Opposing mechanisms mediate morphine- and cocaine-induced generation of silent synapses

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Exposures to cocaine and morphine produce similar adaptations in nucleus accumbens (NAc)-based behaviors, yet produce very different adaptations at NAc excitatory synapses. In an effort to explain this paradox, we found that both drugs induced NMDA receptor–containing, AMPA receptor-silent excitatory synapses, albeit in distinct cell types through opposing cellular mechanisms. Cocaine selectively induced silent synapses in D1-type neurons, likely via a synaptogenesis process, whereas morphine induced silent synapses in D2-type neurons via internalization of AMPA receptors from pre-existing synapses. After drug withdrawal, cocaine-generated silent synapses became ‘unsilenced’ by recruiting AMPA receptors to strengthen excitatory inputs to D1-type neurons, whereas morphine-generated silent synapses were likely eliminated to weaken excitatory inputs to D2-type neurons. Thus, these cell type–specific, opposing mechanisms produced the same net shift of the balance between excitatory inputs to D1- and D2-type NAc neurons, which may underlie certain common alterations in NAc-based behaviors induced by both classes of drugs.

Exposure to drugs of abuse reshapes future behaviors, partially by altering excitatory synapses in the NAc1. However, despite triggering many similar NAc-based behavioral consequences, such as locomotor sensitization, conditioned reward, and self-administration and relapse, exposure to stimulant versus opiate drugs induces distinctly different adaptations at NAc excitatory synapses. This cellular-behavioral disconnection is exemplified in cocaine- and morphine-exposed rodents, in which the density of dendritic spines, postsynaptic structures of excitatory synapses, is increased in the NAc after withdrawal from cocaine, but is decreased after withdrawal from morphine2,3. These opposite synaptic consequences, which may represent new synapse formation and synapse elimination, respectively4,5, raise two questions. How are the opposing synaptic modifications achieved by cocaine and morphine? And do the opposing synaptic modifications result in similar or contrasting functional alterations of the NAc?

To address these questions, we focused on drug-induced generation of silent synapses. Silent glutamatergic synapses contain functional NMDA receptors (NMDARs), with AMPA receptors (AMPArs) being either absent or highly labile6,7. In theory, silent synapses can be generated either by delivering NMDARs to new synaptic locations or internalizing AMPARs from pre-existing synapses, and they can subsequently be either stabilized by recruiting AMPARs or eliminated by synapse turnover6,7. Previous studies have found that exposure to cocaine generates silent synapses in the NAc shell (NAcSh) by synaptic insertion of new NMDARs8–10. Further analyses indicate that cocaine-generated silent synapses share core features of nascent synapses, likely corresponding to cocaine-induced, newly generated dendritic spines4,5,11. Our results support a scheme in which exposure to morphine also generates silent synapses in the NAcSh, but through internalization of AMPARs from pre-existing synapses. Furthermore, although cocaine-induced generation of silent synapses occurs preferentially in dopamine D1 receptor–expressing (D1R) medium spiny neurons (MSNs) in the NAcSh, morphine-induced silent synapses are enriched in D2R MSNs. After withdrawal, a portion of cocaine-generated silent synapses matured by recruiting AMPARs to strengthen glutamatergic input to D1R MSNs, whereas morphine-generated silent synapses were likely pruned away, resulting in weakened glutamatergic input to D2R MSNs. In several rodent models of drug of addiction, D1R and D2R NAcSh MSNs mediate opposing behavioral effects12–14. Thus, the opposing, but cell type–specific, synaptic modifications triggered by exposure to cocaine versus morphine both increase the ratio of excitatory inputs to D1R over D2R MSNs, potentially leading to the same functional shift of the NAc and thereby to common NAc-based behavioral adaptations.

RESULTS
Exposure to morphine generates silent synapses
We previously demonstrated that repeated exposure to cocaine (intraperitoneal (i.p.) injection, 15 mg/kg/d for 5 d, 1-d withdrawal) generated silent synapses in NAcSh MSNs in rats8,11. We found exposing rats to morphine, with a dosing regimen (i.p. 10 mg/kg per d for 5 d, 1-d withdrawal) that typically induces locomotor sensitization and conditioned place preference15,16, also generated silent synapses in

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NAcSh MSNs. Specifically, we first compared the trial-to-trial coefficient of variation (CV) of AMPAR- and NMDAR-mediated excitatory postsynaptic currents (EPSCs), which were isolated at −70 and +50 mV, respectively, and measured on the basis of their distinct kinetics (Fig. 1a). The CV is generally inversely proportional to the number of functional synapses and their release probability\(^1\).\(^7\) Thus, for a given set of synapses mixed with silent (NMDAR only) and nonsilent synapses, the total number of NMDAR-containing synapses (measured at +50 mV) should be greater than the number of AMPAR-containing synapses (measured at −70 mV; NMDAR-only synapses are blocked by Mg\(^{2+}\) at this voltage), resulting in reduced ratio of CV of NMDAR (CV-NMDAR) EPSCs to CV of AMPAR (CV-AMPAR) EPSCs. After 1-d withdrawal from morphine, the CV-NMDAR/CV-AMPAR ratio was decreased in NAcSh MSNs in rats (Fig. 1b–d). It is unlikely that a depolarization-induced suppression of presynaptic release may have contributed to the decrease in CV, as a reduction of release probability would predict an increase, rather than a decrease, in CV at depolarized membrane potentials. Thus, these results suggest that exposure to morphine generates silent synapses in these neurons.

We next used the minimal stimulation assay, in which low-intensity stimulations were applied to a small number of synapses, eliciting intermittent successful or failed EPSCs over the trials. Because silent synapses only respond to presynaptic transmitter release at depolarized membrane potentials, but not at near-resting potentials as a result of Mg\(^{2+}\) block of NMDARs, the failure rate at −70 mV should be higher after withdrawal (5-d data taken from b). Data in a–i were collected when rats were 50 d old. Right, summary showing morphine-induced generation of silent synapses in 70–80-d-old rats ($t_{(11)} = 3.00$, $P = 0.01$, t test). Error bars represent s.e.m. *$P < 0.05$ and **$P < 0.01$.

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**Figure 1** Exposure to morphine generates silent synapses in NAc MSNs. (a) Example traces ($n = 13$ cells) showing pharmacological separation of AMPAR and NMDAR EPSCs. At 35 ms (arrow), the amplitude of dual-component EPSCs was primarily attributable to NMDAR-mediated current. (b,c) Examples and plots of AMPAR (at −70 mV) and NMDAR (at +50 mV) EPSCs from saline- ($n = 7/6$ cells/rats; b) and morphine-exposed ($n = 6/4$ cells/rats; c) rats after 1-d withdrawal for CV assay. (d) Summary showing a significantly decreased CV-NMDAR/CV-AMPAR ratio in NACSh MSNs in morphine-exposed rats ($t_{(11)} = 2.67$, $P = 0.02$, t test). (e–g) Example EPSCs elicited by minimal stimulations at +50 and −70 mV and their trial plots from saline-exposed ($n = 9/6$ cells/rats; e), cocaine-exposed ($n = 10/7$ cells/rats; f) and morphine-exposed ($n = 13/11$ cells/rats; g) rats. (h) Summary showing the increased percentage of silent synapses in NACSh MSNs after 1-d withdrawal from cocaine or morphine ($t_{(2,28)} = 4.85$, $P = 0.02$, one-way ANOVA with Bonferroni post-test). (i) Left, summary showing that silent synapses were gradually generated during exposure to morphine and declined after withdrawal (5-d data taken from b). Data in a–i were collected when rats were 50 d old. Right, summary showing morphine-induced generation of silent synapses in 70–80-d-old rats ($t_{(11)} = 3.00$, $P = 0.01$, t test). Error bars represent s.e.m. *$P < 0.05$ and **$P < 0.01$. 

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**Table 1** Summary of silent synapses among all recorded synapses. (A) Basal levels. (B) 1 d exposure. (C) 2 d exposure. (D) 3 d exposure. (E) 5 d exposure. (F) 7 d withdrawal. (G) Morphine. (H) Saline. (I) Cocaine. (J) Morphine.
Morphine generates silent synapses via AMPAR internalization

Cocaine-induced generation of silent synapses is accompanied by synaptic insertion of GluN2B-containing NMDARs. Other features suggest that cocaine-generated silent synapses are newly formed. Consistent with this hypothesis, we observed prolonged decay kinetics of NMDAR EPSCs in rat NAcSh MSNs 1 d after cocaine administration, and increased sensitivity of NMDAR EPSCs to the GluN2B-selective antagonist Ro256981 (200 nM), suggesting an increase in the relative weight of synaptic GluN2B NMDARs. However, such changes were not detected after morphine exposure, suggesting that morphine-induced generation of silent synapses is not mediated by insertion of GluN2B NMDARs to new synapses. We then examined whether morphine-induced generation of silent synapses was mediated by internalization of AMPARs from pre-existing synapses. We injected the rats intravenously with a Tat-GluA23Y scrambled peptide with saline or a Tat-GluA23Y scrambled peptide with cocaine. Although the GluA23Y peptide with saline did not affect cocaine- or morphine-induced generation of silent synapses, the GluA23Y peptide with cocaine prevented morphine-induced, but not cocaine-induced, generation of silent synapses. Consistent with this hypothesis, we observed prolongation of decay kinetics of NMDAR EPSCs in NAcSh MSNs 1 d after saline (Fig. 2a), decay kinetics of NMDAR EPSCs was measured by the half-decay time, the time elapsed from the peak amplitude to half peak amplitude. (Fig. 2b, c) Example traces and summary showing that co-administration of GluA23Y prevented morphine-induced, but not cocaine-induced, generation of silent synapses. Morphine (11/7) than in saline-exposed (10/7) rats (F2,10 = 15.71, P = 0.00, two-way ANOVA with Bonferroni post-test). Following Ro256981, AP5 (50 μM) was applied. Calibration bars represent 10 ms, 5 pA. (Fig. 2d, e, f) Summary showing that co-administration of scrambled peptide did not affect cocaine- or morphine-induced generation of silent synapses (F2,23 = 6.15, P = 0.01, one-way ANOVA with Bonferroni post-test). (Fig. 2g, h) Example traces evoked by minimal stimulation and their trial plots in NAcSh MSNs from rats 1 d after 5-d co-administration of GluA23Y with saline (19/12 cells/rats), cocaine (20/10 cells/rats) or morphine (10/7 cells/rats). (Fig. 2i, j) Summary showing that co-administration of GluA23Y prevented morphine-induced, but not cocaine-induced, generation of silent synapses (F2,46 = 3.44, P = 0.04, one-way ANOVA with Bonferroni post-test). Error bars represent s.e.m. *P < 0.05.

Figure 2 Morphine-induced generation of silent synapses is mediated by AMPAR internalization. (a) Example traces (n = 38 cells) showing that the decay kinetics of NMDAR EPSCs was measured by the half-decay time, the time elapsed from the peak amplitude to half peak amplitude. (b) Example NMDAR EPSCs in NAcSh MSNs 1 d after saline (n = 13/7 cells/rats), cocaine (n = 11/6 cells/rats) or morphine (n = 14/6 cells/rats) administration. (c) Summary showing that exposure to cocaine, but not morphine, prolonged the decay kinetics of NMDAR EPSCs in NAcSh MSNs 1 d after saline administration. (d) Example traces showing that Ro256981 (200 nM) inhibited NMDAR EPSCs in NAcSh MSNs greater in cocaine-exposed rats (n = 10/6 cells/rats) than in saline-exposed (n = 12/6 cells/rats) or morphine-exposed (n = 11/7 cells/rats) rats (F2,810 = 15.71, P = 0.00, two-way ANOVA with Bonferroni post-test). Following Ro256981, AP5 (50 μM) was applied. Calibration bars represent 10 ms, 5 pA. (e) Example EPSCs evoked by minimal stimulation and their trial plots in NAcSh MSNs from rats 1 d after 5-d co-administration of GluA2 scrambled peptide with saline (n = 10/5 cells/rats), cocaine (n = 8/4 cells/rats) or morphine (n = 8/5 cells/rats). (f) Summary showing that co-administration of scrambled peptide did not affect cocaine- or morphine-induced generation of silent synapses (F2,23 = 6.15, P = 0.01, one-way ANOVA with Bonferroni post-test). (g) Example EPSCs evoked by minimal stimulation and their trial plots in NAcSh MSNs from rats 1 d after 5-d co-administration of GluA23Y with saline (n = 19/12 cells/rats), cocaine (n = 20/10 cells/rats) or morphine (n = 10/7 cells/rats). (h) Summary showing that co-administration of GluA23Y prevented morphine-induced, but not cocaine-induced, generation of silent synapses (F2,46 = 3.44, P = 0.04, one-way ANOVA with Bonferroni post-test). Error bars represent s.e.m. *P < 0.05.
Figure 3 Alterations in spine morphology of rat NAc MSNs 1 d after morphine or cocaine administration. (a) Example image (n = 12/6 slices/rats) showing a NAcSh MSN labeled with DiI. Arrow indicates a secondary dendrite. (b) Example images (n = 12/6 slices/rats) showing that spines located on secondary dendrites were sampled for spine density analysis. (c,d) Examples of different spine types (n = 12/6 slices/rats) along a dendrite (c) or individually presented (d). Blue, magenta, yellow and red arrows indicate stubby, long-thin, mushroom-like and filopodia-like spines, respectively. (e) Example images from saline-exposed (n = 17 rats), cocaine-exposed (n = 16 rats) or morphine-exposed (n = 18 rats) rats without co-administration of peptides, with co-administration of scrambled peptide or with co-administration of GluA23Y. (f) Summary showing that total spine density was selectively increased in cocaine-exposed rats and co-administration of GluA23Y did not alter this effect (F(2,42) = 44.91, P = 0.00). (g) Summary showing that density of filopodia-like spines was increased in cocaine- and morphine-exposed rats; co-administration of GluA23Y prevented morphine-induced, but not cocaine-induced, increases in filopodia-like spines (F(2,42) = 44.91, P = 0.00). (h) Summary showing that density of mushroom-like spines was not affected 1 d after cocaine or morphine administration (F(2,42) = 7.22, P = 0.00). (i) Summary showing that density of long-thin spines was increased in cocaine-exposed rats, but decreased in morphine-exposed rats, and these effects were prevented by co-administration of GluA23Y (F(2,42) = 27.56, P = 0.00). (j) Summary showing that density of stubby spines was not changed by exposure to cocaine or morphine, and was not affected by co-administration of GluA23Y (F(2,42) = 0.97, P = 0.39). Two-way ANOVA with Bonferroni post-test was used in all above statistics. Error bars represent s.e.m. *P < 0.05 and **P < 0.01.

peptide each time they received cocaine or morphine (Online Methods). GluA2-containing AMPARs are enriched at NAc excitatory synapses19. The synthetic Tat-tagged peptide (EEEGKGYNVYG877) can translocate into neurons and block experience-dependent AMPAR endocytosis with minimal effects on constitutive AMPAR trafficking or basal synaptic transmission20, and these functional specificities have been confirmed in NAc MSNs21. Co-administration of the control (scrambled) peptide Tat-GluA2 (VYKGYGYNE) (1.5 nmol/g), which does not affect AMPAR trafficking21, did not affect the basal level of silent synapses (in saline-exposed rats) and did not affect cocaine- or morphine-induced generation of silent synapses in NAcSh MSNs (Fig. 2e,f). However, co-administration of Tat-GluA23Y (1.5 nmol/g), which prevents AMPAR internalization, prevented morphine-induced, but not cocaine-induced, generation of silent synapses (Fig. 2g,h). Note that Tat-GluA23Y treatment appeared to also lower the percentage of silent synapses in cocaine-exposed rats, suggesting that there are additional mechanisms for cocaine-induced generation of silent synapses. Collectively, these results suggest that opposing mechanisms, namely insertion of NMDARs to new synapses versus internalization of AMPARs from pre-existing synapses, are employed by cocaine versus morphine, respectively, to generate silent synapses in the NAcSh.
Morphine-induced spine elimination in rats

Cocaine-induced generation of silent synapses in the NAcSh is accompanied by an increased density of MSN spines, and a molecular manipulation that prevents cocaine-induced generation of silent synapses prevents cocaine-induced increase in spine density. These and other results lead to the speculation that cocaine-generated silent synapses are new synapses and new spines of NAc MSNs. On the other hand, the MSN spine density is decreased after morphine withdrawal and AMPAR internalization-mediated generation of silent synapses can be an initial step toward synapse and spine elimination. We therefore used the peptides verified above to explore the relationship between cocaine- and morphine-generated silent synapses and spine morphology. We injected rats with Tat-GluA2Y or scrambled peptide intravenously each time that they received cocaine or morphine.

Using Dil staining, we focused on secondary dendrites of rat NAcSh MSNs, which densely express at least four types of spines: filopodia-like spines, which are long dendritic protrusions without apparent heads; mushroom-like spines, which are protrusions with a diameter of spine heads greater than twice that of their necks; long-thin spines, which are protrusions with spine heads 1–2-fold larger in diameter than their necks; and stubby spines, which are short and thick protrusions without detectable heads. Although not absolute, a heuristic theme of spine morphology is that mushroom-like spines are more mature and stable postsynaptic structures enriched in AMPARs, whereas filopodia-like and long-thin spines are transitional postsynaptic structures with shorter life times and fewer or no AMPARs.

We observed an increase in total spine density on rat NAcSh MSNs 1 d after cocaine administration, as reported previously. This increase appeared to be primarily attributable to increases in filopodia-like and long-thin spines, but not mushroom-like or stubby spines (Fig. 3g–j). These effects were intact when scrambled peptide or GluA23Y was co-administered with cocaine (Fig. 3e–j), suggesting that cocaine-induced spinogenesis was independent of AMPAR internalization.

In stark contrast, total spine density on NAcSh MSNs was not altered 1 d after morphine administration (Fig. 3a–d), and the proportion of filopodia-like spines was increased and the proportion of long-thin spines was decreased, suggesting a conversion of long-thin spines to filopodia-like spines (Fig. 3e–j). These potential spine-weakening effects were prevented when GluA23Y, but not scrambled, peptide was co-administered with morphine (Fig. 3e–j), suggesting an essential role of AMPAR internalization in this process.

The cocaine-induced increase in total spine density on NAcSh MSNs persisted 21–28 d after cocaine administration, and this increase was not only attributable to increases in filopodia-like and long-thin spines, but also to mushroom-like spines (Fig. 4). Thus, some immature spines were strengthened and matured after cocaine withdrawal. These effects of cocaine were intact in rats that were co-administered GluA23Y during cocaine exposure.

In contrast, 21–28 d after morphine administration, total spine density on NAcSh MSNs was decreased, which was primarily attributable to the loss of long-thin spines (Fig. 4). These results suggest that some weakened spines observed 1 d after morphine administration were eliminated after long-term withdrawal. Furthermore, co-administration of GluA23Y during morphine exposure prevented all these effects observed 21–28 d after morphine withdrawal, suggesting that preventing the initial AMPAR internalization and synaptic weakening prevents subsequent synapse elimination after morphine withdrawal.
Silent synapse–based remodeling of NAc circuits

We used transgenic mice in subsequent experiments to examine cell type–specific expression of silent synapses and behavioral consequences. We therefore used mice to perform the same experiments as described above (Figs. 3 and 4), and detected similar effects of cocaine and morphine on NAcSh MSN spines (Fig. 5). These data confirm that preventing AMPAR internalization prevents morphine-induced spine elimination.

Do the opposing processes of cocaine- and morphine-induced generation of silent synapses lead to the same or different functional alterations in the NAcSh? To address this question, we examined cocaine- and morphine-induced generation of silent synapses in D1R and D2R NAcSh MSNs using a mouse line in which D1R MSNs are genetically tagged with tdTomato24. Using genetically labeled mice, previous studies indicate that the presence and absence of fluorescence signals predict D1R versus D2R MSNs24,25. The percentage of

Figure 5 Drug-induced alterations in spine morphology of mouse NAc MSNs. (a–f) 1-d withdrawal. (a) Example NAcSh dendrites from saline-exposed (n = 16 mice), cocaine-exposed (n = 21 mice) or morphine-exposed (n = 25 mice) mice with or without co-administration of peptides. (b) Summary showing that density of total spines was increased in cocaine-exposed mice (F(2,53) = 62.09, P = 0.00). (c) Summary showing that density of filopodia-like spines was increased in cocaine- and morphine-exposed mice, and morphine-induced increase was prevented by GluA23Y co-administration (F(2,53) = 87.71, P = 0.00). (d) Summary showing that density of mushroom-like spines was not affected in cocaine- or morphine-exposed mice (F(2,53) = 4.42, P = 0.02). (e) Summary showing that density of long-thin spines was increased in cocaine-exposed mice (F(2,53) = 31.49, P = 0.00). (f) Summary showing that the density of stubby spines was not altered in any experimental group (F(2,53) = 0.81, P = 0.45). (g–i) 21-d withdrawal. (g) Example NAcSh dendrites from saline-exposed (n = 13 mice), cocaine-exposed (n = 15 mice) or morphine-exposed (n = 17 mice) mice. (h) Summary showing that density of total spines was increased in cocaine-exposed mice, but decreased in morphine-exposed mice, and GluA23Y co-administration prevented the morphine effects (F(2,36) = 60.97, P = 0.00). (i) Summary showing that density of filopodia-like spines was not affected in cocaine- or morphine-exposed mice (F(2,36) = 0.33, P = 0.72). (j) Summary showing that density of mushroom-like spines was increased in cocaine-exposed mice (F(2,36) = 20.65, P = 0.00). (k) Summary showing that density of long-thin spines was increased in cocaine-exposed mice (F(2,36) = 37.17, P = 0.00). (l) Summary showing that density of stubby spines was not altered in any experimental group (F(2,36) = 1.03, P = 0.37). Two-way ANOVA with Bonferroni post-test was used in all above statistics. Error bars represent s.e.m., *P < 0.05 and **P < 0.01.
silent synapses in tdTomato-positive (+) NAcSh MSNs (operationally defined as D1R MSNs) was increased in cocaine-exposed mice 1 d after the 5-d drug procedure, but not in morphine-exposed mice (Fig. 6a,b). In contrast, the percentage of silent synapses in tdTomato-negative (−) MSNs (operationally defined as D2R MSNs) was not affected in cocaine-exposed mice, but was increased in morphine-exposed mice (Fig. 6c,d). Thus, exposure to cocaine versus morphine preferentially generates silent synapses in NAcSh D1R and D2R MSNs, respectively.

If cocaine-induced generation of silent synapses involves a synaptogenesis-like process, the potential maturation of these silent synapses by recruiting AMPARs after prolonged withdrawal should strengthen the overall excitatory synaptic strength in D1R NAcSh MSNs. On the other hand, if morphine-induced generation of silent synapses is a transition toward synapse elimination, the potential elimination after prolonged withdrawal should weaken the overall excitatory synaptic strength in D2R NAcSh MSNs. These changes would therefore lead to a common circuitry consequence: an increase
in the ratio between excitatory synaptic inputs to D1R MSNs over D2R MSNs after prolonged drug withdrawal. To test this possibility, we simultaneously recorded EPSCs from D1R and D2R NAcSh MSNs in response to the same presynaptic stimulation after 21-d withdrawal from cocaine or morphine exposure. On average, the single stimulation should normalize presynaptic factors, allowing the dual recording to reveal potentially different excitatory synaptic strengths in D1R versus D2R MSNs (Fig. 6c). Following the same presynaptic stimulation, EPSCs were elicited in both D1R and D2R MSNs (Fig. 6f). The ratio of excitatory inputs to D1R over D2R MSNs (EPSCD1/EPSCD2), which was measured by the peak amplitudes of EPSCs in D1R MSNs over the peak amplitudes of EPSCs in D2R MSNs, was increased in both cocaine- and morphine-exposed mice (Fig. 6f,g).

Behavioral correlates

D1R and D2R NAc MSNs have distinct roles in drug-induced locomotor responses and drug-conditioned learning. Our results suggest that co-administration of GluA23Y prevented silent synapse–mediated synaptic weakening selectivity in D2R MSNs following exposure to morphine. We therefore used the GluA23Y-based manipulations to explore the role of this cell type–specific synaptic remodeling in both morphine-induced and cocaine-induced locomotor responses and conditioned place preference (CPP).

In locomotor tests, mice with co-administration of GluA23Y scrambled peptide or without co-administration of a peptide similarly exhibited progressively increased locomotor responses during the 5-d morphine procedure (10 mg/kg/d) compared with saline-injected mice (Fig. 7a,b). Likewise, 21 d after the 5-d procedure, a challenge injection of morphine (10 mg/kg) induced similar locomotor responses in all morphine-exposed mice, including mice receiving co-administration of GluA23Y scrambled peptide or no peptide (Fig. 7c). Similar to morphine, a challenge injection of cocaine (15 mg/kg) on 21 d after the 5-d cocaine procedure (15 mg/kg per d) induced similar locomotor responses in mice with co-administration of GluA23Y scrambled peptide or no peptide during the 5-d procedure (Supplementary Fig. 1a,b). We next examined the acute effects of GluA23Y on cocaine- or morphine-induced locomotor responses. We gave mice GluA23Y or scrambled peptide 21 d after the 5-d cocaine or morphine procedure without co-administration of GluA23Y (Supplementary Fig. 1c,d,f), followed by a challenge injection of cocaine (15 mg/kg) or morphine (10 mg/kg), respectively. The challenge drug–induced locomotor responses were similar in mice with co-administered GluA23Y or scrambled peptides in both cocaine- and morphine-exposed mice (Supplementary Fig. 1e,g). Collectively, these results suggest that AMPAR internalization–mediated generation of silent synapses is not required for cocaine- or morphine-induced locomotor responses.

In CPP tests, mice received daily, alternating conditioning for 40 min both with drug (saline control, cocaine, or morphine) and with saline, separated by 6 h, for 5 d (Fig. 7d and Online Methods). Immediately before the chamber pairing, mice received bilateral intra-NAcSh administration of GluA23Y or scrambled peptide. 21 d after the 5-d conditioning (day 28), the mice spent more time in the cocaine- or morphine-paired chambers compared to before conditioning. The increase of time in drug-paired chambers was similar in cocaine-exposed mice treated with GluA23Y or scrambled peptide, indicating that preventing AMPAR internalization–mediated generation of silent synapses did not prevent cocaine-induced CPP (Fig. 7e). However, morphine-induced CPP was selectively disrupted in GluA23Y-administered, but not scrambled peptide-administered, mice (Fig. 7e). These results suggest that silent synapse–based remodeling of NAcSh circuits is required for morphine-conditioned learning.

DISCUSSION

We found that silent synapses can be intermediate, transitional structures in both synapse formation and elimination; they are used after exposure to cocaine versus morphine to differentially remodel excitatory inputs to NAcSh MSNs, resulting in similar circuitry consequences. These results can explain some of the common behavioral responses to stimulant and opiate classes of addictive drugs in the face of very different molecular-cellular adaptations.

Generation and elimination of spines and synapses

After long-term withdrawal from cocaine or morphine, increased or decreased spine densities are observed, respectively, in the NAc, which are indicative of opposing synaptic remodeling. The morphine-induced decrease in spine density may represent synapse elimination, and our results suggest that this process is achieved through two steps,
the initial silencing and weakening of existing synapses via internalization of AMPARs, followed by pruning of silent and weakened synapses. Abundant in the developing brain, silent synapses are often nascent, immature synapses; they are highly unstable, and can either mature and stabilize after frequent use or be eliminated if not used6,7. Thus, synapse formation, maturation and elimination are connected mechanisms that function together to form new circuits and craft them from generalized connections into dedicated ones during brain development. In light of this, we hypothesize that developmental mechanisms of synapse elimination are awakened after morphine exposure, and generation of silent synapses is the first step toward synapse elimination.

Internalization of AMPARs

Reflecting morphine-induced generation of silent synapses, the CV of AMPAR EPSCs was increased relative to the CV of NMDAR EPSCs (Fig. 1), suggesting decreased AMPAR-mediated responses. Furthermore, GluA23Y, which selectively blocks experience-dependent endocytosis of GluA2-containing AMPARs with minimal effects on constitutive AMPAR trafficking, prevented morphine-induced generation of silent synapses (Fig. 2). These results suggest that morphine-induced generation of silent synapses is mediated by internalization of AMPARs from pre-existing synapses, likely through experience-dependent synaptic plasticity (for example, long-term depression). Experience-dependent synaptic plasticity is highly pathway-specific: only synapses that are activated by experience are subject to plastic changes. Thus, it is reasonable to postulate that the primary pharmacological effects of morphine on AMPAR internalization22 together with the circuit-specific effects of morphine-induced emotional and motivational experience produce cell type–specific generation of silent synapses.

Although these and our previous results suggest that cocaine-induced generation of silent synapses is predominately mediated by insertion of GluN2B NMDARs to new synapses, these results do not rule out the possibility that a portion of silent synapses, likely in certain types of neurons or in certain projections, are generated through internalization of AMPARs. Indeed, recent results suggest that in ~3% of NAc neurons that are activated by cocaine re-exposure after cocaine withdrawal, silent synapses are generated independent of GluN2B NMDARs, and likely through internalization of AMPARs28. Echoing this finding, although GluA23Y did not prevent cocaine-induced generation of silent synapses, the level of cocaine-generated silent synapses was lower in GluA23Y-treated rats than in control rats (14% versus 23%; Fig. 2f,h). Thus, exposure to cocaine or morphine likely triggers both synaptogenesis and synapse elimination processes—at different synapses, in different afferents or in different cell types, with synaptogenesis effects predominating in cocaine-exposed animals and synapse elimination effects predominating in morphine-exposed animals. In either case, generation of silent synapses can serve as a transitional step to achieve the ultimate synaptic remodeling, that is, synapse formation or elimination.

Silent synapses and dendritic spines

Our results indicate that the densities of thin spines, including filopodia-like or long-thin spines, increased following drug-induced generation of silent synapses and declined to basal levels following the disappearance of silent synapses. Furthermore, administration of GluA23Y, which prevented morphine-induced generation of silent synapses, prevented morphine-induced increases in thin spines. These correlative results suggest that the increased thin spines are likely the neuronal substrates of drug-generated silent synapses. This notion is consistent with several key features of thin dendritic spines that were determined previously.

First, although a small number of nonsynaptic spines exist29, and mushroom-like spines tend to have better-defined presynaptic boutons30,31, extensive evidence indicates that even the thinnest spines often express signature synaptic proteins, make synapses with presynaptic boutons, and respond functionally to locally uncaged glutamate32–35. Thus, most drug-generated thin spines are likely postsynaptic structures of true synapses.

Second, it has been consistently observed that the volume of the spine head is positively correlated with the size of the postsynaptic density and the content of AMPARs23,36, with AMPARs being abundant in mushroom-like spines, but sparse or absent in long-thin and filopodia-like spines37. In contrast, the number of NMDARs is much less dependent on spine size36,38,39. Thus, compared with mushroom-like spines, a portion of thin spines is more likely to be the neuronal substrates of silent synapses in the NAcSh after short-term withdrawal from cocaine or morphine.

Third, although the currently used categorization of dendritic spines is heuristic with respect to understanding their structure-function relationship, extensive electron microscopic studies have shown that dendritic spines constitute a structural continuum rather than clear-cut categories23. Particularly for thin spines, it is most likely that they are a mixture of AMPAR-absent and AMPAR-sparse synapses; these two populations of synapses are morphologically similar, but functionally distinct. As such, although thin spines in cocaine- and morphine–pre-exposed animals share morphological similarities, they may have different receptor compositions, functionalities or cellular fates.

Fourth, time-lapse studies in the neocortex have shown that, although some thin spines are persistent, the majority of thin spines are transient, either disappearing or stabilizing into mushroom-like spines40. These results suggest that most thin spines are intermediate structures, mediating the transmission from weak, newly generated synapses toward matured synapses, or from matured synapses toward weak synapses that can be eventually eliminated. We found that morphine-generated thin spines disappeared after withdrawal, with the time course approximately consistent with the disappearance of silent synapses (Figs. 3 and 4). These transient thin spines may correspond to morphine-generated silent synapses. After 21-d withdrawal from cocaine, however, the density of thin spines remained high, despite the disappearance of silent synapses (Figs. 1, 3 and 4). We speculate that, during cocaine withdrawal, some thin spines have already recruited AMPARs, but have remained morphologically thin. This speculation is consistent with the in vivo analysis of the adult somatosensory cortex, in which, unlike in vitro preparations41, the time course of final spine maturation lasts for days to weeks34.

Balance between D1R and D2R circuits

D1R- and D2R MSNs are the two major neuronal subtypes in the NAc with distinct, and often opposing, circuitry and behavioral functions3,13,36,42. Previous studies revealed a monomodal distribution of the percentage of silent synapses across randomly recorded NAcSh MSNs in cocaine-exposed rats, suggesting that exposure to cocaine generates silent synapses in both D1R and D2R MSNs11. Using D1-tdTomato mice, we found a clear separation between D1R and D2R MSNs in cocaine- and morphine-induced generation of silent synapses. In addition, we did not detect a substantially higher percentage of silent synapses in D1R or D2R MSNs in cocaine- or morphine-exposed mice (Fig. 6) than in randomly sampled MSNs in rats (Fig. 1). These seemingly discrepant results may be reconciled.
by two technical considerations. First, the estimated percentage of silent synapses in drug-exposed animals is the sum of basal and drug-generated silent synapses, and thus not exclusively attributable to drug effects. As such, an increase in the drug-generated portion does not result in the same magnitude of increase in overall percentage of silent synapses, particularly in certain experimental conditions in which the basal silent synapse levels are high. Second, when the data for D1R and D2R MSNs in Figure 6 were pooled together as total NAcSh MSNs, the percentage of silent synapses was still significantly higher after exposure to cocaine or morphine (saline, 8.0 ± 3.1%; cocaine, 27.6 ± 4.6%; morphine, 23.2 ± 4.3%; F(2,56) = 5.8, P = 0.01, one-way ANOVA, P = 0.01 saline versus cocaine, P = 0.04 saline versus morphine, Bonferroni post-test). Based on these considerations, cell type-specific generation of silent synapses is a bona fide conclusion, at least at a semiquantitative level.

Excitatory synapses on NAc D1R and D2R MSNs undergo differential molecular and cellular adaptations after exposure to cocaine and differentially contribute to cocaine-induced behaviors. For example, the density of dendritic spines and the frequency of miniature EPSCs are selectively and persistently increased in D1R NAc MSNs after exposure to cocaine and morphine. Furthermore, accumulation of ΔFosB after cocaine withdrawal is also selectively observed in D1R MSNs, and overexpression of ΔFosB increases the number of immature spines in D1R, but not D2R, NAc MSNs. In addition, the ratio of synaptic strength on D1R versus D2R MSNs is increased in certain excitatory projections to the NAc after exposure to cocaine. These results are consistent with our findings that D1R MSNs are primarily targeted by cocaine for synaptogenesis and strengthening of excitatory synapses. On the other hand, our current results demonstrate a distinct form of silent synapse-based synaptic remodeling selectively in D2R NAcSh MSNs after morphine exposure, which may decrease the overall excitatory drive to these neurons. Consistent with this notion, recent findings have shown that withdrawal from morphine decreases the frequency, but not the amplitude, of miniature EPSCs in D2R NAc MSNs, an effect that can result from synapse elimination. Given that the two subtypes of NAc MSNs often exert antagonistic effects on acute drug-related behaviors—namely activation of D1R MSNs or inhibition of D2R MSNs enhance behavioral responses to drugs of abuse, the differential effects of cocaine and morphine perceptively produce an equivalent circuitry consequence, a shift in the balance between excitatory inputs to D1R versus D2R NAc MSNs.

In parallel with generation of silent synapses and potential synaptic elimination in D2R NAc MSNs, recent findings have revealed that exposure to morphine also alters excitatory synapses on D1R MSNs. For example, withdrawal from morphine increases the ratio of AMPAR- to NMDAR-mediated EPSCs in D1R MSNs, suggesting a potentiation of these synapses. These effects are thought to be mediated by synaptic insertion of new, GluA2-lacking AMPARs, and are therefore unlikely to be prevented by GluA2-interacting peptide GluA2Y. These effects of morphine in D1R MSNs may contribute to the persisting morphine-induced locomotor responses in GluA2Y-administered mice (Fig. 7 and Supplementary Fig. 2), supporting the notion that a D1R NAc MSN circuit predominates in drug-induced locomotor responses.

Using cell type-specific expression of tetanus toxin, recent studies found that inhibiting the output of D1R, but not D2R, NAc MSNs impairs the acquisition of cocaine- or palatable food-induced CPP as well as acquisition of reward-directed operant tasks. We found that preventing silent synapse-based remodeling of a D2R NAc MSN circuit disrupted morphine-induced CPP (Fig. 7), revealing a new role for D2R MSN-specific synaptic remodeling in conditioned drug responses. Silent synapse-based D2R MSN-specific circuit remodeling may contribute to morphine-induced CPP at several levels. First, similar to D1R MSN-oriented effects by cocaine, D2R MSN-selective synaptic weakening by morphine caused the same direction of shift between the excitatory inputs to D1R versus D2R MSNs, and this shift may serve as an essential step for acquiring conditioned responses. Second, D2R MSN-specific circuit remodeling may also contribute to the retention of acquired CPP. Previous studies revealed that a similar morphine procedure induces CPP that persists for >12 weeks. Such long-term CPP-related memories must be retained by mechanisms that are sufficiently rigid and durable to persist despite different forms of memory dissipation processes over time. One consequence of silent synapse-mediated synaptic remodeling is the reduced driving force, and thus reduced functional output, of D2R NAc MSNs. It has been observed that animals with inhibited output of D2R NAc MSNs are rigidly locked to established reward-conditioned responses and resistant to updated conditioning. Thus, silent synapse-mediated weakening of the D2R NAc MSN circuit may contribute to the stabilization of morphine-induced CPP. Third, activation of D2R NAc MSNs induces conditioned place aversion. Reduced activity of D2R NAc MSNs after silent synapse-mediated circuitry remodeling may therefore also decrease the morphine- morphine withdrawal-associated anhedonic effects, facilitating CPP acquisition or retention.

Our findings leave unanswered at least three major questions. First, NAc D1R and D2R MSNs receive excitatory projections from several brain regions. Is the cell type-specific generation of silent synapses also projection specific? Second, does the cell type-specific rule hold after contingent exposure to cocaine and morphine? Third, our results, as well as other published findings, support the notion that D1R versus D2R NAc MSNs have distinct or opposing roles in mediating the relatively acute effects of drugs of abuse. Do D1R and D2R MSNs also have distinct roles after chronic drug exposure and withdrawal and do D1R and D2R MSN circuits coordinate in mediating certain behaviors? Answering these questions may reveal a common circuit-based mechanism underlying shared features of the addictive state.

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

N.M.G., S.S., W.J.W., Y.H.H., E.J.N., Y.T.W., O.M.S. and Y.D. designed the experiments and analyses. N.M.G., S.S., W.J.W., D.J. and Z.L. conducted the experiments and data analyses. N.M.G., Y.H.H., E.J.N., O.M.S. and Y.D. wrote the manuscript.

COMPETING FINANCIAL INTEREST

The authors declare no competing financial interests.

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Repetitive systematic injections of saline, cocaine or morphine. Before drug administration or molecular manipulation, rats or mice were allowed to acclimate to their home cages for >5 d. For drug treatment, we used a 5d repeated drug administration procedure. In all electrophysiological and morphological experiments (Figs. 1–6), once per day for 5 d, rats or mice were taken out of the home cages at 7:00–9:00 a.m. for an i.p. injection of either (-)-coca HCl (15 mg/kg in saline), (−)-morphine sulfate pentahydrate (10 mg/kg in saline), or the same volume of saline, and then placed back to the home cage. In locomotor experiments (Fig. 7), subcutaneous (s.c.), rather than i.p., injections were used for systematic delivery of morphine, and i.p. injections were used for saline and cocaine. These doses and routes of drug delivery were selected as they produce equivalent degrees of locomotor sensitization for the two drugs. Animals were randomly selected for each drug treatment. Contextual cues associated with drug injection were intentionally not provided. Cocaine-, morphine- or saline-treated animals were then used for electrophysiological recordings ~24 h after the last injection. For time course studies, animals received 1-, 2-, 3- or 5-d treatment and electrophysiological recordings were taken ~24 h following the last injection. In other experiments, rats receiving a 5-d daily injection procedure of morphine or saline were used on withdrawal day 7 (Fig. 1). In experiments involving long-term withdrawal, animals were killed for imaging or electrophysiological studies 21–28 d after the last drug injection.

Preparation of NAc slices. For acute slices, rats or mice were decapitated following isoflurane anesthesia. Coronal slices (250 µm) containing the NAc were prepared on a VT1200S vibratome (Leica) in 4 °C cutting solution containing (in mM): 135 N-methyl-D-glutamate, 1 KCl, 1.2 KH₂PO₄, 0.5 CaCl₂, 1.5 MgCl₂, 20 choline-HCO₃, and 11 glucose, saturated with 95% O₂ / 5% CO₂ at 37 °C for 30 min and then allowed to recover for 30 min at 20–22 °C before experimentation.

Electrophysiological recordings. Whole-cell recording. All recordings were made from slices of NAcSh that were prepared using a fine-pipette of medium thickness. Brains were removed and postfixed in the same fixative for 1 h. After brief wash in PBS, slices were mounted in aqueous medium prolong (Invitrogen) and were collected in PB-saline and mounted before DiI labeling. DiI fine crystals (Invitrogen) were delivered under a dissecting microscope onto the surface of slices using a fine brush, controlled by a micromanipulator. DiI was allowed to diffuse in PBS for 48 h at 4 °C, and then labeled sections were fixed in 4% PFA at 20–22 °C for 1 h. After brief wash in PBS, tissues were mounted in aqueous medium prolong (Invitrogen).

Imaging dendritic spines. Dendritic spines of NAcSh MSNs were labeled and imaged as described previously. Briefly, rats or mice were perfused transcardially (20 ml/min) with 0.1 M sodium phosphate buffer (PB), followed by 200 ml of 1.5% paraformaldehyde (PFA, wt/wt) in 0.1 M PB. The use of 1.5% rather than the traditional 4% PFA was critical in obtaining maximal dye filling of small diameter spines. Brains were removed and postfixed in the same fixative for 1 h at 20–22 °C before coronal slices of 100-µm thickness were prepared. The slices were collected in PB-saline and mounted before DiI labeling. DiI fine crystals (Invitrogen) were delivered under a dissecting microscope onto the surface of slices using a fine brush, controlled by a micromanipulator. DiI was allowed to diffuse in PBS for 48 h at 4 °C, and then labeled sections were fixed in 4% PFA at 20–22 °C for 1 h. After brief wash in PBS, tissues were mounted in aqueous medium prolong (Invitrogen).

An Olympus confocal microscope was used to image the labeled sections. DiI was excited using the Helium/Neon 559-nm laser line. The entire profile of each Dil-positive neuron to be quantified was acquired using a 60× oil-immersion objective. After the neuron was scanned and confirmed as an NAcSh MSN, its dendrites were focused using a 60× oil-immersion objective and scanned at 0.44 µm intervals along the z-axis for a maximum of 200 planes; the final image of each dendrite was obtained by stacking all planes. Analyses were performed on two-dimensional projection images using the software ImageJ (NIH). Based
on previous results23-3, secondary dendrites were preferentially sampled. For each neuron, one or two dendrites of 20 µm in length were analyzed. For each group, 5–10 neurons per animal were analyzed. We operationally divided the spines into four categories31,44: i) mushroom-like spines were dendritic protrusions with a head diameter >0.5 µm or ≥2× the neck diameter; ii) stubby spines were dendritic protrusions with no discernible head and with a length ≤0.5 µm; iii) filopodia-like spines were dendritic protrusions with no discernible head and with a length > 0.5 µm; and iv) long-thin spines were dendritic protrusions with a head diameter < 2× the neck diameter. The density of long-thin spines can also be obtained by subtracting mushroom, stubby, and filopodia spines from the total spines.

Drug-induced locomotor responses. During light cycles, mice were allowed to habituate to the commercially available locomotor chamber (18.0″ L × 9.5″ W × 12.0″ H) (Camden Instruments, Loughborough, England) 1 h/d for 2 d before the day 0 injection. Locomotor activity was measured using infrared photobeams for 1 h and the average distance traveled (m) over 4 15-min bins was presented34. Mice were immediately returned to their home cage after each session. 21 d after the 5-d procedure, mice received challenge drug injections with ascending doses. Cocaine and morphine were delivered through i.p. and s.c. injections, respectively. For experiments involving peptides, GluA23Y or scrambled peptide (30 pmol in 1 µL) was injected into the tail vein ~20 min before saline, cocaine or morphine injection.

Conditioned place preference. Chambers. The mouse CPP chamber (Med Associates) was consisted of three compartments, separated by manual guillotine doors. The three compartments had distinct characteristics: the center compartment (2.85″ × 5″ × 5″) had gray walls and floor, the two choice compartments (6.6″ × 5″ × 5″) differed in wall color (white versus black with white stripes) and floor texture (stainless steel floor versus stainless steel grid). After each test, compartments were thoroughly cleaned with a scent-free soap solution. Each compartment was illuminated with a dim light situated in the laminate top. Mouse locations were identified by automated data collection software (Med Associates) using infrared photobeam strips that recorded the time spent in each compartment.

Preconditioning. During light cycles, mice were habituated to the testing room for 30 min before each session. Mice were then placed in the center compartment with free access to all three compartments for 20 min. Time spent (seconds) in each compartment was recorded.

Conditioning. 24 h after preconditioning, mice received a 5-d conditioning training. Drug-paired compartments were randomly assigned55. For test involving peptides, mice received bilateral intra-NAcSh (AP, +1.75; ML, ±0.6; DV, −3.5 mm) infusion of GluA23Y or scrambled peptide (30 pmol in 1 µL) through preinstalled guide cannula. Mice were then habituated to the testing room for 30 min, before conditioning experiments. During conditioning, mice received an injection of saline or cocaine (i.p. 15 mg/kg) or morphine (i.p. 10 mg/kg) and were placed into one compartment for 40 min in the morning and to the opposite compartment in the afternoon56. Morning and afternoon sessions were separated by 6 h.

Post-conditioning. 24 h and 21 d after the last conditioning day (days 6 and 28), mice were habituated to the testing room for 30 min and then placed in the center compartment, where they were allowed to move freely for 20 min. CPP scores were calculated as time spent in the drug-paired side minus the time spent on the same side during the preconditioning day57.

Drugs. Picrotoxin was purchased from Sigma-Aldrich. (–)-Morphine sulfate pentahydrate and (–)-cocaine HCl were provided by the National Institute on Drug Abuse Drug Supply Program.

Data acquisition and statistics. In electrophysiological experiments, we used 152 rats and 70 D1-tomato mice. In locomotor tests, we used a total of 136 C57bl/6 mice, with 7 of them excluded before data collection due to death during drug withdrawal. In CPP tests, we used 70 mice, among which 13 were removed from data analysis due to inaccurate cannula placement or strong preference to one chamber before conditioning. In imaging experiments, we used 113 rats with 9 of them excluded from data collection due to death, and 119 C57bl/6 mice, with 12 of them excluded from data collection due to death.

All results are shown as mean ± s.e.m. Each experiment was replicated in 3–12 animals. The data collection was randomized. Data were obtained and analyzed by experimenters who did not know the types of treatments of the animals. No data points were excluded unless specified, and the only exclusion standard is the health condition of the animal. Data from the repeated experiments for the same sub-study were pooled together for statistics. Technical replicates were used for some of the key experiments. Sample size for each experiment was determined either based on our previous experience with similar experiments or those that have been routinely used in similar studies published in this journal (refs. 5, 9). Sample size was presented as n/m, where “n” refers to the number of cells, cell pairs, or dendrites examined, and “m” refers to the number of animals. In electrophysiological experiments, 1–4 recordings were performed using slices from a single animal. In morphological experiments, 3–10 dendrites were assessed from a single animal. Animal-based statistics were performed and reported for all results. In electrophysiological and morphological experiments, we used the averaged value of a parameter from all cells/dendrites from an animal to represent the parameter of this animal. Normal distribution was assumed for all statistics but this was not formally tested. Variance was estimated for most major results and no significant difference was found between control and manipulation groups. Statistical significance was assessed using the t-test, or one- or two-way ANOVA as specified. Two-tail tests were performed for all studies.

A Supplementary Methods Checklist is available.

Data Availability. The data that support the findings of this study are available from the corresponding author upon request.