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In Vivo Cocaine Experience Generates Silent Synapses

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SUMMARY

Studies over the past decade have enunciated silent synapses as prominent cellular substrates for synaptic plasticity in the developing brain. However, little is known about whether silent synapses can be generated postdevelopmentally. Here, we demonstrate that highly salient in vivo experience, such as exposure to cocaine, generates silent synapses in the nucleus accumbens (NAc) shell, a key brain region mediating addiction-related learning and memory. Furthermore, this cocaine-induced generation of silent synapses is mediated by membrane insertions of new, NR2B-containing *N*-methyl-*D*-aspartic acid receptors (NMDARs). These results provide evidence that silent synapses can be generated de novo by in vivo experience and thus may act as highly efficient neural substrates for the subsequent experience-dependent synaptic plasticity underlying extremely long-lasting memory.

INTRODUCTION

Abundant in the developing brain, silent synapses are glutamatergic synapses in which *N*-methyl-*D*-aspartic acid receptor (NMDAR)-mediated excitatory postsynaptic currents (EPSCs) are relatively stable, whereas alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated responses are highly labile (Isaac et al., 1995; Liao et al., 1995; Petralia et al., 1999). Upon activation of NMDARs, silent synapses can be unsilenced by acquiring stable AMPAR activity, leading to long-term potentiation (LTP) of glutamatergic synaptic transmission (Isaac et al., 1995; Liao et al., 1995). Whereas unsilencing of silent synapses in the developing brain has been one of the most efficient mechanisms underlying experience-dependent synaptic plasticity in vitro (Groc et al., 2006; Kerchner and Nicoll, 2008; Marie et al., 2005), little is known as to whether silent synapses are generated during in vivo learning processes. Here, we demonstrate that highly salient in vivo experience can generate silent synapses de novo.

Cocaine addiction has been conceptualized as an extremely durable form of memory (Gerdeman et al., 2003; Hyman et al., 2006), which is, in part, mediated by experience-dependent synaptic plasticity in the nucleus accumbens (NAc) (Hyman et al., 2006; Wolf, 2002). The NAc shell has been closely tied to motivational mechanisms (Kelley, 2004) and has been implicated in a variety of addiction-related molecular, cellular, and behavioral alterations (Hyman et al., 2006; Wolf, 1998). Taking advantage of cocaine exposure as a strong memory inducer, we examined whether silent synapses could be generated in the NAc shell. We observed that exposure to cocaine generated a large proportion of silent synapses in the NAc shell, and these silent synapses were formed by membrane insertion of new, NR2B-containing NMDARs. Collectively, our results show that in vivo experience can generate silent synapses de novo, and these newly generated silent synapses may transiently provide highly efficient plasticity substrates (Marie et al., 2005) for subsequent experience-dependent, long-lasting synaptic plasticity.

RESULTS

Two independent assays revealed that exposure to cocaine increased the number of silent synapses in NAc shell medium spiny neurons (NAc MSNs) All rats were at postnatal day 30–32 when receiving injection unless otherwise indicated. First, we compared the coefficient of variation (CV) of the AMPAR EPSCs and NMDAR EPSCs measured at −80 mV and +40 mV, respectively; an increase in silent synapses would be detected as a decrease in the ratio of CV-NMDAR:CV-AMPAR (Kullmann, 1994; Marie et al., 2005). Following a withdrawal of 1 or 2 days from a 5-day cocaine procedure, the ratio of CV-NMDAR:CV-AMPAR in NAc neurons was decreased (saline: CV-AMPAR, 0.22 ± 0.02; CV-NMDAR, 0.20 ± 0.03; ratio, 0.99 ± 0.12; n = 11 cells/6 rats; cocaine: CV-AMPAR, 0.26 ± 0.03; CV-NMDAR, 0.14 ± 0.01; ratio, 0.62 ± 0.07; n = 14/7; $p < 0.01$ versus saline-ratio, Figure 1C). We then used the minimal stimulation technique to estimate the percentage of silent synapses among total synapses by comparing the failure rates of EPSCs at −80 mV and +40 mV (Figures 1D–1F). The failure rates were not different in saline-treated rats (−80 mV, 50.9% ± 3.1%; +40 mV, 47.7% ± 3.3%; n = 22/12) but were significantly different in cocaine-treated rats (−80 mV, 60.9% ± 3.6%; +40 mV, 44.6% ± 3.2%; n = 25/14; $p < 0.05$, t test). The percentage of silent synapses among total synapses (% silent synapses) was estimated by

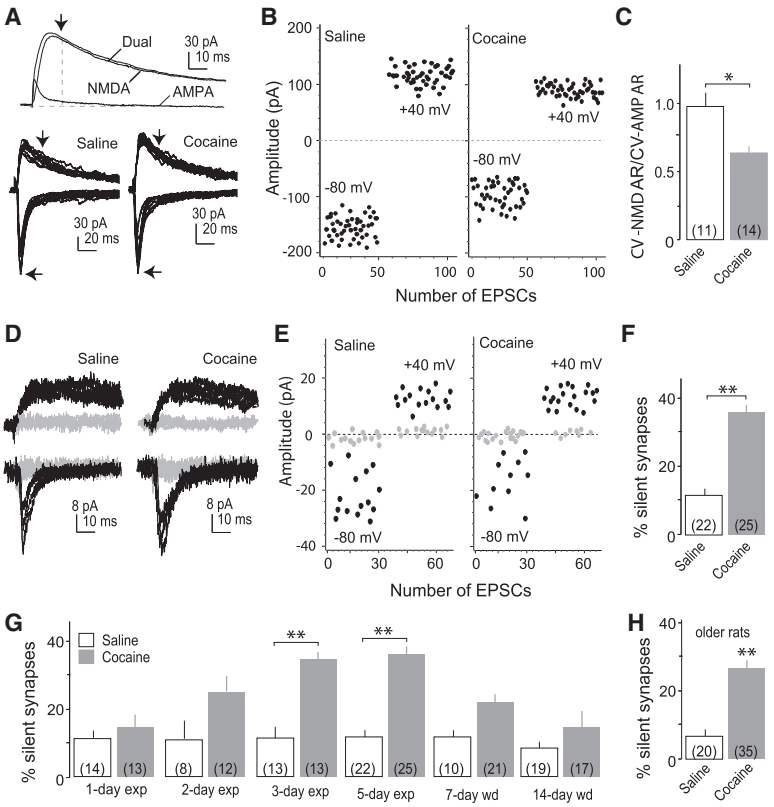

Figure 1. In Vivo Cocaine Experience Generated Silent Synapses in NAc MSNs

(A) Example traces showing 10 consecutive EPSCs at +40 and −80 mV from saline- and cocaine-treated rats, respectively. AMPAR EPSC peaks were measured at −80 mV (arrows); NMDAR EPSC amplitude was measured at +40 mV and 35 ms following the peak of AMPAR EPSCs (arrows). Dual EPSCs at +40 mV could be pharmacologically separated into AMPAR and NMDAR components. At 35 ms (arrow), the amplitude of the dual EPSC was primarily attributable to NMDAR EPSCs (95.2% ± 5.3% of dual EPSC, n = 8). (B) Plots of AMPAR and NMDAR EPSC amplitudes in example NAc MSNs (50 consecutive traces). (C) Grouped data showing significantly decreased ratio of CV-NMDAR:CV-AMPAR in NAc MSNs of cocaine-treated rats. (D) Example traces from the minimal stimulation assays showing 10 consecutive responses (successful or failed) at +40 and −80 mV from saline- and cocaine-treated rats. (E) Plots of responses in (D) (30 consecutive traces). (F) Grouped data showing significantly increased percentage of silent synapses in NAc MSNs by in vivo exposure to cocaine. (G) Grouped data showing that silent synapses were gradually generated in the NAc during exposure to cocaine and declined during withdrawal. (H) Grouped data showing that silent synapses were also generated in NAc in older (~65-day-old) rats following 1-day withdrawal from the 5-day cocaine procedure. (n) = number of cells; *p < 0.05; **p < 0.01.

the following equation: fraction of silent synapses = 1 − Ln-(F_{-80mV})/Ln(F_{+40mV}) (Isaac et al., 1995; Liao et al., 1995; Marie et al., 2005), where F_{-80mV} and F_{+40mV} are failure rates at −80 and +40 mV, respectively (see Experimental Procedures). The % silent synapses in NAc MSNs was significantly higher in cocaine-treated rats than in saline-treated rats (saline, 10.9% ± 2.1%, n = 22/14; cocaine, 35.6% ± 3.6%, n = 25/14; p < 0.01, t test, Figure 1F). Using the same approach, we detected that silent synapses were generated gradually during repeated exposure to cocaine and declined after long-term withdrawal (1-day saline, 10.5% ± 3.0%, n = 14/4; 1-day cocaine, 14.1% ± 4.2%, n = 13/5; 2-day saline, 9.2% ± 2.3%, n = 8/3; 2-day cocaine, 22.8% ± 4.7%, n = 12/5; 3-day saline, 10.3% ± 3.1%, n = 13/4; 3-day cocaine, 33.9% ± 5.8%, n = 13/5; 7-day withdrawal: saline, 7.2% ± 2.8%, n = 10/4; cocaine, 24.3% ± 3.8%, n = 21/6; p < 0.05; 14-day withdrawal: saline, 9.1% ± 2.7%, n = 19/4; cocaine, 17.0% ± 3.4%, n = 17/5; p = 0.78; Figure 1G). Furthermore, cocaine-induced generation of silent synapses in the NAc was also observed in older rats (~65 days old, first cocaine injection at postnatal day 60) (saline, 7.0% ± 1.8%, n = 20/3; cocaine, 27.0% ± 3.5%, n = 35/5; p < 0.01; Figure 1H). Note that the basal level (saline-treated amount) of silent synapses in NAc MSNs tends to be lower in older rats (7.0 ± 1.8, n = 20; ~36 days old, 10.4 ± 1.9, n = 30; p = 0.10), presumably due to developmental regulation (Durand et al., 1996; Hsia et al., 1998; Kerchner and Nicoll, 2008; Liao and Malinow, 1996). Nonetheless, both the CV and minimal stimulation analyses suggest that in vivo experience with cocaine generated silent synapses in NAc MSNs.

In theory, silent synapses can be produced by removing/disabling AMPARs from existing synapses or adding new NMDARs to new synaptic locations. We next examined the surface levels of NMDARs. NMDARs in the forebrain are mainly composed of the obligatory NR1 subunits along with NR2A and NR2B subunits (Monyer et al., 1994). The surface and total levels, as well as the surface:total ratio, of NR2B subunits were significantly increased in cocaine-treated rats (measured at withdrawal day 1; surface: saline, 1.04 ± 0.12, n = 17 rats; cocaine, 2.06 ± 0.48, n = 15; p < 0.05; total: saline, 0.98 ± 0.05, n = 22; cocaine, 1.30 ± 0.13, n = 23; p < 0.05; surface:total: saline, 1.00 ± 0.11, n = 17; cocaine, 1.99 ± 0.36, n = 15; p < 0.05, Figures 2A and 2B), whereas NR2A subunits were not significantly altered (surface: saline, 1.00 ± 0.12, n = 10; cocaine, 1.31 ± 0.12, n = 7; p = 0.09; total: saline, 1.00 ± 0.07, n = 14; cocaine, 1.23 ± 0.15, n = 15; p = 0.19; surface:total: saline, 1.00 ± 0.07, n = 10; cocaine, 1.45 ± 0.20, n = 7; p = 0.12, Figures 2A and 2C). Furthermore, the surface level and the surface:total ratio of NR1 subunits were increased in the NAc tissues from cocaine-treated rats (surface: saline, 0.95 ± 0.07, n = 17; cocaine, 1.22 ± 0.10, n = 15; p < 0.05; total: saline, 1.00 ± 0.05, n = 25; cocaine, 1.04 ± 0.09, n = 25; p > 0.4; surface:total: saline, 1.00 ± 0.08, n = 17; cocaine, 1.38 ± 0.14, n = 13; p < 0.05, Figures 2A and 2D). Thus, NR2B-containing NMDARs were selectively inserted into the cell surface upon cocaine administration. In addition, the selective increase in the total level of NR2B, but not NR1, subunits implies that cocaine-induced upregulation of NR2B subunits begins at the protein synthesis level;

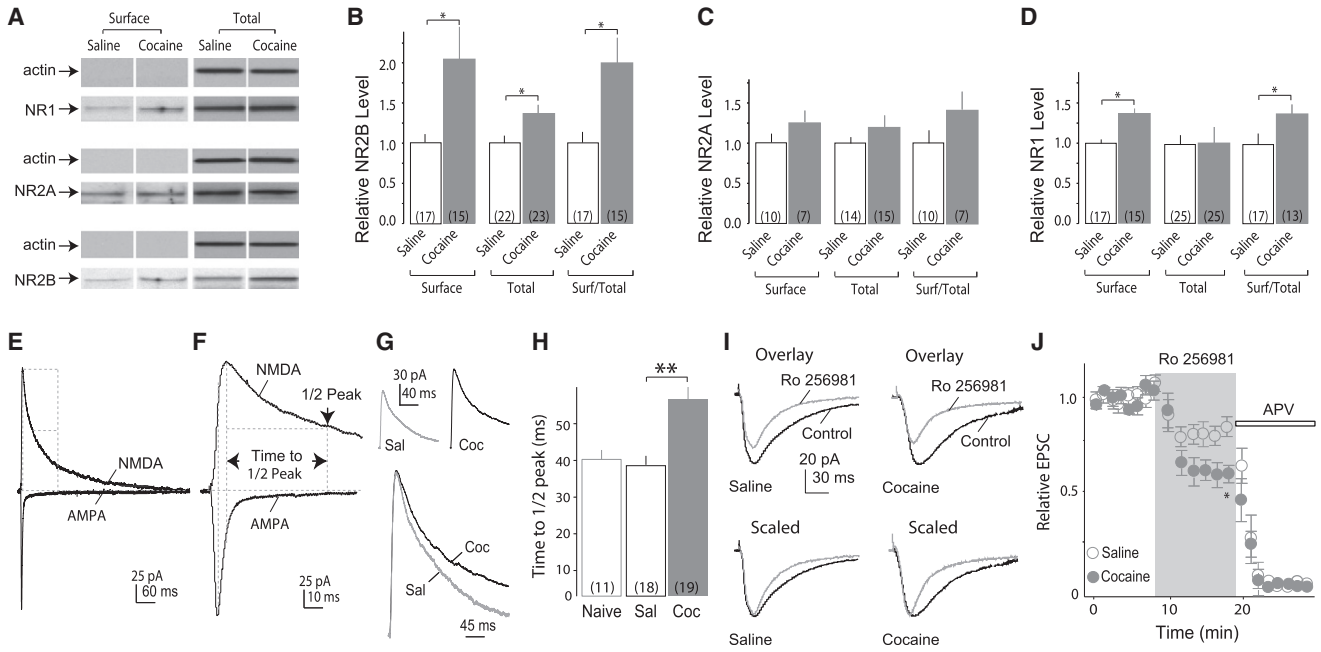

Figure 2. In Vivo Exposure to Cocaine Increased the Number of Synaptic NR2B-Containing NMDARs

(A–D) Examples and summarized results from western blot assays showing that in vivo cocaine experience selectively increased the NR1/NR2B type of NMDARs on the cell surface of NAc MSNs. (n) = number of rats. (E and F) Example EPSCs recorded at +40 and −80 mV shown on a slow (E) and a fast (F) timescale. The half-decay time $T_{1/2}$ was defined as the time elapsed from the peak to one-half peak of NMDAR EPSCs. (G and H) Example traces and grouped data showing significantly slower decay and longer $T_{1/2}$ of NMDAR EPSCs in NAc MSNs from cocaine-treated rats, compared to saline controls. (I and J) Example and summarized results showing that the Ro256981-mediated inhibition of NMDAR EPSCs was significantly greater in NAc MSNs from cocaine-treated rats than those from saline-treated rats. APV (50 μM) was applied at the end of each experiment to indicate no contamination from other conductances. (n) = number of cells; *$p < 0.05$; **$p < 0.01$.

the newly synthesized NR2B subunits may then be assembled to functional NMDARs by recruiting pre-existing NR1 subunits, which, unlike NR2 subunits, are often overabundant intracellularly (Wenthold et al., 2003).

We next tested whether NR2B-containing NMDARs were increased at synaptic locations by biophysical and pharmacological assays. Because NR2B-containing NMDARs exhibit slower decay kinetics than their NR2A-containing counterparts (Cull-Candy and Leszkiewicz, 2004), we measured the decay kinetics of NMDAR EPSCs in NAc MSNs. We observed that the half-decay time (estimated by the time elapsed from the EPSC peak to half of peak amplitude, or $T_{1/2}$ [Barria and Malinow, 2002, 2005]), was significantly longer in cocaine-treated rats on day 1 during withdrawal ($T_{1/2}$ in ms: naive, 40.1 ± 2.4, n = 11/6; saline, 38.2 ± 2.8, n = 18/10; cocaine, 57.6 ± 3.3, n = 19/10; $F_{(2, 47)} = 13.42$, $p < 0.01$, one-factor ANOVA; $p < 0.01$, cocaine versus saline or naive, Bonferroni posttest; Figures 2E–2H, see Supplemental Data available online for alternative measurements). Furthermore, in NAc MSNs from cocaine-treated rats, the sensitivity of NMDAR EPSCs to the NR2B-selective antagonist Ro256981 (200 nM) was increased (inhibition at 9 min during antagonist perfusion: saline, 27% ± 3%, n = 8/5; cocaine, 42% ± 3%, n = 7/5; $p < 0.05$, t test; holding potential [V_H]: −40 mV; Figures 2I and 2J).

The above results suggest that cocaine-induced generation of silent synapses was mediated by selective recruitment of NR2B-containing NMDARs into the new synaptic locations. To test this, we aimed to detect the cocaine-induced, newly recruited NMDARs by monitoring NR1 subunit trafficking. Using in vivo viral-mediated gene transfer within the NAc of anesthetized rats, we expressed a mutant NR1 subunit (mNR1-GFP, NR1a with N598R mutation; wild-type NR1-GFP [wtNR1-GFP] and GFP alone used as controls), which decreased the Mg^{2+}-binding affinity (Barria and Malinow, 2002). Thus, the mNR1-containing NMDARs, once delivered to the synapse, can be detected as NMDAR EPSCs at near-resting potentials (Barria and Malinow, 2002). We established a quantifiable parameter to measure the synaptic delivery of mNR1-containing NMDARs. At a holding voltage of −55 mV, where the Mg^{2+}-block of NMDARs is incomplete (Jahr and Stevens, 1990), presynaptic stimulation elicited a dual EPSC mediated by both AMPARs and NMDARs (Figure 3A). Because AMPAR activation and inactivation are substantially faster than those of NMDARs, the peak current (defined as "0 ms") was mainly attributable to AMPARs, and the slow tail current (measured at 35 ms) was mainly attributable to NMDARs (Figure 3A). By contrast, at a holding potential of −90 mV, where the Mg^{2+}-block of NMDARs is maximal (Jahr and Stevens, 1990), little APV-sensitive current was observed, and the tail current at 35 ms was negligible (Figure 3A). Therefore, we defined the ratio of the current amplitude at 35 ms to the current amplitude at 0 ms (I_{35ms}/I_{0ms}) as an indicator for the number of synaptic NMDARs that were not blocked by Mg^{2+}. As a control,

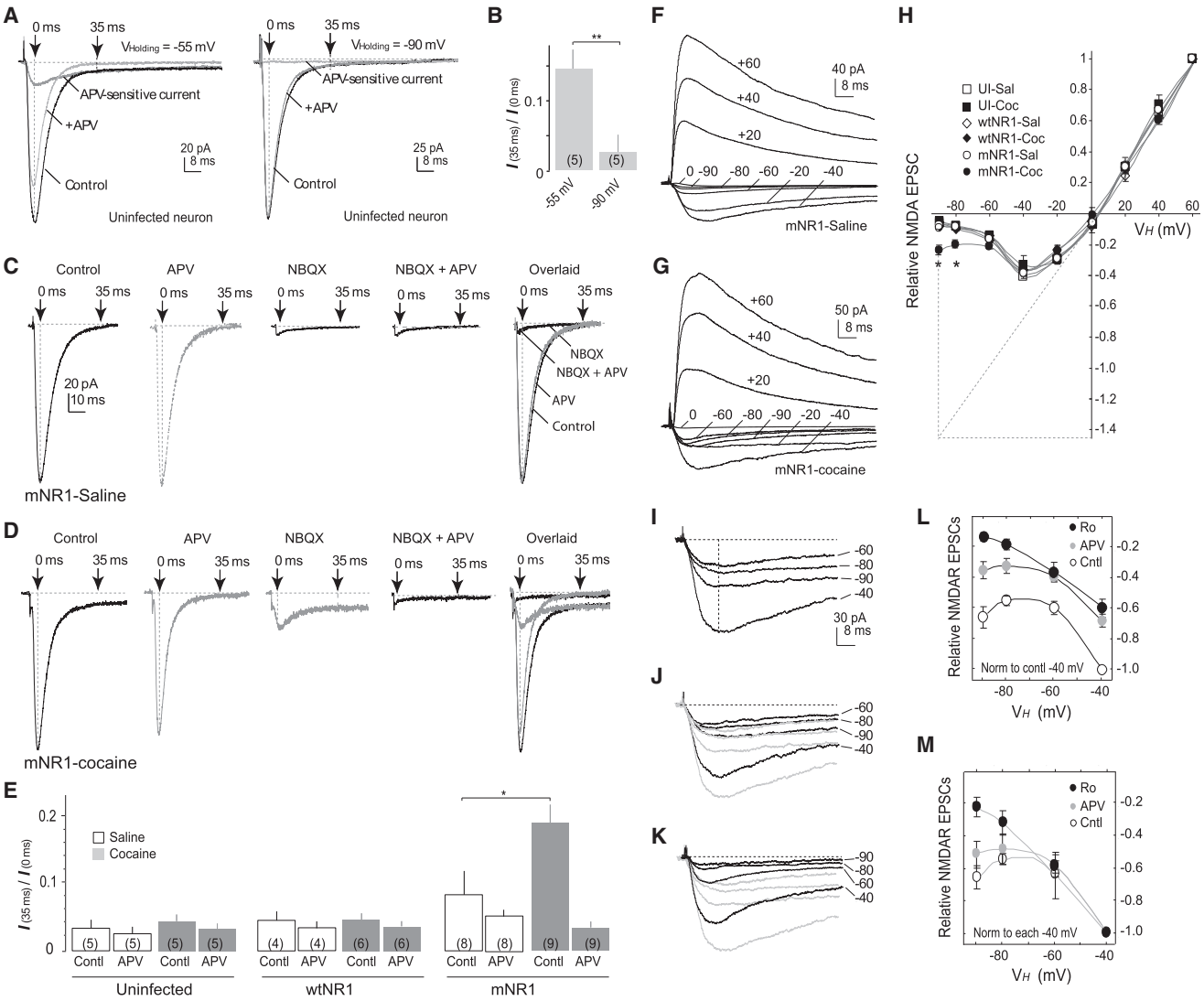

Figure 3. Exposure to Cocaine Induced Synaptic Insertion of New, NR2B-Containing NMDARs

(A) Example EPSCs from NAc MSNs at −55 mV and −90 mV. Note the slowly decaying component at −55 mV, which was attributable to NMDARs (sensitive to APV). (B) Grouped data showing the detection of Mg^{2+} unblocked NMDARs by I_{35}/I_{0ms}. (C and D) Example EPSCs at −90 mV in mNR1-expressing NAc MSNs from saline- and cocaine-treated rats. (E) Grouped data showing that I_{35}/I_{0ms} at −90 mV was increased in mNR1-expressing MSNs by cocaine exposure. (F–H) Example and summarized *I-V* relationship of NMDAR EPSCs in differently manipulated MSNs (normalized to +60 mV). Dashed line represents a hypothetical linear *I-V* curve (by extrapolating the linear segment of *I-V* curve at depolarized voltages), in which the theoretical current by total NMDARs at −90 mV was ~−1.4, whereas the measured current was ~−0.28. (I–K) Example NMDAR EPSCs (at −40, −60, −80, and −90 mV) of an mNR1-expressing MSN from a cocaine-treated rat (I, also grayed traces in J and K), differentially inhibited by subsequent applications of a low concentration of APV (J) and 200 nM Ro256981 (K). A washout was applied between applications of APV and Ro256981. (L and M) Summarized (L) and renormalized (M) *I-V* curves of NMDAR EPSCs show that the mNR1-expressing MSNs from cocaine-treated rats conducted a significant amount of current at hyperpolarized voltages, and this current was differentially inhibited by applications of 0.5 μM APV and 200 nM Ro256981. (n) = number of cells; *p < 0.05; **p < 0.01.

I_{35ms}/I_{0ms} at −55 mV (0.145 ± 0.031, n = 5/3) was significantly higher than that at −90 mV (0.018 ± 0.022, n = 5/3, p < 0.01, Figure 3B).

We then stereotaxically injected viral vectors into the NAc of anesthetized rats and ~6 hr later started cocaine administration (see Experimental Procedures). All subsequent recordings were performed at −90 mV to maximally exclude the involvement of endogenous NMDARs. Exposure to cocaine significantly increased I_{35ms}/I_{0ms} in mNR1-expressing NAc MSNs, and application of APV abolished this increase (mNR1-cocaine-control, 0.19 ± 0.03, n = 9/7; mNR1-cocaine-APV, 0.033 ± 0.003, n = 9/7; $F_{(5, 70)}$ = 16.69, p < 0.01, two-factor ANOVA; p < 0.05, mNR1-cocaine versus all others in Figure 3E, Bonferroni posttest). In contrast, I_{35ms}/I_{0ms} in mNR1-expressing NAc MSNs from saline-treated rats was not increased, suggesting that without cocaine administration, the transiently expressed mNR1 subunits were minimally delivered to the postsynaptic membrane (mNR1-saline-control, 0.08 ± 0.04, n = 8/6; mNR1-saline-APV,

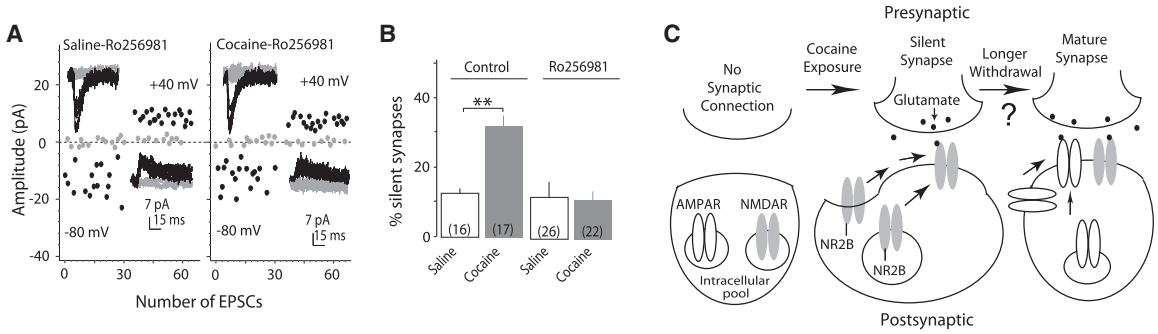

Figure 4. Inhibition of NR2B-Containing NMDARs Erased Cocaine-Generated Silent Synapses

(A) Minimal stimulation assays in NAc MSNs perfused with Ro256981 from saline- and cocaine-treated rats. (B) Grouped data showing that cocaine-generated silent synapses in NAc MSNs could not be detected when the NR2B-containing NMDARs were inhibited by application of Ro256981. (C) A diagram describing the hypothetical cellular process of cocaine-induced generation of silent synapses. (n) = number of cells; **p < 0.01.

0.046 ± 0.004, n = 8/6; Figures 3C–3E). Moreover, I_{35ms}/I_{0ms} in uninfected (UI) or wtNR1-expressing NAc MSNs was also not increased, and not affected by application of APV, suggesting that cocaine treatment by itself does not change the Mg^{2+}-block of wild-type NMDARs (UI-saline-control, 0.030 ± 0.002, n = 5/3; UI-saline-APV, 0.026 ± 0.003, n = 5/3; UI-cocaine-control, 0.046 ± 0.006, n = 5/3; UI-cocaine-APV, 0.043 ± 0.004, n = 5/3; wtNR1-saline-control, 0.045 ± 0.007, n = 4/3; wtNR1-saline-APV, 0.032 ± 0.007, n = 4/3; wtNR1-cocaine-control, 0.044 ± 0.006, n = 6/4; wtNR1-cocaine-APV, 0.036 ± 0.002, n = 6/4, Figure 3E). Together, these results suggest that following exposure to cocaine, new NMDARs were recruited to the synaptic membrane of NAc MSNs.

Consistent with the change in I_{35ms}/I_{0ms}, the current-voltage relationship (*I-V* curves) of NMDAR EPSCs was also altered at near-resting potentials in mNR1-expressing NAc MSNs in rats treated with cocaine (Figures 3F–3H). Under physiological conditions, the *I-V* curves of NMDAR EPSCs exhibit a strong rectification at hyperpolarized potentials due to Mg^{2+} blockade. This rectification was partially lost in mNR1-expressing NAc MSNs from cocaine-treated rats (normalized current amplitude, −80 mV: mNR1-saline, −0.11 ± 0.02, n = 8/7; mNR1-cocaine, −0.19 ± 0.02, n = 8/7; p < 0.05; −90 mV: mNR1-saline, −0.08 ± 0.02, n = 8/7; mNR1-cocaine, −0.28 ± 0.04, n = 8/7; p < 0.05, Figure 3H). These results suggest that new, Mg^{2+}-resistant mNR1-containing NMDARs were delivered to synapses upon cocaine exposure, and allowed us to estimate the percentage of newly recruited mNR1-containing NMDARs among the total synaptic NMDARs. Extrapolating the linear portion of the *I-V* curve at depolarized voltages generated a theoretical linear *I-V* curve at hyperpolarized voltages (dashed line in Figure 3H). At −90 mV, the theoretical amplitude of total NMDAR EPSC was ~−1.4 if all NMDARs conducted current (whereas the actual amplitude of EPSC mediated by wild-type NMDARs was ~0). In cocaine-treated rats expressing mNR1, the current amplitude was ~−0.28 at −90 mV. Thus, assuming that the single-channel conductance was not altered, the newly inserted mNR1-containing receptors could contribute to ~20% (0.28/1.4) of the total synaptic NMDARs in cocaine-treated rats (Figure 3H).

To determine whether the newly recruited mNR1-containing NMDARs are NR2B enriched, we examined the *I-V* curve in the presence of the NR2B-selective antagonist Ro256981. We focused on the *I-V* curve from −40 to −90 mV, a segment that exhibited rectification. In mNR1-expressing MSNs from cocaine-treated rats, application of Ro256981 (200 nM) not only decreased the amplitudes of NMDAR EPSCs (normalized current amplitude, −40 mV: control, −1.0, Ro256981, −0.60 ± 0.06, p < 0.05; −60 mV: control, −0.59 ± 0.08; Ro256981, −0.36 ± 0.06, p < 0.05; −80 mV: control, −0.55 ± 0.03; Ro256981, −0.19 ± 0.04, p < 0.05; −90 mV: −0.66 ± 0.07; Ro256981, −0.13 ± 0.06, p < 0.05, n = 6/5, Figures 3I–3K), but also appeared to decrease the cocaine-induced downward bend in the *I-V* curve (Figure 3L). By contrast, although a low concentration (0.5 μM) of APV, an NMDAR antagonist inhibiting both NR2A- and NR2B-containing receptors with similar selectivity, inhibited the amplitude of NMDAR EPSCs to a similar degree (−40 mV: −0.68 ± 0.04; −60 mV: −0.39 ± 0.04; −80 mV: −0.32 ± 0.05; −90 mV: −0.35 ± 0.05; n = 6/5), a substantial downward bend in the *I-V* curve still remained (Figure 3L). When the *I-V* curve in each pharmacological condition was individually normalized, it became apparent that application of Ro256981, but not APV, abolished the cocaine-induced "drift" in the rectification at hyperpolarized voltages (−90 mV: control, −0.66 ± 0.07; APV, −0.52 ± 0.08, p > 0.04; Ro256981, −0.23 ± 0.06, p < 0.05; −80 mV: control, −0.55 ± 0.03; APV, −0.49 ± 0.09; Ro256981, −0.33 ± 0.07, p < 0.05; −60 mV: −0.59 ± 0.06, APV, 0.59 ± 0.07; Ro256981, −0.65 ± 0.14; n = 7/5 or 7/6 in each group, Figure 3M). Together, these results suggest that cocaine-induced, newly recruited synaptic NMDARs were NR2B-containing receptors.

If these new NR2B-containing NMDARs were indeed the basis for cocaine-generated silent synapses, we reasoned that selective inhibition of NR2B-containing NMDARs should prevent the detection of cocaine-generated silent synapses. We thus performed the minimal stimulation assay and observed that the cocaine-induced increase in the percentage of silent synapses in NAc MSNs was abolished by application of Ro256981 (200 nM) (control-saline, 12.2% ± 2.6%, n = 16/10; control-cocaine, 31.7% ± 4.0%, n = 17/9; Ro256981-saline, 11.8% ± 5.7%, n = 26/12; Ro256981-cocaine, 11.4% ± 2.6%, n = 22/12; $F_{(3, 73)} = 3.16$, p < 0.05, one-factor ANOVA; p = 1.00, Ro256981-saline versus Ro256981-cocaine, Bonferroni posttest; Figures 4A and 4B).

DISCUSSION

The present studies show that in vivo cocaine experience generates NMDAR-active/AMPAR-silent excitatory synapses in the NAc shell, a process that appears to be achieved by recruiting new, NR2B-containing NMDARs into new synaptic locations. These results introduce the concept that silent synapses can be produced by in vivo experience. This is significant in multiple ways. *First*, although unsilencing of silent synapses serves as a prominent model for LTP of excitatory synaptic transmission (Isaac et al., 1995; Kerchner and Nicoll, 2008; Liao et al., 1995), silent synapses are not normally abundant in the developed brain (Groc et al., 2006; Kerchner and Nicoll, 2008). Our results show that silent synapses can be generated de novo in the developed brain, providing a conceptual basis for a silent synapse-based mechanism as a potentially common molecular process for synaptic plasticity. *Second*, it has been highly debated whether the "silent" nature of silent synapses originates presynaptically or postsynaptically (Kerchner and Nicoll, 2008) and whether AMPARs are present in silent synapses (Groc et al., 2006; Kerchner and Nicoll, 2008). Our results suggest that for cocaine-generated silent synapses, postsynaptic recruitment of new NMDARs is the key. *Third*, silent synapses are characteristic structures in the developing brain. Thus, a broader view would be that some strong in vivo experiences may selectively "rejuvenate" or "prime" the related neural circuits by generating silent synapses for more robust synaptic plasticity upon subsequent experience.

Particularly for cocaine-induced adaptations at excitatory synapses, the generation and potential maturation of cocaine-generated silent synapses can be conceptualized as a two-phased cascade. Specifically, the generation phase of silent synapses likely starts during the repeated exposure and may last through the early withdrawal period. During this time window, the surface level of AMPAR subunits remains unchanged (Boudreau and Wolf, 2005), suggesting that the cocaine-induced change in synaptic AMPARs, if any, should be small. At a similar time point, the AMPAR/NMDAR ratio at excitatory synapses of NAc MSNs is decreased (Kourrich et al., 2007). Based on the observations of a decrease in the amplitude of miniature AMPAR EPSCs and no detectable change in NMDARs, the decrease in AMPAR/NMDAR ratio was previously attributed exclusively to the decrease in the number/function of AMPARs (Kourrich et al., 2007; Thomas et al., 2001). However, in these studies, only NMDAR mEPSCs from the nonsilent synapses were sampled; the fast rising AMPAR mEPSCs were used to select the dual-component miniature events (Kourrich et al., 2007; Thomas et al., 2001). Thus, NMDARs in silent synapses were largely excluded. On the other hand, when AMPAR/NMDAR ratio was measured, NMDAR EPSCs from both silent and nonsilent synapses were included. Piecing these results together with our present findings, NMDARs from newly generated silent synapses may also contribute to this observed decrease in AMPAR/NMDAR ratio. Nonetheless, once generated, these silent synapses may endow the NAc MSNs with an increased capacity for recruiting AMPARs to strengthen excitatory synaptic transmission (Figure 4C). As such, unsilencing cocaine-generated silent synapses may contribute to the decline of silent synapses during long-term withdrawal (Figure 1G), which may mediate the observed increase in the surface level of AMPARs during long-term withdrawal from cocaine exposure (Boudreau et al., 2007; Boudreau and Wolf, 2005; Conrad et al., 2008) and the increase in AMPAR/NMDAR ratio during long-term withdrawal (Kourrich et al., 2007). Thus, generation of NMDAR-enriched silent synapses may prime excitatory synapses for the subsequent plastic change. Indeed, the idea that drugs of abuse initiate their effects by first inducing NMDAR-oriented metaplasticity has been assessed in the ventral tegmental area (Argilli et al., 2008; Schilstrom et al., 2006). Nonetheless, if the silent-synapse-based metaplasticity is a key component in the cascade of proaddiction cellular adaptations, inhibiting NR2B-containing NMDARs in the NAc, which disables these silent synapses, should prevent the development of some drug-induced behaviors. This prediction is consistent with the findings that NR2B-selective antagonists prevent the acquisition of conditioned place preference to morphine and reinstatement of morphine during withdrawal (Ma et al., 2006, 2007).

It is important to note that changes in NMDAR localization and subunit expression are likely not the only mechanisms that regulate excitatory synapses in the NAc following cocaine administration. The dynamic changes in the synaptic AMPAR/NMDAR ratio (Kourrich et al., 2007; Thomas et al., 2001) and AMPAR surface expression (Boudreau et al., 2007) after re-exposure suggest that AMPARs may also move in and out of pre-existing synapses. Surface expression of atypical AMPAR subunits (Conrad et al., 2008) following long-term withdrawal from cocaine self-administration suggests that not only the number but also the properties of the inserted AMPARs are regulated. The lack of in vivo LTP within the prefrontal cortex-accumbens pathway (Goto and Grace, 2005; Moussawi et al., 2009), but not the hippocampus-accumbens pathway (Goto and Grace, 2005), following long-term withdrawal suggests that the basic machinery underlying synaptic plasticity may also be subjected to cocaine-induced modification within specific pathways; the decreased intra-NAc level of glutamate during withdrawal implicates the involvement of presynaptic alterations (Baker et al., 2003; Szumlinski et al., 2004). Furthermore, cocaine-induced synaptic adaptations including generation/maturation of silent synapses may vary between the NAc shell and core (Martin et al., 2006).

Some data from the present study appear to be at odds with previous results. For example, the cocaine-induced kinetic changes in NMDAR EPSCs in NAc MSNs demonstrated here during short-term withdrawal were not detected during long-term withdrawal (Kourrich et al., 2007). Furthermore, cocaine-induced insertion of new, NR2B-containing NMDARs predicts an increase in surface NR2B-containing NMDARs. However, no change in the whole-cell current induced by bath application of NMDA was detected during long-term withdrawal (Thomas et al., 2001), and the effects of cocaine on NR2B subunits in NAc appear to be highly inconsistent (see summaries in Supplemental Data #4). In addition to potential technical caveats, one possibility is that the NR2B-containing NMDARs that are inserted to create silent synapses are replaced with different forms of NMDARs after longer withdrawal times. If that is the case, the cellular behavior of NMDARs in NAc may be highly dynamic during/after cocaine exposure.

Finally, if cocaine-generated silent synapses are indeed created de novo, these nascent synaptic connections may present an ongoing process of circuitry modification. A leading hypothesis of synaptogenesis suggests that during development, premature synaptic connections are often overabundantly created but then undergo experience-dependent elimination or maturation (Waites et al., 2005). Consequently, only selected, presumably "useful," nascent synapses mature into fully functional, long-lasting synapses (Waites et al., 2005). Therefore, a hypothetical model of our finding is that the initial exposure to cocaine generates an overabundant number of nascent, silent synapses within the NAc in a nonspecific manner. Subsequent stimulations, such as long-term withdrawal, consolidate (unsilence) a selective portion of silent synapses, forming the fully functional connections that enhance the existing neural circuits or that even create new circuits (Figure 4C).

EXPERIMENTAL PROCEDURES

Detailed experimental protocols can be found in the Supplemental Data.

Cocaine Treatment and Slice Preparation

Male Sprague-Dawley rats at 30–32 days old were used for all experiments unless indicated (Figure 1H). The two repeated cocaine procedures were similar to those in earlier studies (Dong et al., 2006). In procedure 1, rats received daily injections of either cocaine HCl (15 mg/kg i.p.) or the same volume of saline for 5 days. In procedure 2 (referred to as the 2.5-day procedure), rats received one injection of cocaine (15 mg/kg) in the afternoon of day 1, and two daily injections (8 hr apart) of cocaine (15 mg/kg) for the following 2 days. Procedure 2 was only used in experiments involving viral expression (Figure 3), in which the in vivo viral injection was performed in the morning of day 1. Rats were then used for electrophysiological recordings or biochemical assays 24–48 hr following the last injection. Preparation of coronal slices was as described previously (Dong et al., 2006). The MSNs in the ventral-medial subregion of the NAc shell were preferentially examined in all experiments.

Virus Preparation and In Vivo Delivery

The wt/mNR1 constructs were described previously (Barria and Malinow, 2002). The cDNA for wtNR1-GFP or mNR1-GFP was cloned into the recombinant, replication-defective sindbis virus backbone vector (pSINrep2S^{726}). The protocol for making sindbis virus was similar to that used previously (Dong et al., 2006; Huang et al., 2008; Marie et al., 2005) except that the toxicity was further minimized by using a new sindbis virus-based vector, pSINrep (nsP2S^{726}). The infected neurons were identified by the GFP signal.

Electrophysiology

Whole-cell voltage-clamp recordings were used with a MultiClamp 700B amplifier (Molecular Devices). The solutions were as described previously (Dong et al., 2006; Huang et al., 2008). To examine NMDAR EPSCs, the extracellular solution contained picrotoxin (0.1 mM) and NBQX (5 μM). Presynaptic stimuli were applied through a bipolar microelectrode. Amplitudes of AMPAR EPSC were calculated by averaging 25 EPSCs at −80 mV and measuring the peak (2 ms window) compared to the baseline (2 ms window). NMDAR EPSC amplitudes were calculated by averaging 25 EPSCs at +40 mV and measuring the amplitude (2 ms window) 35 ms after the EPSC peak compared to the baseline.

The CV analysis was done as previously described (Kullmann, 1994). Briefly, CVs were estimated for epochs of 50 consecutive trials. Sample variances (SVs) were calculated for EPSC amplitudes and for noise sweeps. The CV was calculated as the square root of the difference for the sample variances [SV(EPSC) − SV(Noise)], divided by the mean. For minimal stimulation experiments, the frequency of presynaptic stimulation was set at 0.33 Hz. After obtaining small (>40 pA) EPSCs at −80 mV, stimulation intensity was reduced in small increments to the point that failures versus successes could be clearly distinguished visually. Stimulation intensity and frequency were then kept constant for the rest of the experiment. Failures versus successes were defined visually. Percent silent synapses were calculated using the following equation: $1 - \mathrm{Ln}(F_{-80})/\mathrm{Ln}(F_{+40})$, in which F_{-80} was the failure rate at −80 mV and F_{+40} was the failure rate +40 mV. In the CV and minimal stimulation assays, the types of cells were blinded for the experimenters. The phenotypes of the cells, pharmacological manipulations, and in vivo treatments were decoded only after all data analysis was completed.

Decay kinetics of NMDAR EPSCs was assessed using the time from the peak amplitude to one-half peak amplitude of the EPSC (Barria and Malinow, 2002, 2005). The NMDAR EPSC used for analysis was obtained by averaging 20–30 consecutive individual EPSCs. Alternative measurements were also applied and similar results were obtained (see Supplemental Data).

Western Blot Analysis of Surface NMDAR Subunits from NAc Shell Slices

The NAc shell was isolated from acute slices, washed twice in ice-cold aCSF, and then incubated in 1 mg/ml NHS-SS-biotin (Pierce, Rockford, IL) for 30 min at 4°C to biotinylate surface proteins as described previously (Huang et al., 2008). After being washed with aCSF containing 1 μM lysine, slices were homogenized and sonicated in lysis buffer containing proteinase and phosphatase inhibitors (20 mM Tris, 50 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM EGTA [pH 7.4]), followed by mixing for 30 min at 4°C. The homogenates were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants were incubated at 4°C for 2 hr with Neutravidin-linked beads (Pierce) to capture biotinylated surface protein. After being washed three times with lysis buffer, the surface proteins were eluted with protein sample buffer containing DTT and subjected to western blotting. The total NMDAR subunit levels were normalized to the actin level in each sample.

Data Acquisition, Analysis, and Statistics

In experiments of CV assays, minimal stimulation assays, and western blot assays, all data were obtained blindly. In experiments involving measuring the decay kinetics of NMDAR EPSCs (Figure 2) and mNR1-containing NMDARs (Figure 3), ~75% of the data were obtained blindly. All results are shown as mean ± SEM. Statistical significance was assessed using the two-tail t test or ANOVA.

SUPPLEMENTAL DATA

Supplemental data for this article include Supplemental Results, Supplemental Experimental Procedures, Supplemental Discussion, three figures, and one table and can be found at http://www.cell.com/neuron/supplemental/S0896-6273(09)00460-7.

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