine group (at –80 mV: 55.8 ± 4.2%; at +40 mV: 39.8 ± 5.6%; P < 0.05), but not for neurons from the repeated saline group (at –80 mV: 48.8 ± 4.9%; at +40 mV: 47.6 ± 5.2%; P > 0.05). In the 6–11 d withdrawal groups, failure rates did not differ between neurons from the repeated cocaine group (at –80 mV: 57.3 ± 4.2%; at +40 mV: 54.1 ± 4.4%; P > 0.05) and neurons from the repeated saline group (at –80 mV: 53.9 ± 7.6%; at +40 mV: 53.8 ± 7.0%; P > 0.05).

When the proportion of silent synapses were calculated for each group, we observed a significant interaction of repeated cocaine exposure and withdrawal duration (F_{1,34} = 7.2, P < 0.01). In the 1–2 d withdrawal groups, post hoc tests revealed a significantly higher proportion of silent synapses in neurons from the repeated cocaine group than in the repeated saline group (P < 0.01; Fig. 7). However, the proportion of silent synapses was not significantly different between repeated cocaine and repeated saline groups following 6–11-d withdrawal (P > 0.05). Thus, in randomly selected (presumably GFP+) neurons, the increase in silent synapses after repeated cocaine was observed only at 1–2 d, and not 6–11 d, after cocaine withdrawal.

A previous study found that silent synapses in the general population of neurons (presumably GFP+) 1–2 d after repeated cocaine had increased levels of NR2B-containing NMDARs. We assessed whether the silent synapses that we observed in GFP+ neurons 6–11 d after repeated cocaine (repeated cocaine group) also contained NMDARs with NR2B subunits. Ro-256981, a selective antagonist of NR2B-containing NMDA receptors, did not differentially alter evoked NMDAR EPSC amplitudes in GFP+ or GFP- neurons (F_{5,35} = 0.63, P = 0.98; Fig. 8a). NR2B-containing NMDARs also exhibit slower decay kinetics than their NR2A-containing counterparts. Thus, we measured the half-decay times of NMDAR EPSCs 6–11 d after repeated cocaine. NMDAR EPSC half-decay times did not differ between GFP+ and GFP- neurons (GFP+, 72.3 ± 7.7 ms; GFP-, 68.6 ± 4.3 ms; t_{5} = 0.42, P > 0.05; Fig. 8b). Overall, our data indicate that increased silent synapses in GFP+ neurons from cocaine-sensitized mice are not associated with altered NR2B subunit expression.

**DISCUSSION**

Repeated cocaine administration to rats in a novel environment induces context-specific sensitization of cocaine-induced locomotion, where the sensitized response is expressed only in the drug-paired environment, and not in a non-paired environment. We found that sensitization of mice in a novel environment enhanced cocaine-induced activation of a small number of sparsely distributed accumbens neurons that expressed GFP as an indicator of strong activation. The high degree of DARPP-32 and GFP coexpression (96% of GFP+ neurons) 1–2 d after repeated cocaine indicates that these silent synapses are different from those found on randomly selected neurons 1–2 d after withdrawal from cocaine.
neurons activated by cocaine test injections in naive mice (acute cocaine group). We previously found that these strongly activated neurons are selected by stimuli in the drug-paired environment and represent a neuronal ensemble that encodes the learned association between drug and environment that is necessary for context-specific sensitization. From our present results, we hypothesize that the GFP+ neurons forming this ensemble undergo unique synaptic alterations that likely have a role in the learned associations underlying context-specific sensitization.

The synaptic alterations found in these strongly activated GFP+ neurons are distinct from those described in previous studies that examined randomly selected accumbens neurons regardless of their activation state following cocaine exposure. Neurons in these studies likely correspond to the less-activated majority of neurons that did not express GFP in our study, and we confirmed these prior observations by showing that AMPAR/NMDAR ratios and sEPSCs were increased in GFP+ neurons from cocaine-sensitized mice. Although these global alterations have been hypothesized to have a direct role in sensitization, our previous study revealed that alterations in these non-activated neurons likely have a lesser role than the selectively activated GFP+ neurons in context-specific cocaine sensitization.

Silent synapses contain functional NMDARs, but lack functional AMPARs, resulting in an absence of synaptic transmission near the resting membrane potential where NMDA channels are inactive. Silent synapses are observed at high levels in juvenile animals and are hypothesized to represent sites of future synapse maturation. However, a previous study found that silent synapses can also be induced in adult animals and are likely involved in synaptic remodeling during cocaine exposure. Repeated cocaine injections (3–5 d, once daily) to rats in their home cage transiently induced silent synapses in randomly selected accumbens neurons 1–2 d, but not 7 d, following repeated cocaine. Our results confirm previous findings in that silent synapses were observed in randomly selected neurons 1–2 d, but not 6–11 d, following repeated cocaine. However, as sensitized cocaine-induced locomotion is normally still present 7 d and 6–11 d following repeated cocaine, silent synapses in GFP+ neurons may be a transient role in sensitization, but are likely not necessary for maintenance of sensitization of cocaine-induced locomotion.

In contrast to these results, we observed silent synapses in strongly activated GFP+ neurons up to 11 d following repeated cocaine. Thus, silent synapse expression in the strongly activated GFP+ neurons is detected after prolonged withdrawal from repeated cocaine, in contrast with that found in randomly selected GFP+ neurons. One explanation for silent synapse expression after extended withdrawal is that it occurs following more prolonged or stronger activation of these neurons during each repeated cocaine injection, perhaps as a result of cocaine exposure in a novel environment. In support of this, we and others have shown that Fos is induced more strongly in accumbens when cocaine is given in a novel environment, rather than in the less-arousing home cage. We have also shown that the same set of accumbens neurons is activated by each cocaine injection in a novel environment, which implies repeated activation of the same set of synapses. Thus, stronger activation of GFP+ neurons with each repeated cocaine injection in a novel environment may induce a more persistent increase of the same silent synapses relative to that in the surrounding population of non-activated neurons.

The silent synapses that we observed in GFP+ neurons differed from those seen previously in that NMDARs in silent synapses from randomly selected accumbens neurons contained increased levels of NR2B subunits, whereas those that we observed in GFP+ neurons did not contain increased levels of NR2B. These data suggest that silent synapses on GFP+ neurons, which have been implicated in long-lasting cocaine sensitization in a novel environment, are distinct from those reported previously.

It is also possible that higher levels of silent synapses in GFP+ neurons represent an acute short-lasting alteration resulting from enhanced activation of these neurons following cocaine test injections, and are not a result of a persistent increase following cocaine sensitization. Previously observed cocaine-induced glutamatergic synaptic alterations, including AMPAR surface expression, AMPAR/NMDAR ratios, and silent synapses found in randomly selected accumbens neurons, are thought to represent homeostatic responses to increased excitation during repeated cocaine injections and/or by cocaine test injections. This homeostatic response is also referred to as synaptic scaling, where prolonged increases in synaptic activity result in decreased excitatory synaptic transmission via AMPAR removal and prolonged decreases in synaptic activity result in increased excitatory transmission via AMPAR insertion. In this regard, GFP+ neurons in our study may have received higher levels of excitation shortly after the cocaine test injection, which activated the Fos promoter. But continuing high levels of excitation in these neurons, enabled by prior repeated cocaine-induced synaptic alterations, could have produced a greater degree of synaptic scaling than in the surrounding majority of less strongly activated neurons. This scaling could be a result of the removal of AMPARs from existing synapses, leaving only NMDARs at these sites. Alternatively, the silent synapses observed in the GFP+ neurons may have been generated via acute activity-dependent synaptogenesis. Thus, silent synapses in GFP+ neurons may be a consequence, rather than a cause, of enhanced cocaine-induced activity in these neurons.

A determination of the causal role of silent synapses in cocaine sensitization awaits methodology that permits manipulation of glutamate receptor expression specifically in these critical neurons.

As we cannot identify GFP+ neurons before cocaine test injections, we cannot distinguish whether higher levels of silent synapses are induced during cocaine sensitization or following the last acute cocaine injection. In addition, we do not know if this putative form of synaptic scaling is short- or long-lasting, as electrophysiological recordings from these neurons are constrained by the transient expression of GFP from approximately 2 h to 6–8 h after cocaine injections. This requirement for GFP expression also precludes examining alterations induced in neurons that are selectively inhibited during cocaine-induced locomotion, but still contribute to behavior.

In summary, we found that repeated cocaine injections in a novel environment increased silent synapses on neurons that have been strongly implicated in mediating learned associations between cocaine and the drug-paired environment in context-specific cocaine sensitization. Silent synapses on strongly activated GFP+ neurons appeared to be distinct, as they did not exhibit altered NR2B expression as found in previously described silent synapses in randomly selected accumbens neurons following cocaine sensitization. We hypothesize that GFP+ neurons comprise a neuronal ensemble that is recruited during cocaine sensitization in a novel environment and that increased silent synapses on these neurons represents either an acute adaptive response to increased glutamatergic excitation or are more directly involved in the development of sensitization behavior.

Sensitization is a useful model for examining drug and environment interactions, but it does not model many components of addiction. In the future, it will be important to determine whether silent synapses are similarly induced in accumbens neurons that are selectively activated during cue- and context-induced reinstatement, which are common animal models of drug relapse. Although beyond the scope of our study, it is also possible that similar silent synapses may be
found in neuronal ensembles that encode other reward-context associations, such as those between food reward and the feeding context. However, ensembles encoding food reward–context associations are likely to be distinct from those involved in context-specific cocaine sensitization, either intermingling with each other in the same brain region or located in other brain regions. Finally, our results indicate that the need to distinguish between synaptic alterations induced specifically in neurons activated during behavior and global synaptic alterations induced in the general neuronal population.

METHODS

Methods and any associated references are available in the online version of the paper.  

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AUTHOR CONTRIBUTIONS

E.K., F.C.C. and B.T.H. designed the behavioral experiments. E.K. performed these experiments with F.C.C., R.A.G. and B.T.H. E.K., A.F.H. and C.R.L. designed the electrophysiology experiments. C.R.L. designed and built the spinning disc confocal and electrophysiology apparatuses. E.K. conducted and analyzed the data from the behavior, immunohistochemistry and electrophysiology experiments. E.K., B.T.H. and C.R.L. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Animals protocols were approved by the Animal Care and Use Committee of the National Institute on Drug Abuse Intramural Research Program and were carried out according to US National Institutes of Health guidelines. Fos-GFP mice previously bred onto a C57Bl/6 background were generously donated by A. Barth (Carnegie Mellon University). Heterozygous males continued to be bred in our facilities with wild-type C57Bl/6 females obtained from Charles Rivers. All experimental mice were males heterozygous for the Fos-GFP transgene. Mice were single housed in a temperature- and humidity-controlled environment under a 12-h light:12-h dark cycle, and experiments occurred during the light phase. Water and food were available ad libitum. Mice were habituated to the colony room for at least 5–7 d before experimentation.

Cocaine treatment and behavioral experiments. For behavioral experiments, two groups of mice (mean age 8.1 ± 0.2 weeks old at time of testing) were used: the repeated cocaine group received five injections (once daily) of 15 mg per kg of cocaine (intraperitoneal, NIDA). The acute cocaine group received five saline injections. After each injection, mice were placed in 46 × 25 × 19 cm clear plastic cages in light- and sound-attenuated Optovarimax activity monitors (Columbus Instruments) for 1 h. On test day, 6–11 d after the last repeated injection, mice were habituated to the test environment for 90 min, then injected with saline and placed in the cage for an additional hour, and then injected again with cocaine (20 mg per kg) and placed back in the cage for an additional hour. Locomotion was assessed as distance traveled for 1 h following each injection.

Two more groups of mice were used for the electrophysiology experiments. Mice in the repeated cocaine group (mean age, 7.9 ± 0.2 weeks at time of testing) received five injections of cocaine (once daily, 15 mg per kg) and placed in locomotor activity chambers for 1 h each time. On test day, 6–11 d after the last injection, mice were given a test injection of cocaine (20 mg per kg) and placed back in activity chambers for 1 h, then placed in their home cages for an additional 30 min until decapitation. Mice in the age-matched acute cocaine group (mean age, 7.8 ± 0.2 weeks) did not receive prior repeated cocaine injections, but were injected on test day with 20 mg per kg cocaine and handled similar to mice in the repeated cocaine group.

To assess AMPAR/NMDAR ratios in randomly selected neurons, mice received repeated cocaine or saline injections once daily for 5 d in activity chambers. On test day, 6–11 d later, each mouse received a saline test injection in the activity chamber and was killed 90 min later. To assess silent synapses in randomly selected neurons, mice received repeated cocaine or saline injections once daily for 3–5 d in activity chambers and were killed 1–2 d or 6–11 d later without test injections.

Histochemical procedures. Mice from behavioral experiments were transcardially perfused with 4% paraformaldehyde (wt/vol) 90 min after cocaine test injections and their brains were frozen. We cut 30-µm-thick sections containing the nucleus accumbens as previously described. To assess cocaine-induced GFP expression, free-floating sections from the behavioral experiment (n = 7 per group) were washed in Tris-buffered saline (TBS; 0.025 M Tris-HCl, 0.5 M NaCl, pH 7.5), mounted on chrom-alum/gelatin–coated slides, air-dried and coveredslipped with Aqua-Mount (#TA-030-FM, Thermo Scientific). Fluorescence images of native GFP from left and right hemispheres of accumbens shell from one coronal section were captured with a Zeiss LSM 710 microscope system and ZEN 2009 software (Carl Zeiss Microimaging). To prevent antibody penetration problems from affecting the determination of the proportion of DARPP-32–labeled neurons, we limited the analysis to the upper 6–7-µm focal plane of the tissue. GFP-expressing cells and cells expressing both GFP and DARPP-32 were manually counted.

We assessed coexpression of GFP and Fos in accumbens sections from three mice from the repeated cocaine group using rabbit antibody to c-Fos (1:500 dilution, #c-Fos sc-52, Santa Cruz Biotechnology) and chicken anti-GFP to GFP (1:500 dilution; #GFP-1020, Aves Labs) as primary antibodies and streptavidin Alexa 488–conjugated goat antibody to rabbit IgG (1:200, #A11011, Invitrogen) and biotinylated donkey antibody to chicken IgG (1:200, #703-065-155, Jackson Immunoresearch) and Alexa 568–conjugated goat antibody to rabbit IgG (1:200, #A11011, Invitrogen) as secondary antibodies. Fluorescence images of Fos immunoreactivity and GFP immunoreactivity were captured from left and right hemispheres of accumbens shell from 1–2 coronal sections per mouse. GFP-expressing cells and cells expressing both GFP and Fos were manually counted.

Brain slice preparation. Mice were decapitated 90 min following test injection of cocaine, when GFP expression is near maximal, and their brains were rapidly removed and immersed in near-freezing oxygenated high-sucrose artificial cerebrospinal fluid (aCSF; 194 mM sucrose, 30 mM NaCl, 4.5 mM KCl, 1 mM MgCl2, 26 mM NaHCO3, 1.2 mM NaH2PO4 and 10 mM d-glucose, saturated with 95% O2/5% CO2). To determine AMPAR/NMDAR ratios and the proportion of silent synapses in randomly selected neurons following repeated cocaine or saline, we decapitated mice 90 min after saline injections (AMPAR/NMDAR) or without test injections (silent synapses). Coronal slices (280 µm, ~Bregma +0.98–1.54 mm, Paxinos and Franklin atlas) containing the nucleus accumbens shell (Fig. 2a) were prepared using a vibrating tissue slicer (VTT1000S, Leica Instruments). Hemisectioned brain slices were stored in a holding chamber containing normal aCSF containing 126 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 26 mM NaHCO3, 1.2 mM NaH2PO4 and 11 mM d-glucose for approximately 20 min at 35 °C before imaging and recording. A brain slice was transferred to the recording chamber and superfused with normal aCSF (2 ml min−1) at 30–32 °C. Drugs were added to superfusion using a syringe pump (Razel Scientific Instruments).

Electrophysiological recordings. Neurons were viewed with an Olympus BX51W1 microscope equipped with a spinning disk laser confocal system (PerkinElmer), near-infrared differential interference optics and a 40× water-immersion objective. Images were acquired with a high-resolution CCD camera (Hamamatsu). Confocal imaging of nucleus accumbens GFP+ neurons was conducted with 488-nm laser light and images acquired using the Ultraview RS software (PerkinElmer). GFP signals were then digitally superimposed on to differential interference images of the same neuron. To confirm that recordings were from GFP+ cells they were loaded with Alexa 568 (cat # A10437, Invitrogen) via whole-cell patch pipette. Colocalization of GFP and Alexa 568 fluorescence was confirmed within minutes of break-in (Fig. 2a). Cells not exhibiting GFP fluorescence were considered to be GFP-. From each mouse, data from 1–3 GFP+ and GFP- cells were obtained. All recordings were obtained from the accumbens shell region within 5–6 h of decapitation.

Whole-cell recordings of evoked EPSCs in accumbens shell region were obtained with an Axopatch 200B amplifier (Axon Instruments) and electrodes pulled from borosilicate capillary glass (1.5 mm outer diameter, 0.86 mm inner diameter). Currents were measured in whole-cell mode using pipettes filled with 120 mM CsMeSO4, 5 mM NaCl, 10 mM TEA-Cl, 10 mM HEPES, 4 mM QX-314, 1 mM MgEGTA, 4 mM Mg-ATP, 0.3 mM Na-GTP, 0.1 mM spermine and 0.1 µM Alexa 568 (λ = 7.2). Signals were amplified, filtered at 3–5 kHz, digitized at >10 kHz, and stored on a PC hard drive using WinWCP (courtesy of J. Dempster, University of Strathclyde) via an A/D board (PCI 6024E, National Instruments). Responses were evoked by stimulating in the medial accumbens shell (Fig. 2a) 200–400 µm away from a recorded neuron with single pulses (0.1 ms) at 0.033 Hz, from a Formvar insulated Ni-chromium wire (180 µm in diameter) bipolar electrode. Series resistance was monitored using ~10-mV voltage steps (200 ms) and only cells maintaining stable access (<10% change) were included in the analyses. EPSCs were measured in aCSF containing picrotin (100 µM, Sigma) to block GABAA receptor–mediated inhibitory postsynaptic currents. Neurons were voltage-clamped at ~70 mV except where noted. sEPSCs were measured in aCSF containing picrotin (100 µM, Sigma) to block GABAA receptor–mediated inhibitory postsynaptic currents. Neurons were voltage-clamped at ~70 mV except where noted. sEPSCs were measured in aCSF containing picrotin (100 µM, Sigma) to block GABAA receptor–mediated inhibitory postsynaptic currents. Neurons were voltage-clamped at ~70 mV except where noted. 

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currents were acquired using WinEDR (courtesy of J. Dempster, University of Strathclyde) and analyzed using miniAnalysis (Synaptosoft).

AMPAR/NMDAR ratios were measured from the averages of ten EPSCs evoked at +40 mV with and without 50 µM d(-)-2-amino-5-phosphonovaleric acid (d-AP5, Ascent). Average responses recorded during d-AP5 application (AMPAR-only EPSC) were subtracted from responses recorded without d-AP5 (AMPAR + NMDAR) to determine the NMDAR EPSC. The peak AMPAR EPSC was divided by the peak NMDAR EPSC to yield the AMPAR/NMDAR ratio. To determine whether sEPSCs were action potential independent, 1 µM tetrodotoxin (TTX, Sigma) was applied for 10 min and mean sEPSC frequency and amplitude were compared to a pre-TTX control period.

In some experiments, AMPA (1 µM, Tocris Bioscience) was applied for 10 min and holding current (V_{hold} = –70 mV) was measured every 30 s. AMPAR rectification indices were calculated by obtaining the ratio of mean evoked peak AMPAR EPSCs from three responses at –80 mV divided by mean peak AMPAR EPSCs at +40 mV (ref. 25). To assess GluR2 lacking AMPA receptors, 25 µM NASPM (Sigma) was applied for 15 min while measuring evoked EPSCs at –80 mV before and during NASPM application.

Minimal stimulation assay experiments were performed as previously described30,31 from epochs of 30 consecutive trials at –80 mV and +40 mV. The percentage of silent synapses was calculated using the equation27,32

\[ I = \frac{\ln(\% \text{ failures at } -80 \text{ mV})}{\ln(\% \text{ failures at } +40 \text{ mV})} \]

The CV analysis was performed as previously described30,31,49. We calculated the CVs from epochs of 30 consecutive trials at –80 mV for AMPAR-mediated currents and at +40 mV for NMDAR-mediated currents. The peak responses of NMDAR EPSCs were measured approximately 35 ms after the onset of the AMPAR EPSC at –80 mV. The CV was calculated for AMPAR- and NMDAR-mediated currents using the equation

\[ CV = \frac{\sqrt{\text{SV}(\text{EPSC}) - \text{SV}(\text{noise})}}{\text{mean}(\text{EPSC})}, \]

where SV = sample variance. The observer was blind to the cell type (EPSC) when determining successes or failures used to calculate the percentage of silent synapses.

To assess NR2B containing NMDA receptors, a selective antagonist33 (Ro-256981, 1 µM, Sigma) was applied for 20 min and evoked EPSCs obtained at +40 mV before and during application were compared. The time to half-decay for NMDAR-mediated EPSCs was obtained while holding the cell at +40 mV in aCSF containing 100 µM picrotoxin (Sigma) and 20 µM DNQX (Sigma).

**Statistics.** Group data are presented as mean ± s.e.m. The number of cells and animals used for each experiment are shown in the figure legends, and were established on the basis of the magnitude of the effect and the robustness of the statistical model. Paired Student’s t tests were used for cocaine-induced locomotor activity, cocaine-induced GFP expression and time to half-decay measurements. A two-factor ANOVA with GFP expression and repeated or acute cocaine as factors, followed by Bonferroni’s post hoc test, was used for sEPSC parameters, AMPAR/NMDAR ratios, AMPAR rectification, difference in failure rates at –80 mV and +40 mV, proportion of silent synapses, and CV-NMDAR/CV-AMPAR experiments. For difference in failure rates between –80 mV and +40 mV, and percentage of silent synapses from neurons in the repeated cocaine and saline groups withdrawn at 1 d and 6–11 d, data were analyzed using a two-factor ANOVA with repeated cocaine and withdrawal duration as factors, followed by Bonferroni’s post hoc test. For the TTX experiment, data were analyzed using a two-factorial ANOVA with GFP and TTX as factors. For AMPAR/NMDAR ratios in randomly selected neurons following repeated cocaine and saline injections, data were analyzed using a one-way ANOVA. Two-factor ANOVAs with repeated measures were used for AMPA, NASPM and Ro-256981 bath application and paired-pulse ratio experiments with GFP and time as factors. For AMPAR/NMDAR ratios and sEPSC experiments, cells that exhibited frequencies two s.d. from the mean were excluded from analysis. Only two-tailed P values <5% were considered to be significant for all parametric statistical tests used in these studies.

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