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Calcium-permeable AMPA receptors and silent synapses in cocaine-conditioned place preference

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Abstract

Exposure to cocaine generates silent synapses in the nucleus accumbens (NAc), whose eventual unsilencing/maturation by recruitment of calcium-permeable AMPA-type glutamate receptors (CP-AMPARs) after drug withdrawal results in profound remodeling of NAc neuro-circuits. Silent synapse-based NAc remodeling was shown to be critical for several drug-induced behaviors, but its role in acquisition and retention of the association between drug rewarding effects and drug-associated contexts has remained unclear. Here, we find that the postsynaptic proteins PSD-93, PSD-95, and SAP102 differentially regulate excitatory synapse properties in the NAc. Mice deficient for either of these scaffold proteins exhibit distinct maturation patterns of silent synapses and thus provided instructive animal models to examine the role of NAc silent synapse maturation in cocaine-conditioned place preference (CPP). Wild-type and knockout mice alike all acquired cocaine-CPP and exhibited increased levels of silent synapses after drug-context conditioning. However, the mice differed in CPP retention and CP-AMPAR incorporation. Collectively, our results indicate that CP-AMPAR-mediated maturation of silent synapses in the NAc is a signature of drug–context association, but this maturation is not required for establishing or retaining cocaine-CPP.

Keywords AMPA receptor; cocaine; conditioned place preference; nucleus accumbens; silent synapse

Subject Categories Molecular Biology of Disease; Neuroscience

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Introduction

Silent synapses are excitatory synapses expressing stable NMDA-type glutamate receptor (NMDAR)-mediated responses, while AMPA-type glutamate receptors (AMPAR) are either absent or highly labile (Isaac *et al*, 1995; Liao *et al*, 1995; Xiao *et al*, 2004). Enriched in the newborn brain, silent synapses are thought to be initial synaptic contacts, mediating synapse formation, and neural circuit development. Recently, we and others demonstrated that exposure to cocaine generates silent synapses in the nucleus accumbens (NAc), which may initiate a remodeling of NAc neurocircuits (Huang *et al*, 2009; Brown *et al*, 2011; Koya *et al*, 2012). After withdrawal from cocaine self-administration, some of these NAc silent synapses become unsilenced by recruiting calcium-permeable AMPARs (CP-AMPARs; Lee *et al*, 2013). Consequently, this maturation process may lead to additionally remodeled NAc neurocircuits. Silent synapse-based NAc remodeling has been critically implicated in a large repertoire of drug-induced behaviors, ranging from the development of cocaine-induced locomotor sensitization to cue-induced cocaine seeking after drug withdrawal (Conrad *et al*, 2008; Brown *et al*, 2011; Lee *et al*, 2013; Ma *et al*, 2014). However, the role of silent synapses in cocaine-conditioned place preference (CPP), an important and widely used model testing the acquisition and retention of the association between drug rewarding effects and drug-associated contexts, remains unclear.

Cocaine-induced silent synapses resemble nascent synapses, partly by expressing GluN2B-containing NMDARs (Huang *et al*, 2009; Brown *et al*, 2011), such that preferential blockade of GluN2B prevented the development of cocaine-induced locomotor sensitization, suggesting a mechanism involving GluN2B-mediated silent synapse signaling. In mice lacking the signaling scaffold protein PSD-95, the number of silent synapses is elevated, which may serve

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as substrates to facilitate synaptic plasticity and long-term synaptic potentiation (Béïque *et al*, 2006; Carlisle *et al*, 2008; Huang *et al*, 2015a). Consistent with the elevated number of silent synapses, cocaine administration acutely increases locomotor responses in PSD-95 knockout (KO) mice, but the development of locomotor sensitization is prevented (Yao *et al*, 2004). In an alcohol-CPP test, PSD-95 KO mice establish the initial preference, but the long-term retention is impaired (Camp *et al*, 2011). One goal of the current study is to determine whether silent synapses are generated in PSD-95 KO mice by cocaine exposure, and if so, whether these silent synapses mature after drug withdrawal to contribute to cocaine-CPP retention.

While it has been proposed that the disk-large (DLG) membrane-associated guanylate kinases (MAGUKs), especially PSD-95, PSD-93, and SAP102, play redundant roles in regulating synaptic AMPAR numbers (Elias *et al*, 2006), recent results in KO mice show clear behavioral and synaptic differences among these MAGUKs (Carlisle *et al*, 2008; Krüger *et al*, 2013; Nithianantharajah *et al*, 2013). Thus, a correlation of functional synaptic differences with behavioral alterations might enable the causal dissection of cellular events regulating behavior.

Using a 10-day CPP procedure, we examined the role of cocaine-generated NAc silent synapses, the maturation of silent synapses after drug withdrawal, as well as the regulation of these silent synapses by MAGUKs in the acquisition and retention of cue-reward associative memories. Our results suggest that PSD-95, PSD-93, and SAP102 differentially regulate the maturation of cocaine-generated silent synapses in the NAc and that disrupting silent synapse maturation does not disrupt CPP retention.

Results

CP-AMPA receptors as a signature of drug–context association

To elucidate the role of NAc shell silent synapses and CP-AMPAR-mediated maturation of silent synapses in context–cocaine association, we used a CPP procedure. Mice were allowed to explore the open apparatus for an initial pre-test to measure their baseline (BL) place preference score (PPS; see Materials and Methods). For CPP training, one of the compartments was randomly chosen for the conditioned drug stimulus (CS+), in which the mice were placed for 20 min after receiving an i.p. injection of cocaine (20 mg/kg) once every other day. The other compartment was paired with the conditioned control (saline) stimulus (CS−) on the alternating days. Mice were trained with this procedure for a total of 10 days (Fig 1A). One day after the CPP training (withdrawal day 1; WD1), the PPS was measured over a 20-min trial. Control mice, which received saline injections in both chambers during training, showed no preference for either chamber, indicating that the setup of the CPP apparatus per se did not produce side preference (Fig 1B; Table EV1; PPS, saline BL: 522 ± 23 versus saline-WD1: 491 ± 25, $n = 28$, $t(27) = 1.258$, $P = 0.22$). CS+ mice acquired cocaine-CPP as tested on WD1, indicating a positive association of cues with the cocaine experience (Fig 1B; Table EV1; PPS: BL, 529 ± 22 versus WD1, 717 ± 16, $n = 29$, $t(28) = 7.51$, $P < 0.0001$). Different subgroups of mice were then tested for CPP retention at additional time points, WD10-15, WD20-25, WD30-35, or WD40-45. At each time point, the PPS was higher compared to its paired BL (Fig 1B; Table EV1; PPS: BL(WD10), 529 ± 23 versus WD1(WD10), 661 ± 19, $n = 21$, $t(20) = 5.81$, $P < 0.0001$; BL(WD10) versus WD10, 615 ± 22, $n = 21$, $t(20) = 3.45$, $P < 0.01$; BL(WD20), 538 ± 23 versus WD1 (WD20), 711 ± 16, $n = 25$, $t(24) = 6.84$, $P < 0.0001$; BL(WD20) versus WD20, 728 ± 21, $n = 25$, $t(24) = 6.20$, $P < 0.001$; BL(WD30), 512 ± 27 versus WD1 (WD30), 690 ± 16, $n = 27$, $t(26) = 6.51$, $P < 0.0001$; BL(WD30) versus WD30, 629 ± 26, $n = 27$, $t(26) = 3.26$, $P < 0.01$; BL(WD40), 550 ± 25 versus WD1(WD40), 736 ± 25, $n = 20$, $t(19) = 5.87$, $P < 0.0001$; BL(WD40) versus WD40, 745 ± 27, $n = 20$, $t(19) = 5.08$, $P < 0.001$), indicating that our procedure induced a robust cocaine-CPP, which persisted for > 45 days.

Repeated i.p. injections of cocaine generates silent synapses in the NAc shell (Huang *et al*, 2009; Brown *et al*, 2011; Koya *et al*, 2012; Graziane *et al*, 2016). We next tested whether our cocaine-CPP procedure also generates silent synapses. Immediately after the PPS measurement, we used *ex vivo* slice electrophysiology with the minimal stimulation assay (Liao *et al*, 1995; Huang *et al*, 2009), in which a small number of synapses is activated over repeated trials. At a holding potential of −60 mV, we recorded relatively fast excitatory postsynaptic currents (EPSCs), which are mediated only by AMPARs because NMDARs are largely blocked by Mg^{2+} at this membrane potential (Fig 1C–E). At a depolarized holding potential of +40 mV, the shape of the EPSCs was broader due to the composite activity of AMPARs and NMDARs. Since the release probability for glutamatergic synapses onto medium spiny neurons (MSNs) is ~0.5 (Casassus *et al*, 2005; Suska *et al*, 2013), we detected failures at both holding potentials. However, the failure rate at −60 mV was higher compared to that at +40 mV, indicating a fraction of AMPAR-lacking synapses, that is, silent synapses (Liao *et al*, 1995; Huang *et al*, 2009). The fraction of silent synapses among the sampled synapses (%SS) in NAc shell MSNs was increased ~twofold, 24 h after cocaine-CPP training (Fig 1C, D and G; Table EV1; %SS: $F(5, 51) = 7.57$, $P < 0.001$, saline WD1, 24 ± 3.8%, $n/m = 12/46$ versus cocaine WD1, 47 ± 3.0%, $n/m = 14/42$, $P < 0.001$). At WD20 and all withdrawal time points thereafter, the fraction of silent synapses was similar compared to that of saline control mice (Fig 1G; Table EV1; %SS: cocaine WD20, 20 ± 4.9%, $n/m = 6/22$ versus saline WD1, $P > 0.05$, $t(16) = 0.70$, $P = 0.49$; cocaine WD30, 23 ± 5.0%, $n/m = 9/17$ versus saline WD1, $P > 0.05$; cocaine WD40, 23 ± 4.5%, $n/m = 7/16$ versus saline WD1, $P > 0.05$), whereas the fraction of silent synapses at WD10 was between that of WD1 and WD20 (Fig 1C, E and G; %SS: WD10, 36 ± 4.2%, $n/m = 9/29$).

We previously showed that in rats after cocaine self-administration, a portion of the silent synapses are unsilenced by incorporating CP-AMPARs, reflected as an increase in the rectification index of AMPAR EPSCs (Lee *et al*, 2013). After the cocaine-CPP procedure, the rectification index progressively increased until WD30 (Fig 1F and G; Table EV1; $F(5, 48) = 5.34$, $P < 0.001$, saline WD1, 2.0 ± 0.1, $n/m = 9/34$; cocaine WD1, 2.0 ± 0.1, $n/m = 13/39$ versus saline WD1, $P > 0.05$; cocaine WD10, 2.1 ± 0.2, $n/m = 7/23$ versus saline WD1, $P > 0.05$; WD20, 2.5 ± 0.1, $n/m = 6/23$ versus saline WD1, $P > 0.05$; cocaine WD30, 2.74 ± 0.16, $n/m = 7/21$ versus saline WD1, $P < 0.01$). Thus, based on the correlated decline of silent synapses and the increase of CP-AMPARs, withdrawal after cocaine-CPP training likely resulted in a conversion of silent

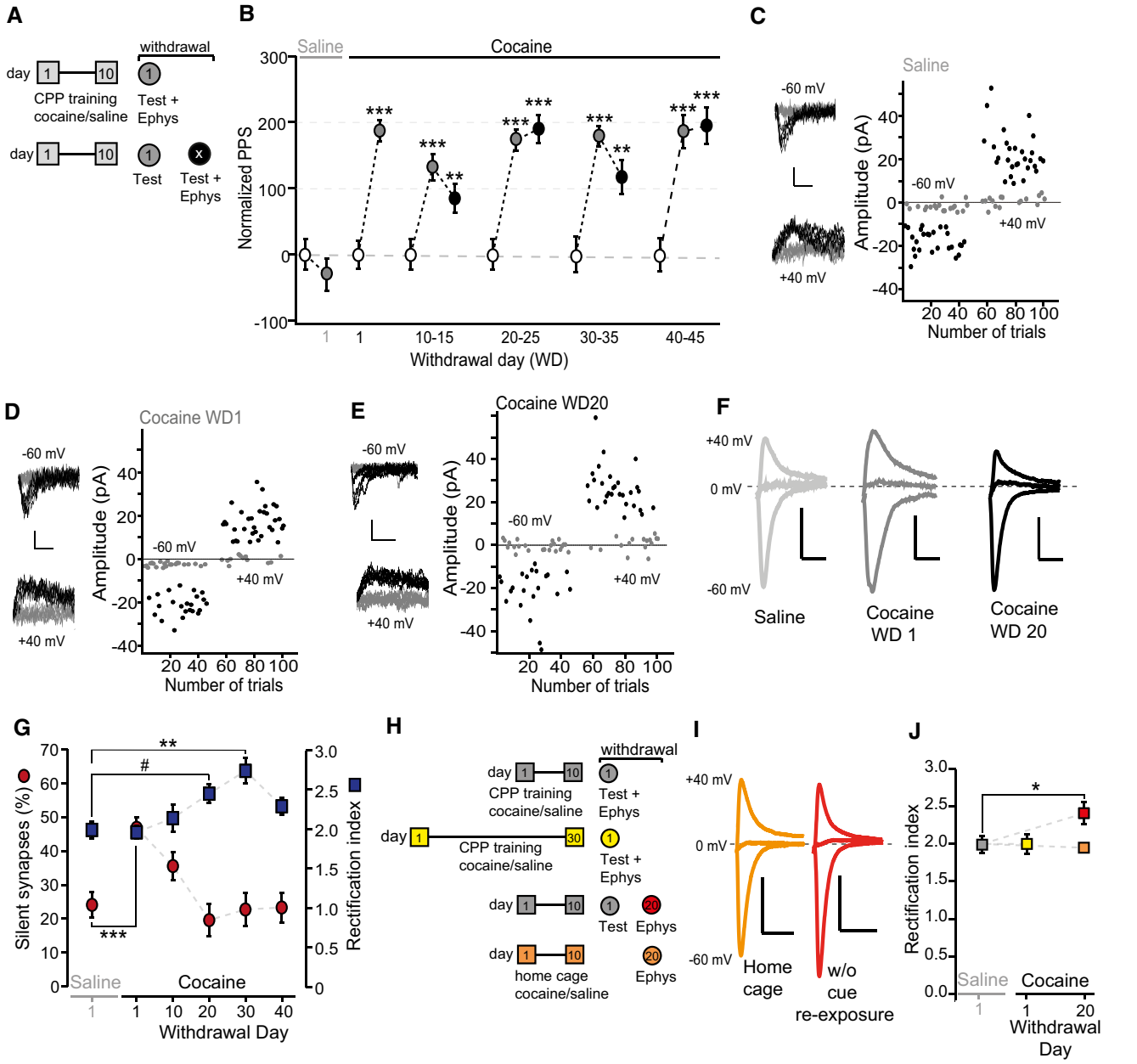


Figure 1. Wild-type mice learn and remember the association with the cocaine-paired chamber in the conditioned place preference (CPP) paradigm.
Withdrawal from cocaine-CPP is accompanied by a decrease in percentage of silent synapses and increase in the rectification index.

A Schematic representation of the timeline of behavior and electrophysiology experiments.

B The place preference score (PPS) is indicated by normalizing to the baseline PPS. Baseline, white circles; withdrawal day 1 (WD1), gray circles (saline WD1, $n = 28$; cocaine WD1, $n = 29$); long-term withdrawal day, black circles (cocaine WD10, $n = 21$; WD20, $n = 25$; WD30, $n = 27$; WD40, $n = 20$).

C–E EPSCs evoked at −60 mV and +40 mV by minimum stimulation (left side) over 100 trials from example recordings from nucleus accumbens (NAc), medium spiny neurons (MSNs) with saline-CPP WD1 (C, scale bar 10 pA, 5 ms), cocaine-CPP WD1 (D, scale bar 10 pA, 5 ms), and cocaine-CPP WD20 (E, scale bar 25 pA, 25 ms). Responses are shown in black and failures in gray.

F Example AMPA EPSCs evoked at −60 mV, 0 mV, and +40 mV for rectification index assay. Scale bar 100 pA, 25 ms.

G Summarized results showing the percentage of silent synapses (red circles; saline WD1, $n/m = 12/46$; cocaine WD1, $n/m = 14/42$; WD10, $n/m = 9/29$; WD20 $n/m = 6/22$; WD30, $n/m = 9/17$; WD40, $n/m = 7/16$) and rectification index (blue squares; saline WD1, $n/m = 9/34$; cocaine WD1, $n/m = 13/39$; WD10, $n/m = 7/23$; WD20, $n/m = 6/23$; WD30, $n/m = 7/21$; WD40, $n/m = 10/40$) in NAc MSNs.

H Scheme for behavior and electrophysiology experiments to check the specificity of increased rectification index in NAc MSNs following cocaine exposure.

I Example AMPA EPSCs evoked at −60 mV, 0 mV, and +40 mV for rectification index assay. Scale bar 100 pA, 25 ms.

J Summarized results showing rectification index increases due to withdrawal from cocaine-CPP (yellow, $n/m = 3/9$) and not due to re-exposure to the CPP apparatus (red, $n/m = 6/18$), but only specifically when CPP assay was performed (orange, $n/m = 5/20$).

Data information: Data are given as means ± s.e.m.; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; paired t-test in (B) or one-way ANOVA with Bonferroni post-test in (G) and (J). $^{\#}P < 0.05$ by Student's t-test in (G).

synapses into CP-AMPAR-containing synapses. Notably, the rectification index at WD40 was reduced again and was not significantly different compared to that of saline control mice (Fig 1G; cocaine WD40, 2.3 ± 0.1, n/m = 10/40 versus saline WD1, $P > 0.05$). The decline of CP-AMPARs at WD40 was not paralleled by an increase in silent synapses, indicating that the affected synapses were not re-silenced and might be pruned or converted to GluA2-containing AMPAR synapses. Thus, withdrawal from cocaine-CPP resulted in a transient upregulation of synaptic CP-AMPARs, an effect contrasting the persistent upregulation of CP-AMPARs in rats after extended-access cocaine self-administration, which lasts beyond WD70 (Wolf & Tseng, 2012).

In adult rats, contingent exposure is required for cocaine-induced accumulation of CP-AMPARs in the NAc, while passive exposure is insufficient (Conrad *et al*, 2008; McCutcheon *et al*, 2011). However, in mice, repeated passive cocaine exposure induces the accumulation of CP-AMPARs (Mameli *et al*, 2009). It remains unclear whether the expression of CP-AMPARs after drug withdrawal is a general adaptation of repeated drug exposure or is specific for contingent (e.g., self-administered) drug exposure. In an attempt to resolve this potential discrepancy, we tested whether the repeated i.p. injections of cocaine per se, or the repeated cocaine injections in the context of CPP training, induce CP-AMPAR upregulation in mice. Similar to our previous cohorts (after CPP training and PPS measurement), the rectification index of the reference group at WD1 was ~2 (Fig 1H and J). To test whether context re-exposure triggers the expression of CP-AMPARs, one cohort of mice received CPP training and a single PPS measure at WD1 before CP-AMPARs were analyzed at WD20 without an additional PPS measure (Fig 1H). The rectification index was higher than that of the reference group of WD1 (Fig 1I and J; $F(3, 13) = 3.13$, $P < 0.05$, cocaine without context exposure WD20 (red), 2.4 ± 0.2, n/m = 6/18 versus saline WD1, $P < 0.05$), indicating that context re-exposure after long-term withdrawal from cocaine-CPP was not required for the expression of CP-AMPARs. To test whether withdrawal was required or whether the onset of CP-AMPAR expression follows a set time course after the initial cocaine administration, one cohort of mice received 30 days CPP training with alternating CS+ and CS−. The PPS was measured at WD1, and CP-AMPARs were measured immediately afterward (Fig 1H and J). The rectification index was similar to that of the reference group on WD1, a result consistent with the requirement of prolonged drug withdrawal for the expression of CP-AMPARs (30 days cocaine-CPP WD1 (yellow), 2.0 ± 0.1, n/m = 3/9 versus saline WD1, $P > 0.05$). In the last cohort, we tested whether CPP training and thus the association of context with drug exposure was required for the expression of CP-AMPARs after prolonged withdrawal. Mice received alternating CS+ and CS− in their home cage without exposure to the CPP apparatus, and CP-AMPARs were measured at WD20 (Fig 1H–J). The rectification index was similar to that of the reference group on WD1 (rectification index cocaine home cage WD20 (orange): 2.0 ± 0.04, n/m = 5/20 versus saline WD1, $P > 0.05$), indicating that repeated non-contingent cocaine administration itself did not trigger the expression of CP-AMPARs in MSNs of the NAc shell, and that pairing non-contingent cocaine with the CPP training context is necessary. Following a similar logic, we also tested whether cocaine-induced silent synapse generation is context dependent or is exclusively a pharmacological effect. Mice received alternating cocaine and saline injections in their home cage and silent synapses were measured at WD1 (Fig EV1). The fraction of silent synapses was similar to that when paired with CPP training (%SS: cocaine with CPP WD1 versus cocaine home cage WD1: 37 ± 4.6%, n/m = 7/14, $t(19) = 1.81$, $P = 0.09$), indicating that the primary pharmacological mechanisms of cocaine are sufficient to generate silent synapses in the NAc.

Together, the above results revealed that generation of silent synapses was induced by repeated cocaine administration, and the time course of CP-AMPAR expression paralleled the reduction in silent synapses. However, CP-AMPAR expression was not induced if the cocaine administration was not associated with CPP training, revealing the induction of CP-AMPARs as a signature of cocaine–cue association.

PSD-95 is required for CPP retention, but not acquisition

We previously showed that PSD-95 KO mice exhibit a high basal level of silent synapses in the visual cortex, and these silent synapses do not undergo experience-dependent maturation (Huang *et al*, 2015a). Partially due to the altered levels of silent synapses and related synaptic properties such as enhanced long-term synaptic potentiation, PSD-95 KO mice exhibit extended juvenile-like plasticity (Migaud *et al*, 1998; Béïque *et al*, 2006; Carlisle *et al*, 2008; Huang *et al*, 2015a), and at the same time, compromised cognitive learning as tested in the Morris water maze (Migaud *et al*, 1998). Subsequent studies show that the acquisition of contextual fear conditioning is normal, but long-term memory retention is impaired (Fitzgerald *et al*, 2014), indicating that PSD-95 is not required for memory acquisition but is required for retention. Given the importance of silent synapse-based NAc circuitry remodeling in drug-induced behavioral learning, we tested cocaine-CPP in PSD-95 KO, in which silent synapses are resistant to maturation. Similar to WT mice and interleaved littermate wild-type controls, saline-injected PSD-95 KOs did not exhibit a preference for either chamber (Fig 2A; Table EV1; PPS: saline BL, 508 ± 33 versus saline WD1, 546 ± 80, n = 8, $t(7) = 0.50$, $P = 0.63$). One day after (WD1) the standard CPP training, PSD-95 KOs exhibited cocaine-CPP (Fig 2A; Table EV1; PPS: cocaine BL, 519 ± 45 versus cocaine WD1, 810 ± 45, n = 11, $t(10) = 3.99$, $P < 0.01$), but the CPP established in these PSD-95 KOs disappeared after 20-days withdrawal (Fig 2A; Table EV1; PPS: cocaine WD20, 531 ± 51 versus BL, n = 10, $t(9) = 0.19$, $P = 0.86$). Thus, PSD-95 knockout does not affect CPP acquisition, but disrupts the retention of cocaine-CPP.

On WD1, the PPS in PSD-95 KOs was higher than that in wild-type mice (PPS: cocaine WD1 (WT) versus cocaine WD1 (PSD-95 KO), $t(34) = 2.60$, $P < 0.05$), which may be due to a quantitative increase in CPP acquisition by PSD-95 KOs. To test this possibility, we trained another cohort of PSD-95 KOs and their wild-type littermate controls with a more stringent CPP training procedure, in which the cocaine pairing was reduced to 5 days with daily alternating i.p. injections of cocaine and saline. After this relatively weak conditioning procedure, the wild-type littermate mice did not acquire cocaine-CPP (PPS: BL (WT), 581 ± 14 versus cocaine WD1 (WT), 535 ± 55, n = 4, $t(3) = 0.68$, $P = 0.54$), but the PSD-95 KO mice did (PPS: BL (PSD-95 KO), 492 ± 32 versus cocaine WD1 (PSD-95 KO), 856 ± 62, n = 4, $t(3) = 7.14$, $P < 0.01$). These results indicate that CPP acquisition was indeed facilitated by PSD-95 KO,

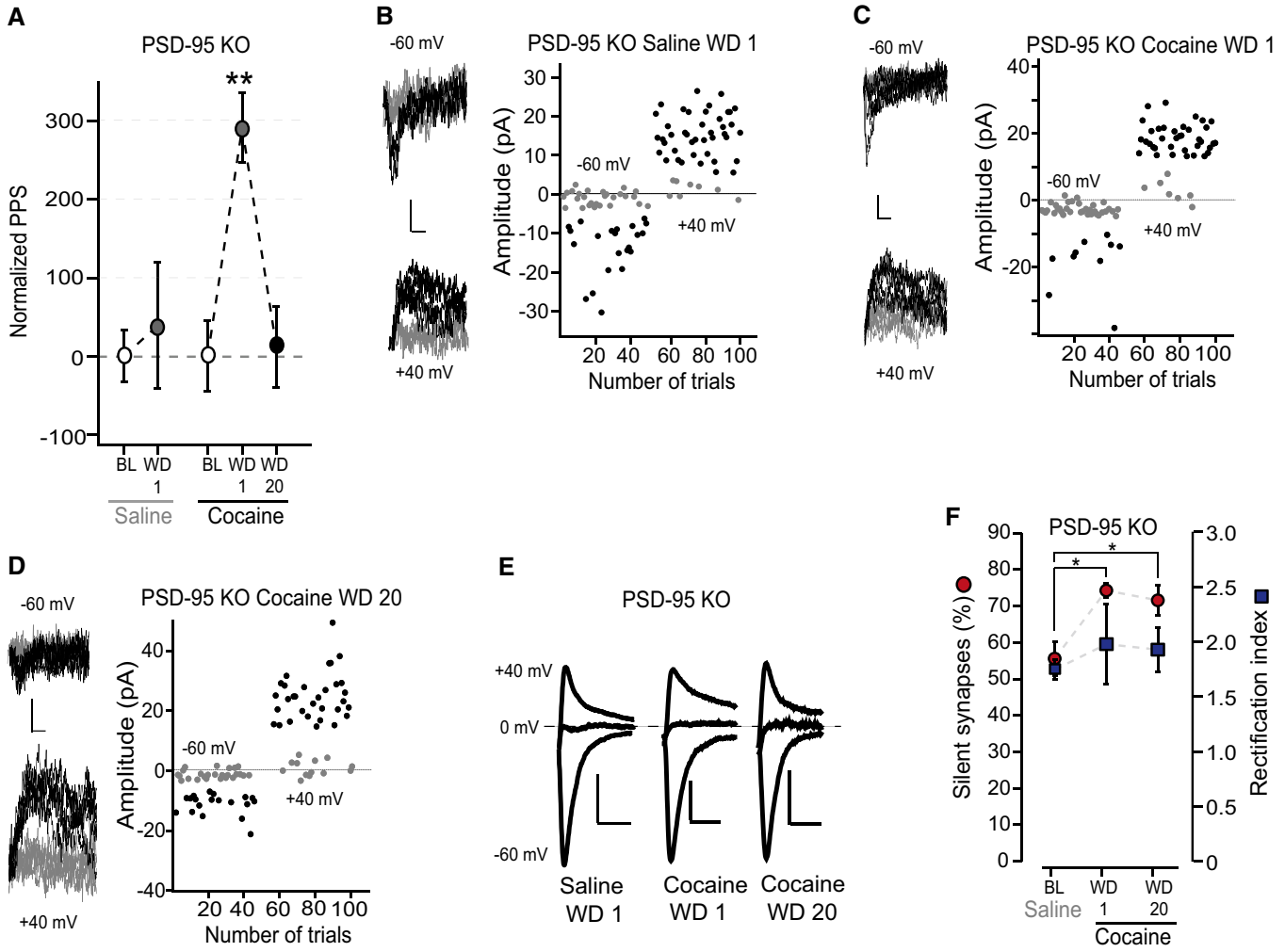


Figure 2. PSD-95 KO mice do not retain the preference for the cocaine-paired chamber in the CPP paradigm and lack maturation of silent synapses in the NAc.

A The place preference score (PPS) is indicated by normalizing to the baseline PPS. Baseline, white circles; withdrawal day 1 (WD1), gray circles (saline, $n = 8$; cocaine, $n = 11$); long-term withdrawal day, black circles (cocaine, $n = 10$).

B–D EPSCs evoked at −60 mV and +40 mV by minimum stimulation (left side) over 100 trials from example recordings from NAc MSNs of PSD-95 KO mice with saline-CPP WD1 (B), cocaine-CPP WD1 (C), and cocaine-CPP WD20 (D). Responses are shown in black and failures in gray. Scale bars 10 pA, 5 ms.

E Example AMPA EPSCs evoked at −60 mV, 0 mV, and +40 mV for rectification index in NAc MSNs of PSD-95 KO mice. Scale bar 50 pA, 20 ms.

F Summarized results showing the percentage of silent synapses (red circles; saline WD1, $n/m = 6/21$; cocaine WD1, $n/m = 4/14$; WD20, $n/m = 5/20$) and rectification index (blue squares; saline WD1, $n/m = 4/24$; cocaine WD1, $n/m = 4/15$; WD20, $n/m = 4/14$) in NAc MSNs of PSD-95 KO mice.

Data information: Data are given as means ± s.e.m.; *$P < 0.05$, **$P < 0.01$; paired t-test in (A) or one-way ANOVA with Bonferroni post-test in (F).

which may partially result from the high basal levels of silent synapses and the related enhancement of synaptic potentiation.

Some mice were used for analysis of silent synapses and AMPAR rectification index immediately after WD1 PPS measurement, and some others were analyzed after the WD20 PPS measurement. In saline-exposed PSD-95 KOs, the fraction of silent synapses in NAc MSNs was ~twofold higher than that in wild-type controls (Fig 2B and F; Table EV1; %SS: saline WD1 (PSD-95 KO), 56 ± 4.6%, $n/m = 6/21$ versus saline WD1 (WT), 24 ± 3.8%, $t(16) = 4.97$, $P < 0.001$). Notably, such a high basal level of silent synapses was also observed in the visual cortex of PSD-95 KOs (Huang *et al*, 2015a), indicating that a large portion of the forebrain excitatory synapses are resistant to experience-dependent maturation. Despite the high basal levels, the fraction of silent synapses in the NAc was further increased in PSD-95 KOs 1d after the cocaine-CPP (Fig 2C and F; Table EV1; %SS: $F(2, 12) = 6.41$, $P < 0.05$, cocaine WD1, 74 ± 1.9%, $n/m = 4/14$ versus saline WD1, $P < 0.05$). 20d after cocaine-CPP, while the fraction of silent synapses in wild-type mice dropped likely through a maturation process, it remained high in PSD-95 KOs (Fig 2D and F; Table EV1; %SS: WD20 (PSD-95 KO), 72 ± 4.1%, $n/m = 5/20$ versus saline WD1 (PSD-95 KO), $P < 0.05$). Thus, similar to the lack of experience-dependent silent synapse maturation in the visual cortex during development (Huang *et al*, 2015a), the cocaine-induced silent synapses in PSD-95 KOs also remained unsilenced after prolonged drug withdrawal.

In saline-exposed PSD-95 KOs, the AMPAR rectification index was similar to that of saline-exposed wild-type mice (Fig 2E and F; Table EV1; rectification index: saline WD1 (PSD-95 KO), 1.8 ± 0.1,

n/m = 4/24 versus saline WD1 (WT), $t(11) = 1.40$, $P = 0.19$). In cocaine-CPP-exposed PSD-95 KOs, the rectification index did not increase on WD20, which would otherwise increase in wild-type mice (Fig 2E and F; Table EV1; rectification index: $F(2, 9) = 0.24$, $P = 0.79$, cocaine WD1, 2.0 ± 0.4, n/m = 4/15 versus saline WD1 (PSD-95 KO), $P > 0.05$; cocaine WD20, 1.9 ± 0.2, n/m = 4/14 versus saline WD1 (PSD-95 KO), $P > 0.05$). In conclusion, these results so far show that the acquisition of cocaine-CPP was correlated with cocaine-induced generation of silent synapses, the expression of CP-AMPARs was correlated with CPP long-term retention, and PSD-95 was required for silent synapse unsilencing during cocaine withdrawal.

PSD-95 is the most abundant paralog in the NAc compared to PSD-93 and SAP102

Loss of function of PSD-95 causes a reduction in AMPAR EPSC amplitudes by ~50% (Nakagawa *et al*, 2004; Elias *et al*, 2006; Schlüter *et al*, 2006), which echoes the high basal fraction of silent synapses after PSD-95 KO or knockdown (Béïque *et al*, 2006; Huang *et al*, 2015a). Additional loss of function of the paralogs PSD-93 and SAP102 further reduced AMPAR EPSC amplitudes, which might reflect the redundant contribution of these paralogs to synaptic properties (Elias *et al*, 2006). To estimate the relative abundance of the three paralogs in the NAc, we used quantitative Western blotting with recombinant reference samples (Fig 3; Krüger *et al*, 2013). We expressed GFP-tagged PSD-93, PSD-95, and SAP102 in HEK293 cells and adjusted the cell lysates to similar signal intensities in Western blot detection with an anti-GFP antibody (Fig 3A). Protein levels of the three paralogs were quantified in samples of mouse NAc punches with paralog-specific antibodies relative to the protein levels of the recombinant proteins (Fig 3A and B). PSD-95 was > threefold more abundant than PSD-93 or SAP102 (Fig 3A–C; $F(2, 15) = 16.08$, $P < 0.001$; PSD-95, 0.36 ± 0.06, $n = 6$ versus PSD-93, 0.10 ± 0.03, $n = 6$, $P < 0.001$; SAP102, 0.06 ± 0.01, $n = 6$ versus PSD-95, $P < 0.001$). Thus, similar to the hippocampus (Krüger *et al*, 2013), PSD-95 is also the predominant paralog in the NAc.

The reduced basal levels of silent synapses in PSD-93 KOs do not prevent cocaine-induced silent synapse generation

The PSD-93 gene is the most complex among the DLG-MAGUKs; it codes for the highest number of N-terminal isoforms (Parker *et al*, 2004; Krüger *et al*, 2013). Consequently, the analysis of PSD-93 KOs has uncovered multiple, and partly conflicting results on PSD-93-mediated regulation of AMPARs. It was consistently observed that PSD-95 and PSD-93 isoforms share some redundancy in regulating AMPAR function, when one is replaced by another or overexpressed (Elias *et al*, 2006; Krüger *et al*, 2013). However, results on the loss of PSD-93 function vary. Some studies observe no effect or increased AMPAR EPSC amplitudes (Tao *et al*, 2003; Carlisle *et al*, 2008; Krüger *et al*, 2013), while other studies report an equivalent effect of PSD-93 and PSD-95 on synaptic AMPARs (Elias *et al*, 2006). Since our recent work uncovered a critical role of PSD-95 in the experience-dependent maturation of silent synapses (Huang *et al*, 2015a; Fig 2), we tested whether PSD-93 provides redundant or unique regulation of cocaine-induced silent synapses and CPP.

Without a preference in saline-CPP for either of the two compartments (Fig 4A; Table EV1; PPS: saline BL, 502 ± 62 versus saline WD1, 551 ± 64, $n = 5$, $t(4) = 1.05$, $P = 0.35$), the PSD-93 KOs acquired cocaine-CPP on WD1 after the 10-d training procedure (Fig 4A; Table EV1; PPS: cocaine BL, 502 ± 18 versus cocaine WD1, 685 ± 28, $n = 12$, $t(11) = 6.09$, $P < 0.0001$). Similar to wild-type mice, the PPS in PSD-93 KOs remained high on WD20 (Fig 4A; Table EV1; PPS: cocaine BL versus cocaine WD20, 623 ± 48, $n = 9$, $t(8) = 2.81$, $P < 0.05$). Thus, both the acquisition and retention of cocaine-CPP were preserved in PSD-93 KOs.

We next analyzed the cellular correlates in MSNs of the NAc shell. Unexpectedly, in PSD-93 KOs, the fraction of silent synapses of saline-exposed PSD-93 KOs was lower than that of wild-type controls (Fig 4B and F; Table EV1; %SS: saline WD1 (PSD-93 KO), 4.8 ± 2.7%, n/m = 5/19 versus saline WD1 (WT), 24 ± 3.8%, $t(15) = 3.08$, $P < 0.01$). Thus, loss of PSD-93 resulted in the opposite effect on silent synapse numbers compared to the loss of PSD-95. Cocaine-CPP training generated silent synapses in PSD-93 KOs (Fig 4C and F; Table EV1; %SS: $F(2, 11) = 8.51$, $P < 0.01$; cocaine WD1, 26 ± 4.5%, n/m = 5/22 versus saline WD1, $P < 0.01$). Similar to wild-type mice, the fraction of silent synapses returned to the basal level on WD20 (Fig 4D and F; %SS: cocaine WD20, 12 ± 4.1%, n/m = 4/12 versus saline WD1, $P > 0.05$). The AMPAR rectification index in PSD-93 KO mice was similar to that of wild-type mice on WD1 (Fig 4E and F; Table EV1; rectification index: saline WD1 (PSD-93 KO), 1.8 ± 0.14, n/m = 6/22 versus saline WD1 (WT), $t(13) = 1.32$, P =0.21). Similar to wild-type mice, it increased on WD20 (Fig 4E and F; rectification index: $F(2, 16) = 3.54$, cocaine WD1, 1.9 ± 0.1, n/m = 5/17 versus saline WD1, $P > 0.05$; cocaine WD20, 2.2 ± 0.1, n/m = 8/23 versus saline WD1, $P < 0.05$). Thus, despite the reduced basal level of silent synapses, exposure to cocaine generated silent synapses in PSD-93 KOs, and these cocaine-generated silent synapses were likely unsilenced by CP-AMPAR incorporation after cocaine withdrawal. These results reveal that PSD-93 was not required for CPP acquisition or retention, although the overall fraction of silent synapses was reduced.

SAP102 KO prevents synaptic expression of CP-AMPARs after cocaine withdrawal but does not affect cocaine-CPP retention

Previous studies show redundancy and functional interaction between SAP102 and PSD-95 in regulating synaptic AMPAR numbers (Elias *et al*, 2006; Bonnet *et al*, 2013). Here, we tested the role of SAP102 in cocaine-induced silent synapse generation and CPP. SAP102 KOs did not exhibit a preference for either of the two compartments after saline-CPP (Fig 5A; Table EV1; PPS: saline BL, 523 ± 85 versus saline WD1, 488 ± 59, $n = 6$, $t(5) = 0.32$, $P = 0.76$). The 10-d cocaine-CPP training resulted in an increased PPS on WD1 (Fig 5A; Table EV1; PPS: cocaine BL, 495 ± 26 versus cocaine WD1, 734 ± 29, $n = 19$, $t(18) = 7.27$, $P < 0.0001$). Similar to wild-type mice, the PPS in SAP102 KOs remained increased on WD20 (Fig 5A; Table EV1; PPS: cocaine WD20, 662 ± 38 versus cocaine BL, $n = 15$, $t(14) = 3.59$, $P < 0.01$), revealing that the retention of CPP was not affected by the loss of SAP102. In saline-exposed SAP102 KOs, the basal fraction of silent synapses was similar to that of wild-type mice (Fig 5B and F; Table EV1; %SS: saline WD1 (SAP102 KO), 33 ± 4.8%, n/m = 6/14 versus saline WD1

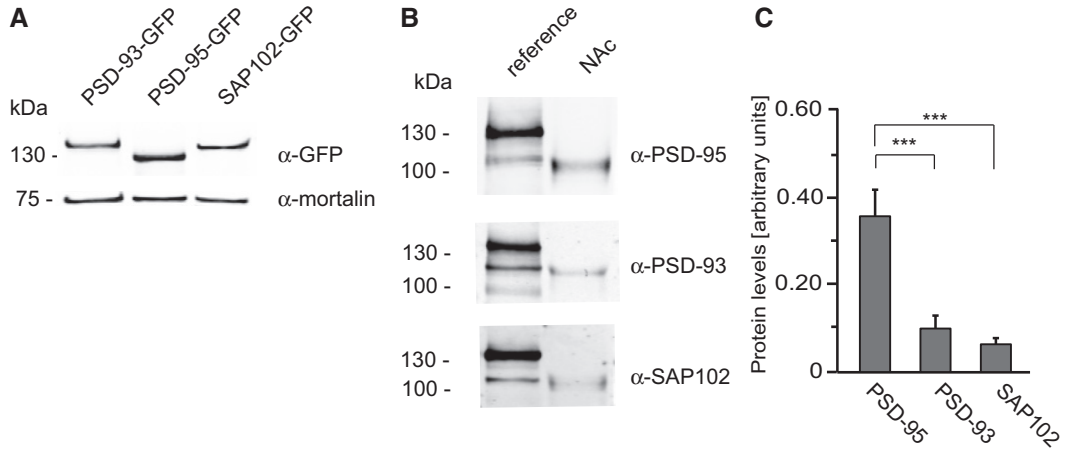


Figure 3. PSD-95 is the predominant protein of the DLG-MAGUK family in the nucleus accumbens (NAc) of WT mice.

A Lysates of transfected HEK293 cells loaded to have similar quantities of the recombinant GFP-tagged proteins PSD-95, PSD-93, and SAP102 as seen by GFP immunoreactivity. Mortalin was used as the loading control.

B Abundance of endogenous DLG-MAGUK proteins in NAc punches of WT mice. The corresponding GFP-tagged recombinant protein from transfected HEK cell lysates has been used as the reference.

C Quantification of the protein levels of DLG-MAGUK proteins in the NAc, after normalization to the reference ($n = 6$). Data are given as means ± s.e.m.; ***$P < 0.001$; one-way ANOVA with Bonferroni post-test.

(WT), $t(16) = 1.33$, $P = 0.20$). Cocaine-CPP training generated silent synapses, detected on WD1 (Fig 5C and F; Table EV1; %SS: $F(2, 15) = 6.47$, $P < 0.01$, cocaine WD1, 56 ± 6.5%, $n/m = 4/13$ versus saline WD1, $P < 0.05$). However, similar to PSD-95 KOs, the fraction of silent synapses remained high on WD20 in SAP102 KOs (Fig 5D and F; Table EV1; %SS: cocaine WD20, 57 ± 5.0%, $n/m = 8/16$ versus saline WD1, $P < 0.01$). The AMPAR rectification index in saline-exposed SAP102 KOs was similar to that of wild-type controls (rectification index: saline WD1 (SAP102 KO), 2.1 ± 0.2, $n/m = 7/20$ versus saline WD1 (WT), $t(14) = 2.14$, $P = 0.69$). In cocaine-CPP trained SAP102 KOs, the rectification index remained low on WD1 and WD20 (Fig 5E and F; Table EV1; $F(2, 16) = 0.60$, $P = 0.56$; saline WD1 versus cocaine WD1: 2.1 ± 0.1, $n/m = 4/14$, $P > 0.05$; cocaine WD20: 1.9 ± 0.1, $n/m = 8/31$ versus saline WD1, $P > 0.05$). Thus, in SAP102 KOs, the cocaine-CPP was similar to that of wild-type mice, while the time course of cellular changes was similar to PSD-95 KO mice. While these results are again consistent with a mechanism of silent synapse unsilencing through CP-AMPAR incorporation, which is impaired in SAP102 KOs, they indicate a clear disconnection between silent synapse maturation and cocaine-CPP. Specifically, silent synapse unsilencing through CP-AMPAR incorporation is not essential for CPP retention.

NAc-restricted knockdown of PSD-95 mimicked cellular, but not behavioral changes in PSD-95 KO mice

In KO mice, the gene is deleted throughout synapse development, which might cause homeostatic adaptations that mask the normal physiological response. To circumvent this potential caveat, we used recombinant adeno-associated virus vectors (AAV) to restrict the gene manipulation within the NAc *in vivo* at a time point when neural networks have already matured. We focused on PSD-95 because the cellular changes paralleled the behavioral changes in the PSD-95 KO mice, that is, the lack of CP-AMPAR synaptic accumulation correlated with the lack of CPP retention. 2-3 weeks before CPP training, we injected an AAV (Fig 6A and B), which expressed an shRNA against PSD-95 (AAV-sh95) or a control vector (AAV-ctr; Fitzgerald *et al*, 2014; Huang *et al*, 2015a). The transduction area for each mouse was post hoc analyzed on a fluorescence stereo microscope. Only mice in which > 50% of the bilateral NAc shell was covered by fluorescence were included in the final analysis (Fig 6B). The colocalization of GFP fluorescence and DAPI nuclear staining revealed that the AAV8 serotype transduced the NAc cells with high efficiency. The cocaine-CPP training resulted in a positive PPS on WD1, and the PPS remained increased on WD20 in both AAV-sh95 and AAV-ctr mice (Fig 6C; Table EV1; PPS: BL (AAV-ctr), 523 ± 21 versus cocaine WD1 (AAV-ctr), 687 ± 21, $n = 16$, $t(15) = 6.02$, $P < 0.0001$; cocaine WD20 (AAV-ctr), 616 ± 30 versus BL (AAV-ctr), $n = 12$, $t(11) = 2.70$, $P < 0.05$; BL(AAV-sh95), 529 ± 17 versus cocaine WD1 (AAV-sh95), 691 ± 19, $n = 26$, $t(25) = 7.90$, $P < 0.0001$; cocaine WD20 (AAV-sh95), 721 ± 29 versus BL (AAV-sh95), $n = 13$, $t(12) = 4.30$, $P < 0.01$). Thus, in contrast to PSD-95 KO mice, local NAc AAV-sh95 expression did not impair CPP retention.

Electrophysiological analysis revealed that the fraction of silent synapses and AMPAR rectification index of AAV-ctr transduced MSNs at WD20 was similar to that of non-injected mice (Fig 6D; %SS: cocaine WD20 (AAV-ctr), 18 ± 2.8%, $n/m = 4/14$ versus cocaine WD20 (WT non-injected), $t(8) = 0.30$, $P = 0.77$; rectification index: cocaine WD20 (AAV-ctr), 2.9 ± 0.3, $n/m = 4/12$ versus cocaine WD20 (WT non-injected), $t(8) = 1.63$, $P = 0.14$), indicating that AAV transduction by itself did not alter the cellular response to cocaine. In AAV-sh95-transduced neurons, the fraction of silent synapses was increased on WD1 and remained high on WD20 (Fig 6E; Table EV1; %SS: cocaine WD1 (AAV-sh95), 60 ± 4.9%, $n/m = 5/13$ versus cocaine WD20 (AAV-sh95),

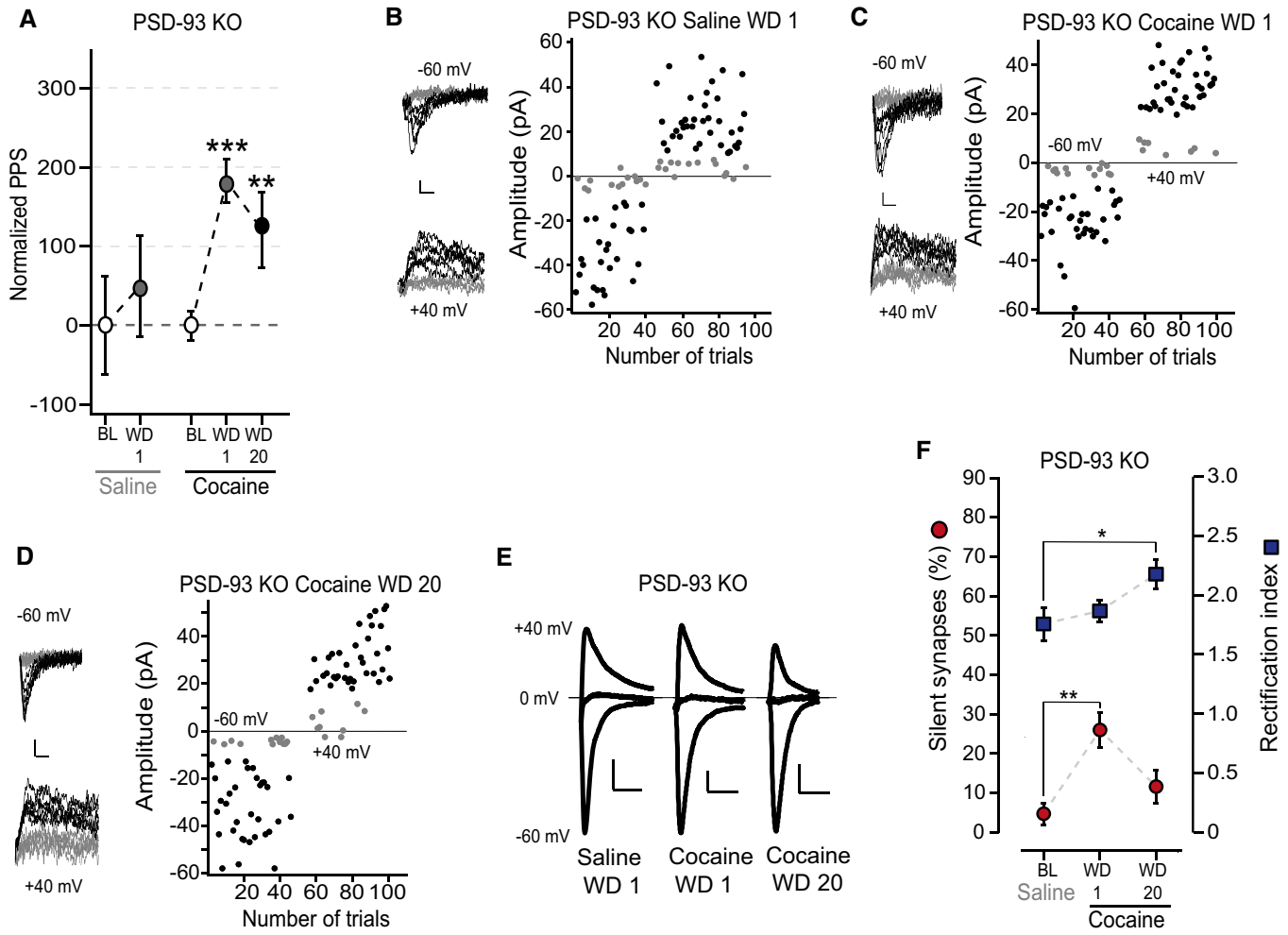


Figure 4. PSD-93 KO mice have lower baseline number of silent synapses in the NAc. Silent synapses induced by cocaine-CPP mature during withdrawal and the mice have long-term retention of their place preference in the CPP paradigm.

A The place preference score (PPS) is indicated by normalizing to the baseline PPS. Baseline, white circles; withdrawal day 1 (WD1), gray circles (saline, $n = 5$; cocaine, $n = 12$); long-term withdrawal day, black circles (cocaine, $n = 9$).

B–D EPSCs evoked at −60 mV and +40 mV by minimum stimulation (left side) over 100 trials from example recordings from NAc MSNs of PSD-93 KO mice with saline-CPP WD1 (B), cocaine-CPP WD1 (C), and cocaine-CPP WD20 (D). Responses are shown in black and failures in gray. Scale bars 10 pA, 5 ms.

E Example AMPA EPSCs evoked at −60 mV, 0 mV, and +40 mV for rectification index in NAc MSNs of PSD-93 KO mice. Scale bar 50 pA, 20 ms.

F Summarized results showing the percentage of silent synapses (red circles; saline WD1, $n/m = 5/19$; cocaine WD1, $n/m = 5/22$; WD20, $n/m = 4/12$) and rectification index (blue squares; saline WD1, $n/m = 6/22$; cocaine WD1, $n/m = 5/17$; WD20, $n/m = 8/23$) in NAc MSNs of PSD-93 KO mice.

Data information: Data are given as means ± s.e.m.; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; paired t-test in (A) or one-way ANOVA with Bonferroni post-test in (F).

48 ± 6.7%, $n/m = 8/20$, $t(11) = 1.22$, $P = 0.25$; cocaine WD20 (AAV-sh95) versus cocaine WD20 (AAV-ctr), $t(10) = 3.10$, $P < 0.05$). Similar to PSD-95 KO mice, the AMPAR rectification index in AAV-sh95 transduced MSNs did not increase between WD1 and WD20 (Fig 6D; Table EV1; rectification index: cocaine WD1 (AAV-sh95), 2.4 ± 0.2, $n/m = 4/11$ versus cocaine WD20 (AAV-sh95), 2.1 ± 0.2, $n/m = 10/20$, $t(12) = 1.10$, $P = 0.29$; cocaine WD20 (AAV-sh95) versus cocaine WD20 (AAV-ctr), $t(12) = 2.50$, $P < 0.05$). These results reveal that impairing the withdrawal-induced increase in CP-AMPARs in NAc MSNs did not impair CPP retention.

SAP102 levels are increased in PSD-95 KOs, and the functional interaction between PSD-95 and SAP102 (Cuthbert *et al*, 2007; Bonnet *et al*, 2013) might mask the behavioral consequence of PSD-95 knockdown. To address this possibility, we injected the AAV-sh95 or AAV-ctr into the NAc of SAP102 KOs (Fig 6A). The cocaine-CPP training resulted in a positive PPS on WD1, and the high PPS remained on WD20 in both AAV-sh95 and AAV-control mice (Fig 6C; Table EV1; PPS: BL(SAP102 KO + AAV-ctr), 538 ± 44 versus cocaine WD1 (SAP102 KO + AAV-ctr), 795 ± 24, $n = 8$, $t(7) = 5.90$, $P < 0.001$; cocaine WD20 (SAP102KO + AAV-ctr), 882 ± 29 versus BL (SAP102KO + AAV-ctr), $n = 8$, $t(7) = 5.32$, $P < 0.01$; BL (SAP102 KO + AAV-sh95), 528 ± 25 versus cocaine WD1 (SAP102 KO + AAV-sh95), 706 ± 22, $n = 19$, $t(18) = 5.05$, $P < 0.0001$; cocaine WD20 (SAP102 KO + AAV-sh95), 691 ± 47 versus BL (SAP102 KO + AAV-sh95), $n = 10$, $t(9) = 3.59$, $P < 0.01$).

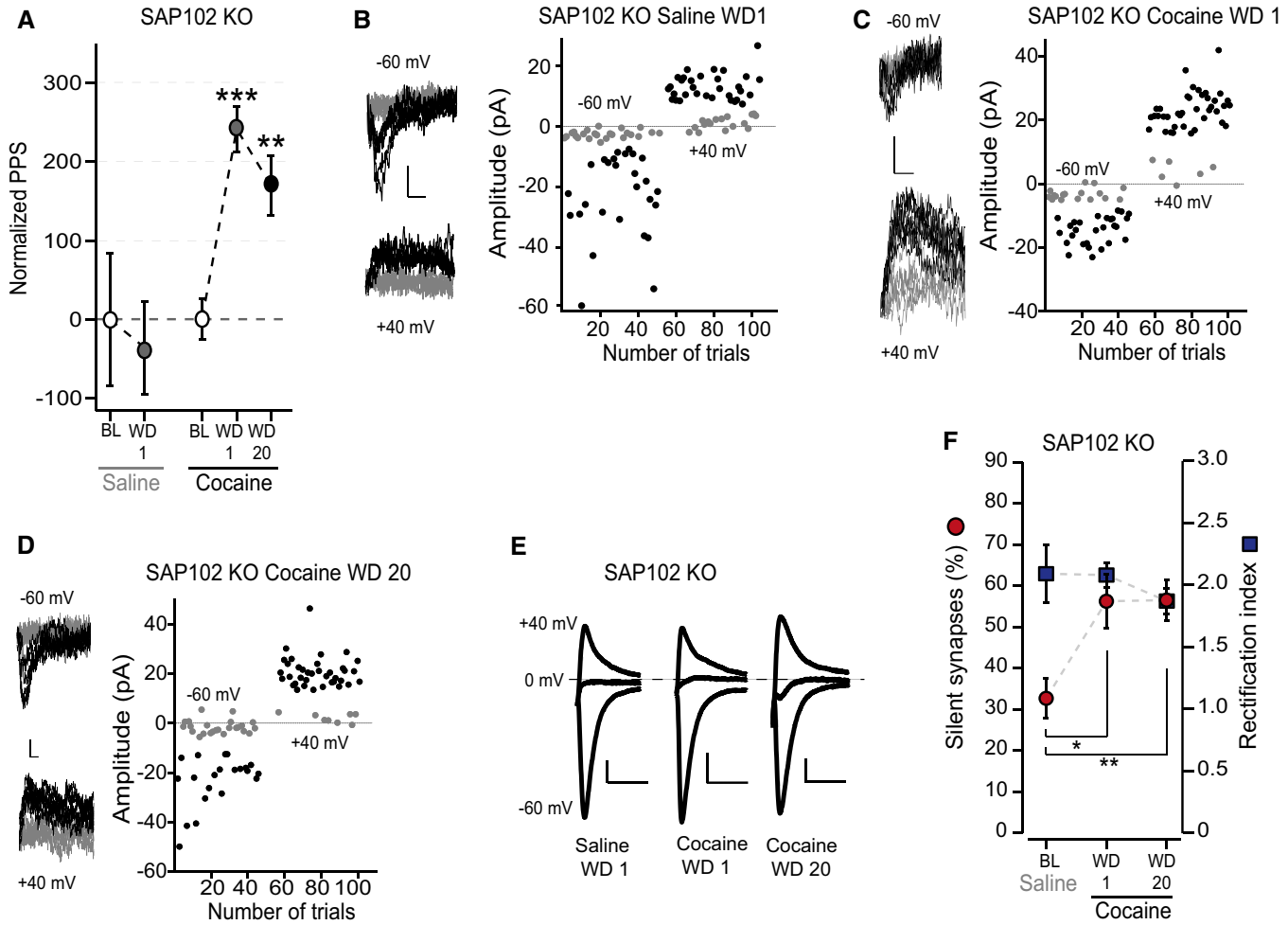


Figure 5. SAP-102 KO mice retain the preference for the cocaine-paired chamber in the CPP paradigm despite maturation of cocaine-induced silent synapses in the NAc.

A The place preference score (PPS) is indicated by normalizing to the baseline PPS. Baseline, white circles; withdrawal day 1 (WD1), gray circles (saline, $n = 6$; cocaine, $n = 19$); long-term withdrawal day, black circles (cocaine, $n = 15$).

B–D EPSCs evoked at −60 mV and +40 mV by minimum stimulation (left side) over 100 trials from example recordings from NAc MSNs of SAP102 KO mice with saline-CPP WD1 (B), cocaine-CPP WD1 (C), and cocaine-CPP WD20 (D). Responses are shown in black and failures in gray. Scale bars 10 pA, 5 ms.

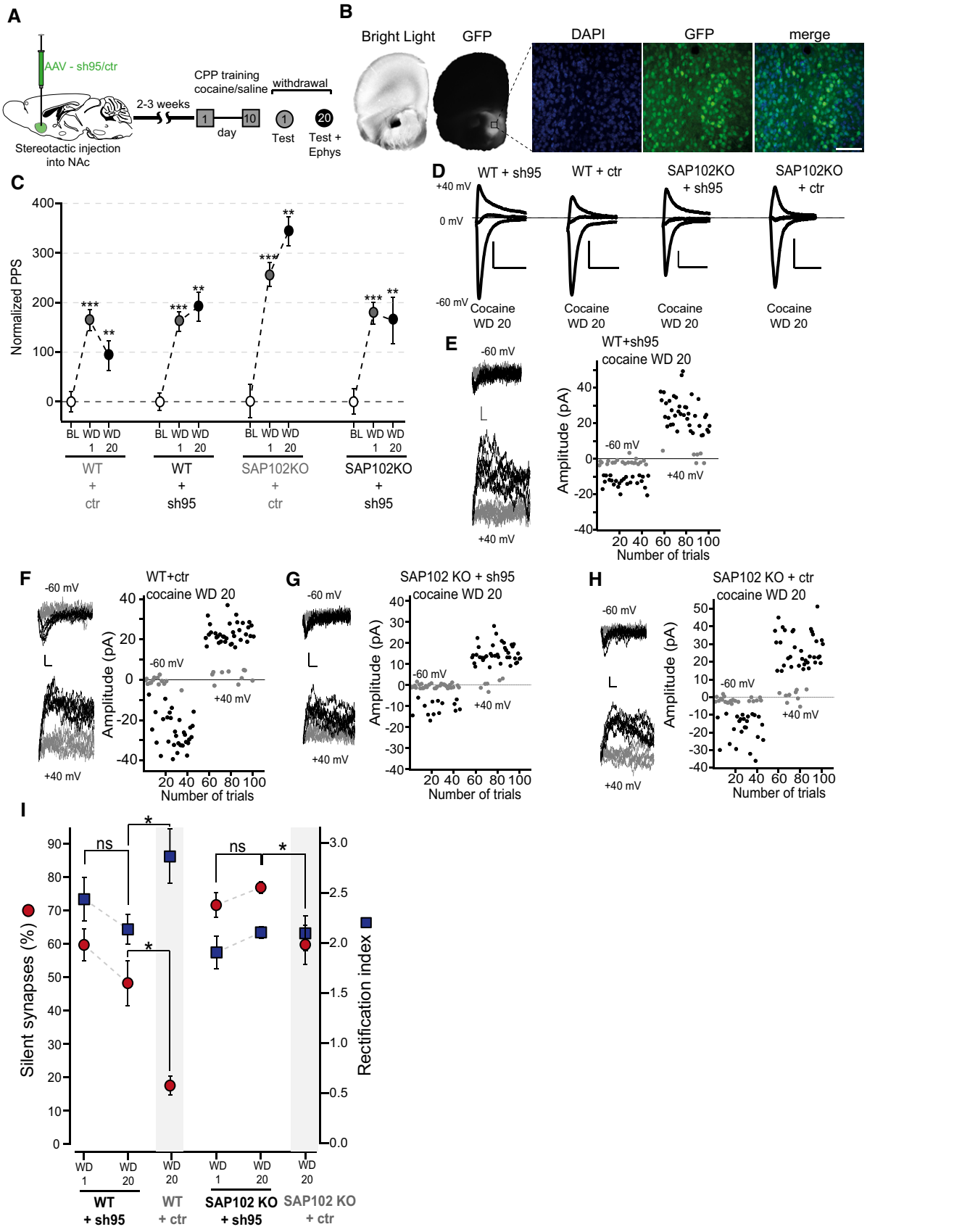
E Example AMPA EPSCs evoked at −60 mV, 0 mV, and +40 mV for rectification index in NAc MSNs of SAP102 KO mice. Scale bar 50 pA, 20 ms.

F Summarized results showing the percentage of silent synapses (red circles; saline WD1, $n/m = 6/14$; cocaine WD1, $n/m = 4/13$; WD20, $n/m = 8/16$) and rectification index (blue squares; saline WD1, $n/m = 7/20$; cocaine WD1, $n/m = 4/14$; WD20, $n/m = 8/31$) in NAc MSNs of SAP102 KO mice.

Data information: Data are given as means ± s.e.m.; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; paired t-test in (A) or one-way ANOVA with Bonferroni post-test in (F).

Thus, the potential functional compensation between PSD-95 and SAP102 within the NAc is not directly involved in CPP retention.

The electrophysiological analysis revealed that both the fraction of silent synapses and AMPAR rectification index did not change between WD1 and WD20 (Fig 6D and I; Table EV1; %SS: cocaine WD1 (SAP102 KO + AAV-sh95), 71 ± 3.7%, $n/m = 6/20$ versus cocaine WD20 (SAP102 KO + AAV-sh95), 76 ± 1.8%, $n/m = 5/17$, $t(9) = 1.20$, $P = 0.26$; rectification index: cocaine WD1 (SAP102 KO + AAV-sh95), 1.9 ± 0.2, $n/m = 3/11$ versus cocaine WD20 (SAP102 KO + AAV-sh95), 2.1 ± 0.1, $n/m = 4/14$, $t(5) = 1.30$, $P = 0.25$), indicating that the lack of either PSD-95 or SAP102 autonomously prevents the CP-AMPAR-dependent unsilencing of silent synapses. Similar to PSD-95 and SAP102 KOs, the rectification index was not different compared to that of WT mice on WD20 (Fig 6D; rectification index: cocaine WD20 (SAP102 KO + AAV-ctr), 2.1 ± 0.2, $n/m = 3/9$ versus cocaine WD20 (SAP102 KO + AAV-sh95), $t(5) = 0.07$, $P = 0.95$). On WD20, the fraction of silent synapses in sh95-expressing MSNs from SAP102 KO was higher compared to that of SAP102 KO + AAV-control (Fig 6G–I; %SS: cocaine WD20 (SAP102 KO + AAV-ctr), 59 ± 5.9%, $n/m = 3/8$ versus cocaine WD20 (SAP102 KO + AAV-sh95), $t(6) = 3.49$, $P < 0.05$), indicating additive effects of PSD-95 and SAP102 on silent synapse maturation. Together, these results revealed that cell-restricted manipulations of PSD-95 reiterated the cellular phenotype in PSD-95 KOs, implying a cell autonomous function of PSD-95 in regulating silent synapse unsilencing and synaptic expression of CP-AMPAR increase. However, unlike the global KO of PSD-95, the NAc-restricted knockdown of PSD-95 did not impair CPP retention.


A
AAV - sh95/ctr
Stereotactic injection into NAc
2-3 weeks
CPP training cocaine/saline
withdrawal
1
10
day
1
Test
20
Test + Ephys
B
Bright Light
GFP
DAPI
GFP
merge
C
Normalized PPS
400
300
200
100
0
BL WD WD
1 20
WT + ctr
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Silent synapses (%)
Rectification index
ns
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WD 1
WD 20
WT + sh95
WT + ctr
SAP102 KO + sh95
SAP102 KO + ctr

Figure 6.

Figure 6. NAc-restricted knockdown of PSD-95 by itself and in combination with SAP102 KO prevents maturation of cocaine-induced silent synapses in the NAc but does not impair long-term retention of CPP memory.

A Schematic representation of the timeline of stereotaxic injection of AAV8 virus (sh95 or control) in the NAc followed by behavior and electrophysiology experiments.

B Bright light and fluorescence microscopy showing the GFP expression from AAV8 virus in the NAc. Confocal images of a selected area of the NAc shell showing infected cells (GFP) among total number of cells (DAPI-stained nuclei). Scale bar 50 μm.

C The place preference score (PPS) is indicated by normalizing to the baseline PPS. Baseline, white circles; withdrawal day 1 (WD1), gray circles (WT+ctr, $n = 16$; WT+sh95, $n = 26$; SAP102KO+ctr, $n = 8$; SAP102KO+sh95, n = 19) ; long-term withdrawal day, black circles (WT+ctr, $n = 12$; WT+sh95, $n = 13$; SAP102KO+ctr, $n = 8$; SAP102KO+sh95, $n = 10$).

D Example AMPA EPSCs evoked at −60 mV, 0 mV, and +40 mV for rectification index in GFP-positive (AAV8-infected) NAc MSNs. Scale bar 50 pA, 20 ms.

E–H EPSCs evoked at −60 mV and +40 mV by minimum stimulation (left side) over 100 trials from example recordings from GFP-positive NAc MSNs with PSD-95 knockdown, cocaine-CPP WD20 (E), ctr AAV, cocaine-CPP WD20 (F), PSD-95 knockdown + SAP102 KO, cocaine-CPP WD20 (G), and SAP102 KO + ctr AAV, cocaine-CPP WD20 (H). Responses are shown in black and failures in gray. Scale bars 10 pA, 5 ms.

I Summarized results showing the percentage of silent synapses (red circles; WT+sh95 WD1, $n/m = 5/13$; WT+sh95 WD20, $n/m = 8/20$; WT+ctr WD20, $n/m = 4/14$; SAP102KO+sh95 WD1, $n/m = 6/20$; SAP102KO+sh95 WD20, $n/m = 5/17$; SAP102KO+ctr WD20, $n/m = 3/8$) and rectification index (blue squares; WT+sh95 WD1, $n/m = 4/11$; WT+sh95 WD20, $n/m = 10/20$; WT+ctr WD20, $n/m = 4/12$; SAP102KO+sh95 WD1, $n/m = 3/11$; SAP102KO+sh95 WD20, $n/m = 4/14$; SAP102KO+ctr WD20, $n/m = 3/9$) in GFP-positive NAc MSNs of stereotaxically injected mice.

Data information: Data are given as means ± s.e.m.; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; paired t-test in (C) or unpaired t-test in (I).

Pharmacological reversal of CP-AMPAR insertion does not impair CPP retention

The lack of NAc CP-AMPAR accumulation after cocaine withdrawal in SAP102 KOs or in wild-type mice with NAc-restricted PSD-95 knockdown indicated that CP-AMPAR induction was not required for CPP retention (Figs 5 and 6). However, CP-AMPARs in the NAc are required for incubation of drug craving (Grimm *et al*, 2001; Conrad *et al*, 2008; Lee *et al*, 2013), and removal of CP-AMPARs by pharmacological enhancement of mGluR1 activity blunts incubation of cocaine craving (Loweth *et al*, 2013). Thus, we used the mGluR1-positive allosteric modulator SYN119 as an alternative approach to test the requirement of NAc CP-AMPARs in CPP retention. Cocaine-CPP training resulted in a positive PPS on WD1 (Fig 7A and B; PPS: BL (vehicle), 501 ± 38 versus cocaine WD1 (vehicle), 752 ± 18, $n = 8$, $t(7) = 6.72$, $P < 0.001$; BL (SYN119), 518 ± 41 versus cocaine WD1 (SYN119), 765 ± 27, $n = 12$, $t(11) = 5.83$, $P < 0.001$). 1 h prior to the PPS measurement on WD20-30, one group of mice received an i.p. injection of SYN119, and the other group received a vehicle injection. The cocaine-CPP was retained in both groups (Fig 7B; Table EV1; PPS: cocaine WD20 (vehicle), 678 ± 44 versus BL (vehicle), $n = 8$, $t(7) = 4.38$, $P < 0.01$; cocaine WD20 (SYN119), 694 ± 47 versus BL (SYN119), $n = 12$, $t(11) = 2.96$, $P < 0.05$). Immediately after the PPS measurements, the mice were sacrificed for electrophysiological assays. In the SYN119 group on WD20-30, the fraction of silent synapses was increased compared to that of the vehicle group (Fig 7D and F; Table EV1; %SS: cocaine WD20-30 (SYN119 treatment), 50 ± 2.8%, $n/m = 11/26$ versus cocaine WD20-30 (vehicle treatment), 25 ± 15%, $n/m = 5/10$, $t(14) = 2.43$, $P < 0.05$). Consistently, the rectification index in the SYN119 group on WD20-30 was reduced compared to that of the vehicle group (Fig 7C and F; rectification index: cocaine WD20-30 (SYN119 treatment), 2.1 ± 0.1, $n/m = 9/23$ versus cocaine WD20-30 (vehicle treatment), 2.7 ± 0.1, $n/m = 5/14$, $t(12) = 2.94$, $P < 0.05$). To test whether SYN119 itself has an effect on silent synapses or CP-AMPARs, we treated a saline group on WD1 with SYN119. Neither the rectification index nor the fraction of silent synapses in the SYN119 group was significantly affected, indicating that mGluR1 activation modulates CP-AMPARs during cocaine withdrawal but not in drug-naive mice (Fig 7G; %SS: saline WD1 (SYN119 treatment), 27 ± 11%, $n/m = 5/19$ versus saline WD1 (WT control), $t(15) = 0.36$, $P = 0.72$; rectification index: saline WD1 (SYN119 treatment), 2.3 ± 0.4, $n/m = 4/13$ versus saline WD1 (WT control), $t(11) = 1.25$, $P = 0.24$). Thus, SYN119 reduced CP-AMPARs after cocaine withdrawal and restored the fraction of silent synapses to an elevated level, similar to that in mice on WD1 after cocaine (% SS: cocaine WD20-30 (SYN119 treatment) versus cocaine WD1 (WT), $t(23) = 0.76$, $P = 0.45$). However, CPP was not affected by the single SYN119 injection prior to the CPP measurement. Together, these results reveal that NAc accumulation of CP-AMPARs is not required for the retention of CPP.

Discussion

Drugs of abuse hijack synaptic plasticity mechanisms in the meso-corticolimbic dopamine system, which regulates reinforcement learning of natural as well as drug rewards (Hyman *et al*, 2006; Kauer & Malenka, 2007; Pierce & Wolf, 2013; Huang *et al*, 2015b). In NAc MSNs, synaptic strengthening after cocaine exposure is partially expressed as CP-AMPAR incorporation (Conrad *et al*, 2008; Mameli *et al*, 2009; Lee *et al*, 2013) likely into cocaine-induced silent synapses (Conrad *et al*, 2008; Mameli *et al*, 2009; Lee *et al*, 2013). We examined the role of cocaine-induced silent synapse maturation in cocaine-CPP, which assesses the acquisition and retention of the contextually conditioned rewarding effects of cocaine. We demonstrate that after withdrawal the cocaine-induced expression of CP-AMPARs in NAc shell MSNs required the association of cocaine with the CPP context, while cocaine administration in the home cage did not induce subsequent CP-AMPAR accumulation in the NAc shell. Using KO mice of the signaling scaffold proteins PSD-95, PSD-93, and SAP102, we found that functional loss of each of the three resulted in strikingly different cellular and behavioral phenotypes, revealing functional differences of these proteins in the regulation of silent synapse maturation and cocaine-induced behavior. These functional differences are the product of the increased synapse proteome complexity arising from genome duplications that generated this family of paralogs (Nithianantharajah *et al*, 2013). The functional specialization of these paralogs allowed us to test the causal relationship between silent synapse maturation in the NAc and CPP retention. In contrast to the critical role of CP-AMPARs for incubation of drug craving, as measured by operant conditioning, CPP from Pavlovian conditioning produced but did not require NAc CP-AMPAR accumulation.

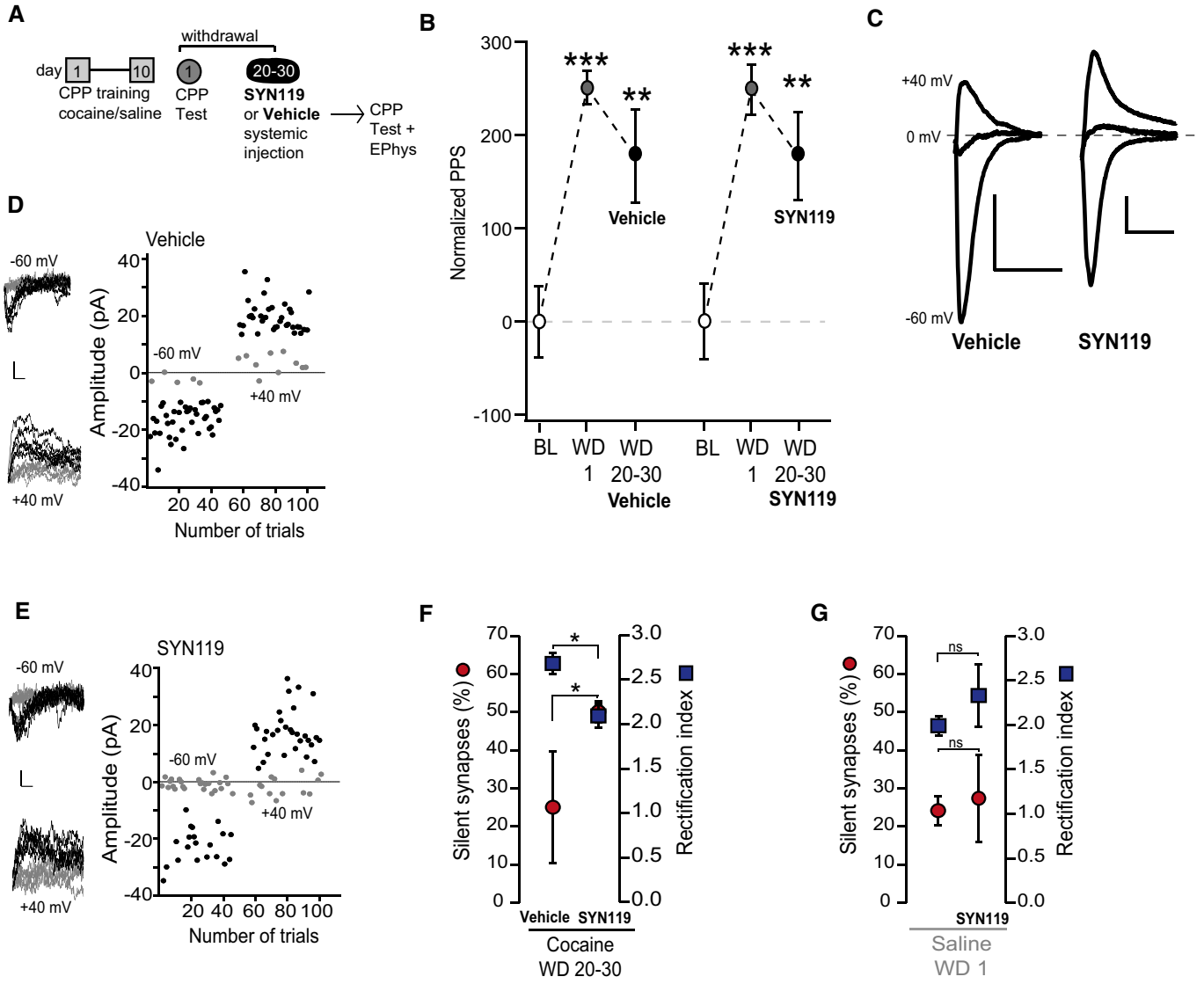

Figure 7. Positive allosteric modulation of mGluR1 by systemic SYN119 injection re-silences the NAc synapses after long-term withdrawal from cocaine-CPP.

A Schematic representation of the timeline of cocaine-CPP protocol followed by SYN119 injection, CPP behavior test, and electrophysiology.

B The place preference score (PPS) is indicated by normalizing to the baseline PPS. Baseline, white circles; withdrawal day 1 (WD1), gray circles (Vehicle, $n = 8$; SYN119, $n = 12$); long-term withdrawal day, black circles (Vehicle, $n = 8$; SYN119, $n = 12$).

C Example AMPA EPSCs evoked at −60 mV, 0 mV, and +40 mV for rectification index in NAc MSNs following vehicle or SYN119 injections. Scale bar 50 pA, 25 ms.

D, E EPSCs evoked at −60 mV and +40 mV by minimum stimulation (left side) over 100 trials from example recordings from NAc MSNs of SAP102 KO mice after vehicle treatment (D) and SYN119 treatment (E). Responses are shown in black and failures in gray. Scale bars 10 pA, 5 ms.

F Summarized results showing the percentage of silent synapses (red circles; Vehicle, $n/m = 5/10$; SYN119, $n/m = 11/26$) and rectification index (blue squares; Vehicle, $n/m = 5/14$; SYN119, $n/m = 9/23$) in NAc MSNs of vehicle or SYN119-treated mice after 20–30 days of withdrawal from cocaine-CPP.

G Summarized results showing the percentage of silent synapses (red circles; SYN119, $n/m = 5/19$) and rectification index (blue squares; SYN119, $n/m = 4/13$) in NAc MSNs of mice treated with saline following SYN119 injection.

Data information: Data are given as means ± s.e.m.; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; paired t-test in (B) or unpaired t-test in (F) and (G).

Different functions of PSD-95-related signaling scaffolds on silent synapse-based synapse remodeling

Loss of function of each of the three signaling scaffolds affected basal levels of silent synapses in NAc MSNs differently and, in some cases, in an opposing manner. Loss of PSD-95 function increased basal levels of silent synapses, and loss of PSD-93 decreased them, while loss of SAP102 did not affect them. However, cocaine-induced silent synapse generation was preserved in all three KOs, as well as when PSD-95 was additionally knocked down in SAP102 KOs. In both PSD-95 and SAP102 KOs, cocaine-generated silent synapses persisted throughout withdrawal and did not mature. Thus, neither PSD-93, nor PSD-95, nor SAP102 is required for silent synapse generation, but each is differently involved synapse maturation.

These findings recapitulate the increased basal level of silent synapses in PSD-95 KOs and indicate a general impairment of silent synapse maturation in the absence of PSD-95. It is also consistent with previous results that experience-dependent silent synapse maturation is impaired in PSD-95 KOs (Huang *et al*, 2015a). Interestingly, in PSD-95 KOs, the basal levels of silent synapses in the visual cortex and the NAc are similar (~50%). Knockdown of PSD-95 in the hippocampus reduces AMPAR-mediated EPSC amplitudes by ~50% (Elias *et al*, 2006; Schlüter *et al*, 2006). Thus, loss of PSD-95 might generally impair silent synapse maturation in ~50% of all synapses. These results also reveal a similarity between experience-dependent silent synapse-based cortical neurocircuit refinements during critical periods and drug-induced silent synapse-based circuit remodeling in the NAc, akin to the rejuvenation hypothesis of drug addiction (Dong & Nestler, 2014; Huang *et al*, 2015b). In both processes, PSD-95 is required for silent synapse maturation, and, as shown in the visual cortex, PSD-95 also stabilizes the mature state (Huang *et al*, 2015a). Notably, cocaine exposure further elevates the fraction of silent synapses (Fig 2), likely by the induction of new spines in the NAc (Robinson *et al*, 2001; Brown *et al*, 2011).

In SAP102 KOs, the basal level of silent synapses was similar to that of wild-type mice, and cocaine-generated silent synapses did not mature, similar to PSD-95 KOs. The similar effect on silent synapse maturation is consistent with the proposed functional redundancy between PSD-95 and SAP102 (Elias *et al*, 2006; Levy *et al*, 2015) or a functional interaction (Bonnet *et al*, 2013). Apparently, despite having higher abundance, PSD-95 was not able to compensate for the functional loss of SAP102. A possible explanation for their distinct mechanistic functions is afforded by the fact that PSD-95 and SAP102 reside in distinct supramolecular complexes of 1.5 and 350 kDa size, respectively (Frank *et al*, 2016). However, the functional similarity in silent synapse maturation cannot explain the differences in the behavioral phenotypes, because CPP retention was impaired in PSD-95, but not SAP102 KOs. Thus, either the PSD-95 phenotype is more penetrant with a synergistic function of the two paralogs on silent synapse maturation or PSD-95 and SAP102 regulate synapse maturation in different synapse types, which differently affect the encoding of CPP retention.

In PSD-93 KOs, the basal level of silent synapses in the NAc was reduced. This result indicates an opposite function of PSD-93 in silent synapse maturation compared to that of PSD-95 and SAP102. A previous study revealed opposing effects of PSD-93 and PSD-95 on long-term synaptic plasticity in the hippocampus (Carlisle *et al*, 2008). It is conceivable that the high availability of plasticity substrates such as silent synapses facilitates LTP in the PSD-95 KOs, while reduction in silent synapses might hamper LTP induction in PSD-93 KOs. Notably, most previous studies did not reveal an impairment of AMPAR-mediated EPSC amplitudes in adolescent PSD-93 KOs (Elias *et al*, 2006; Carlisle *et al*, 2008; Krüger *et al*, 2013), presumably due to the low basal level of silent synapses at that age. Despite the reduced basal level of silent synapses and a potential impairment of LTP, CPP acquisition and retention was normal in PSD-93 KOs.

CP-AMPAR accumulation into cocaine-generated silent synapses was impaired in PSD-95 or SAP102, but not in PSD-93 KOs. The synaptic accumulation of CP-AMPARs was correlated with a decrease of silent synapses during withdrawal, and consequently, cocaine-generated silent synapses remained silent in PSD-95 and SAP102 KOs after withdrawal. Furthermore, if CP-AMPARs were removed by mGluR1 activation in wild-type mice (Fig 7) or by *in vivo* LTD in rats (Lee *et al*, 2013; Ma *et al*, 2014), silent synapses were reinstated. Thus, genetic, pharmacological and optogenetic manipulations consistently reveal a correlation between silent synapse reduction and CP-AMPAR expression and vice versa. The most parsimonious explanation is that cocaine-generated silent synapses are at least partially unsilenced by CP-AMPAR accumulation (Huang *et al*, 2015b).

CP-AMPARs in cocaine-induced behaviors

CP-AMPARs were incorporated into cocaine-induced silent synapses if cocaine exposure was associated with the CPP context (Fig 1) or during operant conditioning with extended-access cocaine self-administration (Conrad *et al*, 2008; McCutcheon *et al*, 2011; Lee *et al*, 2013). In contrast, when cocaine was administered without contextual association, CP-AMPARs were not incorporated, while silent synapse numbers decreased (Fig 1; Huang *et al*, 2009; McCutcheon *et al*, 2011). These results indicate that the context association during cocaine administration was critical for CP-AMPAR accumulation in the NAc. But, the CP-AMPAR accumulation was not required for CPP retention as evidenced by the following results: First, SAP102 KOs acquired and retained CPP similar to wild-type mice (Fig 4), while cocaine-generated silent synapses in the NAc did not mature after cocaine withdrawal and did not accumulate CP-AMPARs. Second, mGluR1 activation before CPP testing removed CP-AMPARs and re-silenced cocaine-generated silent synapses (Fig 7), but did not affect CPP retention. Third, AAV-mediated knockdown of PSD-95 in the NAc also prevented CP-AMPAR accumulation after cocaine withdrawal but did not prevent CPP retention (Fig 6). Thus, disrupting CP-AMPAR accumulation into NAc silent synapses either genetically or pharmacologically did not prevent CPP retention. Because of the global effects of KO and i.p. injections of SYN119, these results also imply that CP-AMPARs in other brain regions are not involved in CPP retention as well. While our results show that CP-AMPARs are not required for CPP acquisition and retention, it is possible that our currently used robust CPP procedure might mask some subtle contribution of CP-AMPARs to CPP.

CPP retention but not acquisition was impaired in PSD-95 KOs. Similarly, previous studies show that long-term retention of contextual fear conditioning is impaired in PSD-95 KOs as well as retention of alcohol-CPP (Camp *et al*, 2011; Fitzgerald *et al*, 2014). Thus, lack of PSD-95 might generally impair long-term retention of associative memories but not initial acquisition or short-term retention. What cellular mechanism mediates this impairment? Because the cellular alterations in SAP102 KOs exhibited normal CPP retention, but impaired CP-AMPAR-mediated silent synapse maturation, and mGluR1-mediated CP-AMPAR removal did not impair CPP retention, it is unlikely that impaired CP-AMPAR accumulation into NAc silent synapses in PSD-95 KOs is responsible for the impaired CPP retention. Furthermore, in PSD-95 KOs and SAP102 KOs, LTP induction in the hippocampus is facilitated, which could explain why acquisition was normal or even enhanced and short-term memory is intact (Migaud *et al*, 1998; Béïque *et al*, 2006; Cuthbert *et al*, 2007; Carlisle *et al*, 2008). This interpretation would also imply that

PSD-95-deficient synapses could be transiently potentiated to code for CPP acquisition, but this potentiation would not persist.

CP-AMPAR removal or pharmacological inhibition in the NAc after cocaine withdrawal disrupts the expression of incubation of cocaine craving as measured in operant paradigms (Conrad *et al*, 2008; Lee *et al*, 2013; Loweth *et al*, 2014; Ma *et al*, 2014). Importantly, the preference for the drug-associated lever was still preserved, so that removing/inactivating CP-AMPARs reduced the intensity of the behavioral response, but not the associative memory. Thus, similar to CPP, the memory of the cue–drug association does not require CP-AMPARs for cocaine self-administration.

Notably, the expression of CP-AMPARs in the NAc after cocaine-CPP was transient and disappeared after WD35, while NAc CP-AMPARs after cocaine self-administration persist beyond WD70 (Wolf & Tseng, 2012). It was recently shown that NAc CP-AMPAR removal blocks the reinstatement of morphine-CPP after extinction (Hearing *et al*, 2016). Thus, synaptic strengthening, including CP-AMPAR incorporation in the NAc, might regulate the initial establishment or reinstatement of an association in drug-related behaviors. Consequently, CPP acquisition is blocked if the NAc shell is lesioned (Ito *et al*, 2008) or the transcription factor Npas2 is knocked down in the NAc (Ozburn *et al*, 2015). But since LTP and short-term memory are intact in PSD-95 KO mice, initial changes in the NAc might have already occurred, which are the basis for CPP acquisition, and their persistence is not required for CPP retention.

In conclusion, we uncovered differential functions of the paralogs of the PSD-95-related signaling scaffold family on silent synapse-based circuitry remodeling in the NAc. We further defined that NAc CP-AMPARs are a signature of drug-associated behaviors but are not required for their retention.

Materials and Methods

Mice

Male wild-type mice of C57Bl6/J background (Charles River), PSD-95 KO mice, PSD-93 KO mice, and SAP102 KO mice were generated as previously described (McGee *et al*, 2001; Yao *et al*, 2004; Cuthbert *et al*, 2007), intercrossed and separated by crossing into a C57Bl6/J background. Mice were group-housed 2–5 per cage and used at 4–6 weeks of age at the start of the experiments. The animals were housed under a 12-h light/dark cycle with controlled temperature and humidity and were provided food and water *ad libitum*. All procedures were performed during the light cycle. All experimental procedures were approved by the Animal Care and Use Committees of the University Medical School Göttingen, the Lower Saxony State Office for Consumer Protection and Food Safety, or the University of Pittsburgh.

Conditioned place preference

CPP was performed as described previously (Roux *et al*, 2003). During the initial habituation period, mice were given sham intraperitoneal (i.p.) injections for 3–5 days. CPP was measured in an apparatus, containing two conditioning compartments. Each of those compartments (15 × 15 cm) had distinct olfactory cues (a piece of tissue paper with a drop of scented oils—cinnamon or vanilla, hidden in an open Eppendorf tube), tactile cues (texture of the interior-interspersed circular pits or interspersed long linear pits) and visual cues (thick, vertical, black and white stripes versus small, black dots on white background). The two compartments exited through a guillotine door into a connecting small neutral area. One day prior to the start of conditioning, the mice were put into the neutral area and allowed free access to explore both chambers. The location of the mice was monitored for 18 min to calculate a baseline place preference score (PPS): PPS = time in the conditioned compartment × total time/(total time − time in the neutral area). The PPS takes the time spent in the neutral area into account and as such decreases the variance of the measures compared to the measures of time in the conditioning compartments (Roux *et al*, 2003). In the normalized PPS, the PPS was subtracted by the baseline PPS, for the purpose of illustration, so that positive normalized PPS depict preference and negative normalized PPS depict aversion. Mice with a biased basal preference of > 75% for either of the two chambers were excluded from experiments. During the 10-day conditioning phase, a cocaine-paired chamber was randomly assigned for each mouse. Mice received i.p. injections of 20 mg/kg cocaine (Sigma) or saline (Braun) on alternating days and immediately placed in the respective compartment for 20 min. One day after the conditioning phase, we performed the CPP test, during which the mice were given free access to both compartments without cocaine or saline administration. The mice were then either immediately (< 10 min) sacrificed for electrophysiology or returned to their home cages for 10–15, 20–25, 30–35, or 40–45 days of withdrawal. The CPP test was performed again at different withdrawal time points, followed by electrophysiology.

Nucleus accumbens slice preparation

Mice were anesthetized with isoflurane and decapitated. 300-μm-thick coronal brain slices were prepared with a vibratome in ice-cold cutting solution (135 mM N-methyl-D-glucamine, 1 mM KCl, 1.2 mM KH_2PO_4, 1.5 mM $MgCl_2 \cdot 6H_20$, 0.5 mM $CaCl_2 \cdot 2H_2O$, 10 mM D-glucose, and 20 mM choline bicarbonate). The slices were incubated for 10–15 min in oxygenated aCSF solution (119 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 20 mM D-glucose saturated with 95% (v/v) O_2 and 5% (v/v) CO_2) at 35°C and then transferred to room temperature until further use.

Electrophysiology

Standard whole-cell patch-clamp recordings were performed in voltage-clamp mode. The slices were held in a chamber heated to 28–32°C with aCSF perfusion at 2–3 ml/min. Medium spiny neurons of the NAc shell were identified visually with infrared differential interference contrast microscopy. Borosilicate glass pipettes (2–5 MΩ) filled with Cs-based internal solution (117.5 mM $CsMeSO_3H$, 10 mM HEPES, 17.75 mM CsCl, 10 mM TEA-Cl, 0.25 mM EGTA, 10 mM glucose, 2 mM $MgCl_2 \cdot 6H_2O$, 4 mM Na_2ATP, 0.3 mM NaGTP, 5 mM QX-314 chloride, 100 μM spermine, pH 7.1, 290 mOsm) were used for patch-clamp electrophysiological recordings. Electrical stimulation of NAc slices was done using double-barreled glass bipolar electrodes, filled with aCSF. The recordings

were acquired using custom algorithms in Igor Pro (Wavemetrics) with an ELC-03XS amplifier (NPI) and digitized at 10 kHz with an ITC-18 (HEKA). The signals were filtered at 3 kHz, and series and input resistances were monitored throughout the experiment. 100 μM picrotoxin was included in the aCSF to inhibit GABA-A receptors in all recordings. For assessing the proportion of silent synapses, a minimum stimulation protocol was performed (Liao *et al*, 1995; Huang *et al*, 2009). The strength of the electrical stimulation was adjusted so that both successes and failures of synaptic transmission were detected at a holding potential of −60 mV and +40 mV. At either holding potential, a minimum of 50 sweeps were recorded at 0.2 Hz. The holding potential was switched every 20–30 sweeps to monitor stability. The percentage of silent synapses was calculated using the following equation: % silent synapses = $1 - [\ln(F_{-60\ mV})/\ln(F_{40\ mV})]$, where $F_{-60\ mV}$ and $F_{+40\ mV}$ are failure rates at −60 mV and +40 mV, respectively. For recording the AMPAR rectification index, 50 μM D-APV was included in the aCSF to block NMDAR currents. AMPAR excitatory postsynaptic currents (EPSC) were evoked at −60 mV, 0 mV, and +40 mV at 0.1 Hz, and the rectification index calculated using the equation: RI = EPSC amplitude at −60 mV/EPSC amplitude at +40 mV.

Immunoblotting

HEK293 cells were transfected with recombinant GFP-tagged MAGUKs, PSD-95-GFP, PSD-93-GFP, or SAP102-GFP as described previously (Bonnet *et al*, 2013; Krüger *et al*, 2013). Cells were harvested 48 h after transfection in a cell lysis buffer [20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholate, 0.5% Triton X-100, protease inhibitor cocktail (Roche)] and lysed by sonication. The supernatant was collected after centrifugation (5,000 × *g*, 15 min) and used for Western blotting. NAc tissue punches were collected from 500-μm-thick coronal brain slices of wild-type mice, cut with a vibratome in a homogenization buffer (0.32 M sucrose, 4 mM HEPES, pH 7.3). The tissue was lysed by sonication in a lysis buffer (50 mM HEPES, 50 mM NaF, 1 mM Na_3VO_4, pH 7.4 + protease inhibitors). After a brief centrifugation (800 × *g*, 5 min), protein quantification of the supernatant was done using BCA quantification assay. 7.5% Bis-Tris polyacrylamide gels were used for resolving 8 μg of total NAc proteins in each lane, along with equal quantities of recombinant GFP-tagged MAGUKs in HEK cell lysates as reference for quantitation (Krüger *et al*, 2013). After protein transfer onto nitrocellulose membranes, the protein bands were decorated with the following primary antibodies with 1:1,000 dilution: GFP (mouse, Neuromab), Mortalin (mouse, Neuromab) PSD-95 (rabbit, Millipore), PSD-93 (rabbit, Alomone), and SAP102 (mouse, Neuromab). Bands were detected using secondary antibodies goat anti-mouse Alexa 680 (Invitrogen) or goat anti-rabbit IR 800 (Li-COR Biosciences) and visualized as well as quantified using an infrared fluorescence scanner (Krüger *et al*, 2013).

Stereotaxic surgery and viral delivery

Adeno-associated viral vector particles, pseudotyped with serotype 8 (AAV8), that express an shRNA against PSD-95 (sh95) and EGFP were prepared as described previously (Suska *et al*, 2013; Huang *et al*, 2015a). The control (ctr) AAV either expressed EGFP without shRNA (GFP control) or an shRNA against luciferase (Addgene 26701). For *in vivo* AAV delivery, mice were anesthetized using a mixture of fentanyl (0.07 mg/kg), midazolam (7.0 mg/kg), and medetomidine (0.7 mg/kg). The head of the mouse was fixed on a stereotaxic apparatus. 1 μl AAV8 was bilaterally injected into the nucleus accumbens (from bregma: AP, +1.55; ML, ± 0.55; DV, −4.7) using a glass pipette controlled by a microsyringe pump controller. For imaging AAV8-transduced tissues, mice were transcardially perfused with 75 ml of ice-cold 4% paraformaldehyde in 4% sucrose solution and remained in the same solution at 4°C overnight. 300-μm-thick coronal brain slices containing the NAc were obtained and stained with 5 μg/ml DAPI for 15 min. Following washes in 0.01 M phosphate-buffered saline, the slices were mounted onto glass slides and imaged using a stereomicroscope with a 1× objective. Confocal images were acquired using a laser scanning confocal microscope with a 40× objective with a resolution of 1,024 × 1,024 pixels.

SYN119 treatment

SYN119, a positive allosteric modulator of mGluR1, was used as previously described (Loweth *et al*, 2014). SYN119 was dissolved in a vehicle consisting of 10% Tween 80 (Sigma) in saline. Mice received one intraperitoneal injection of vehicle or 20 mg/kg SYN119 at different time points (see Results). Mice were returned to their home cages for 1 h before CPP testing and/or electrophysiology measurements.

Data analysis

All results are shown as mean ± standard error of the mean. Student's *t*-test or one-way ANOVA with Bonferroni post-test was used for statistical analyses. Paired *t*-test was used for CPP data to compare PPS of the same animals under different conditions. For the electrophysiological and behavioral data, animal-based statistics were performed, wherein the data from multiple cells of one animal were averaged to represent this animal. Sample size is presented as n/m, where n is the number of animals used and m is the number of cells.

Expanded View for this article is available online.

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Author contributions

AS, AB, YD, and OMS designed experiments; AS, AB, MP, PAN performed experiments; SGNG and MFO provided research tools and expertise; AS, AB, MP, PAN,

YD, and OMS analyzed and interpreted data; AS, AB, YD, and OMS wrote the manuscript, which was edited by all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Béïque J-C, Lin D-T, Kang M-G, Aizawa H, Takamiya K, Huganir RL (2006) Synapse-specific regulation of AMPA receptor function by PSD-95. *Proc Natl Acad Sci USA* 103: 19535 – 19540

Bonnet SAD, Akad DS, Samaddar T, Liu Y, Huang X, Dong Y, Schlüter OM (2013) Synaptic state-dependent functional interplay between postsynaptic density-95 and synapse-associated protein 102. *J Neurosci* 33: 13398 – 13409

Brown TE, Lee BR, Mu P, Ferguson D, Dietz D, Ohnishi YN, Lin Y, Suska A, Ishikawa M, Huang YH, Shen H, Kalivas PW, Sorg BA, Zukin RS, Nestler EJ, Dong Y, Schlüter OM (2011) A silent synapse-based mechanism for cocaine-induced locomotor sensitization. *J Neurosci* 31: 8163 – 8174

Camp MC, Feyder M, Ihne J, Palachick B, Hurd B, Karlsson R-M, Noronha B, Chen Y-C, Coba MP, Grant SGN, Holmes A (2011) A novel role for PSD-95 in mediating ethanol intoxication, drinking and place preference. *Addict Biol* 16: 428 – 439

Carlisle HJ, Fink AE, Grant SGN, O'Dell TJ (2008) Opposing effects of PSD-93 and PSD-95 on long-term potentiation and spike timing-dependent plasticity. *J Physiol* 586: 5885 – 5900

Casassus G, Blanchet C, Mulle C (2005) Short-term regulation of information processing at the corticoaccumbens synapse. *J Neurosci* 25: 11504 – 11512

Conrad KL, Tseng KY, Uejima JL, Reimers JM, Heng L-J, Shaham Y, Marinelli M, Wolf ME (2008) Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. *Nature* 454: 118 – 121

Cuthbert PC, Stanford LE, Coba MP, Ainge JA, Fink AE, Opazo P, Delgado JY, Komiyama NH, O'Dell TJ, Grant SGN (2007) Synapse-associated protein 102/dlgh3 couples the NMDA receptor to specific plasticity pathways and learning strategies. *J Neurosci* 27: 2673 – 2682

Dong Y, Nestler EJ (2014) The neural rejuvenation hypothesis of cocaine addiction. *Trends Pharmacol Sci* 35: 374 – 383

Elias GM, Funke L, Stein V, Grant SGN, Bredt DS, Nicoll RA (2006) Synapse-specific and developmentally regulated targeting of AMPA receptors by a family of MAGUK scaffolding proteins. *Neuron* 52: 307 – 320

Fitzgerald PJ, Pinard CR, Camp MC, Feyder M, Sah A, Bergstrom HC, Graybeal C, Liu Y, Schlüter OM, Grant SG, Singewald N, Xu W, Holmes A (2014) Durable fear memories require PSD-95. *Mol Psychiatry* 20: 901 – 912

Frank RAW, Komiyama NH, Ryan TJ, Zhu F, O'Dell TJ, Grant SGN (2016) NMDA receptors are selectively partitioned into complexes and supercomplexes during synapse maturation. *Nat Comm* 7: 11264

Graziane NM, Sun S, Wright WJ, Jang D, Liu Z, Huang YH, Nestler EJ, Wang YT, Schlüter OM, Dong Y (2016) Opposing mechanisms mediate morphine- and cocaine-induced generation of silent synapses. *Nat Neurosci* 19: 915 – 925

Grimm JW, Hope BT, Wise RA, Shaham Y (2001) Neuroadaptation. Incubation of cocaine craving after withdrawal. *Nature* 412: 141 – 142

Hearing MC, Jedynak J, Ebner SR, Ingebretson A, Asp AJ, Fischer RA, Schmidt C, Larson EB, Thomas MJ (2016) Reversal of morphine-induced cell-type-specific synaptic plasticity in the nucleus accumbens shell blocks reinstatement. *Proc Natl Acad Sci USA* 113: 757 – 762

Huang YH, Lin Y, Mu P, Lee BR, Brown TE, Wayman G, Marie H, Liu W, Yan Z, Sorg BA, Schlüter OM, Zukin RS, Dong Y (2009) *In vivo* cocaine experience generates silent synapses. *Neuron* 63: 40 – 47

Huang X, Stodieck SK, Goetze B, Cui L, Wong MH, Wenzel C, Hosang L, Dong Y, Löwel S, Schlüter OM (2015a) Progressive maturation of silent synapses governs the duration of a critical period. *Proc Natl Acad Sci USA* 112: E3131 – E3140

Huang YH, Schlüter OM, Dong Y (2015b) Silent synapses speak up: updates of the neural rejuvenation hypothesis of drug addiction. *Neuroscientist* 21: 451 – 459

Hyman SE, Malenka RC, Nestler EJ (2006) Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu Rev Neurosci* 29: 565 – 598

Isaac JTR, Nicoll RA, Malenka RC (1995) Evidence for silent synapses: implications for the expression of LTP. *Neuron* 15: 427 – 434

Ito R, Robbins TW, Pennartz CM, Everitt BJ (2008) Functional interaction between the hippocampus and nucleus accumbens shell is necessary for the acquisition of appetitive spatial context conditioning. *J Neurosci* 28: 6950 – 6959

Kauer JA, Malenka RC (2007) Synaptic plasticity and addiction. *Nat Rev Neurosci* 8: 844 – 858

Koya E, Cruz FC, Ator R, Golden SA, Hoffman AF, Lupica CR, Hope BT (2012) Silent synapses in selectively activated nucleus accumbens neurons following cocaine sensitization. *Nat Neurosci* 15: 1556 – 1562

Krüger JM, Favaro PD, Liu M, Kitlinska A, Huang X, Raabe M, Akad DS, Liu Y, Urlaub H, Dong Y, Xu W, Schlüter OM (2013) Differential roles of postsynaptic density-93 isoforms in regulating synaptic transmission. *J Neurosci* 33: 15504 – 15517

Lee BR, Y-y MA, Huang YH, Wang X, Otaka M, Ishikawa M, Neumann PA, Graziane NM, Brown TE, Suska A, Guo C, Lobo MK, Sesack SR, Wolf ME, Nestler EJ, Shaham Y, Schlüter OM, Dong Y (2013) Maturation of silent synapses in amygdala-accumbens projection contributes to incubation of cocaine craving. *Nat Neurosci* 16: 1644 – 1651

Levy JM, Chen X, Reese TS, Nicoll RA (2015) Synaptic consolidation normalizes AMPAR quantal size following MAGUK loss. *Neuron* 87: 534 – 548

Liao DS, Hessler NA, Malinow R (1995) Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* 375: 400 – 404

Loweth JA, Scheyer AF, Milovanovic M, Lacrosse AL, Flores-Barrera E, Werner CT, Li X, Ford KA, Le T, Olive MF, Szumlinski KK, Tseng KY, Wolf ME (2013) Synaptic depression via mGluR1 positive allosteric modulation suppresses cue-induced cocaine craving. *Nat Neurosci* 17: 73 – 80

Loweth JA, Tseng KY, Wolf ME (2014) Adaptations in AMPA receptor transmission in the nucleus accumbens contributing to incubation of cocaine craving. *Neuropharmacology* 76(Pt B): 287 – 300

Ma Y-Y, Lee BR, Wang X, Guo C, Liu L, Cui R, Lan Y, Balcita-Pedicino JJ, Wolf ME, Sesack SR, Shaham Y, Schlüter OM, Huang YH, Dong Y (2014) Bidirectional modulation of incubation of cocaine craving by silent synapse-based remodeling of prefrontal cortex to accumbens projections. *Neuron* 83: 1453 – 1467

Mameli M, Halbout B, Creton C, Engblom D, Parkitna JR, Spanagel R, Lüscher C (2009) Cocaine-evoked synaptic plasticity: persistence in the VTA triggers adaptations in the NAc. *Nat Neurosci* 12: 1036 – 1041

McCutcheon JE, Wang X, Tseng KY, Wolf ME, Marinelli M (2011) Calcium-permeable AMPA receptors are present in nucleus accumbens synapses after prolonged withdrawal from cocaine self-administration but not experimenter-administered cocaine. *J Neurosci* 31: 5737 – 5743

McGee AW, Topinka JR, Hashimoto K, Petralia RS, Kakizawa S, Aguilera-Moreno A, Wenthold RJ, Kano M, Bredt DS, Kauer FW (2001) PSD-93 knock-out mice reveal that neuronal MAGUKs are not required for development or function of parallel fiber synapses in cerebellum. *J Neurosci* 21: 3085 – 3091

Migaud M, Charlesworth P, Dempster M, Webster LC, Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RGM, Morrison JH, O'Dell TJ, Grant SGN (1998) Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* 396: 433 – 439

Nakagawa T, Futai K, Lashuel HA, Lo I, Okamoto K-I, Walz T, Hayashi Y, Sheng M (2004) Quaternary structure, protein dynamics, and synaptic function of SAP97 controlled by L27 domain interactions. *Neuron* 44: 453 – 467

Nithianantharajah J, Komiyama NH, Mckechanie A, Johnstone M, Blackwood DH, Clair DS, Emes RD, Van De Lagemaat LN, Saksida LM, Bussey TJ, Grant SGN (2013) Synaptic scaffold evolution generated components of vertebrate cognitive complexity. *Nat Neurosci* 16: 16 – 24

Ozburn AR, Falcon E, Twaddle A, Nugent AL, Gillman AG, Spencer SM, Arey RN, Mukherjee S, Lyons-Weiler J, Self DW, Mcclung CA (2015) Direct regulation of diurnal Drd3 expression and cocaine reward by NPAS2. *Biol Psychiatry* 77: 425 – 433

Parker MJ, Zhao S, Bredt DS, Sanes JR, Feng G (2004) PSD93 regulates synaptic stability at neuronal cholinergic synapses. *J Neurosci* 24: 378 – 388

Pierce RC, Wolf ME (2013) Psychostimulant-induced neuroadaptations in nucleus accumbens AMPA receptor transmission. *Cold Spring Harb Perspect Med* 3: a012021

Robinson TE, Gorny G, Mitton E, Kolb B (2001) Cocaine self-administration alters the morphology of dendrites and dendritic spines in the nucleus accumbens and neocortex. *Synapse* 39: 257 – 266

Roux S, Froger C, Porsolt RD, Valverde O, Maldonado R (2003) Place preference test in rodents. *Curr Protoc Neurosci* Chapter 9: Unit 9.15

Schlüter OM, Xu W, Malenka RC (2006) Alternative N-terminal domains of PSD-95 and SAP97 govern activity-dependent regulation of synaptic AMPA receptor function. *Neuron* 51: 99 – 111

Suska A, Lee BR, Huang YH, Dong Y, Schlüter OM (2013) Selective presynaptic enhancement of the prefrontal cortex to nucleus accumbens pathway by cocaine. *Proc Natl Acad Sci USA* 110: 713 – 718

Tao Y-X, Rumbaugh G, Wang G-D, Petralia RS, Zhao C, Kauer FW, Tao F, Zhuo M, Wenthold RJ, Raja SN, Huganir RL, Bredt DS, Johns RA (2003) Impaired NMDA receptor-mediated postsynaptic function and blunted NMDA receptor-dependent persistent pain in mice lacking postsynaptic density-93 protein. *J Neurosci* 23: 6703 – 6712

Wolf ME, Tseng KY (2012) Calcium-permeable AMPA receptors in the VTA and nucleus accumbens after cocaine exposure: when, how, and why? *Front Mol Neurosci* 5: 72

Xiao M-Y, Wasling P, Hanse E, Gustafsson B (2004) Creation of AMPA-silent synapses in the neonatal hippocampus. *Nat Neurosci* 7: 236 – 243

Yao W-D, Gainetdinov RR, Arbuckle MI, Sotnikova TD, Cyr M, Beaulieu J-M, Torres GE, Grant SGN, Caron MG (2004) Identification of PSD-95 as a regulator of dopamine-mediated synaptic and behavioral plasticity. *Neuron* 41: 625 – 638

