Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving

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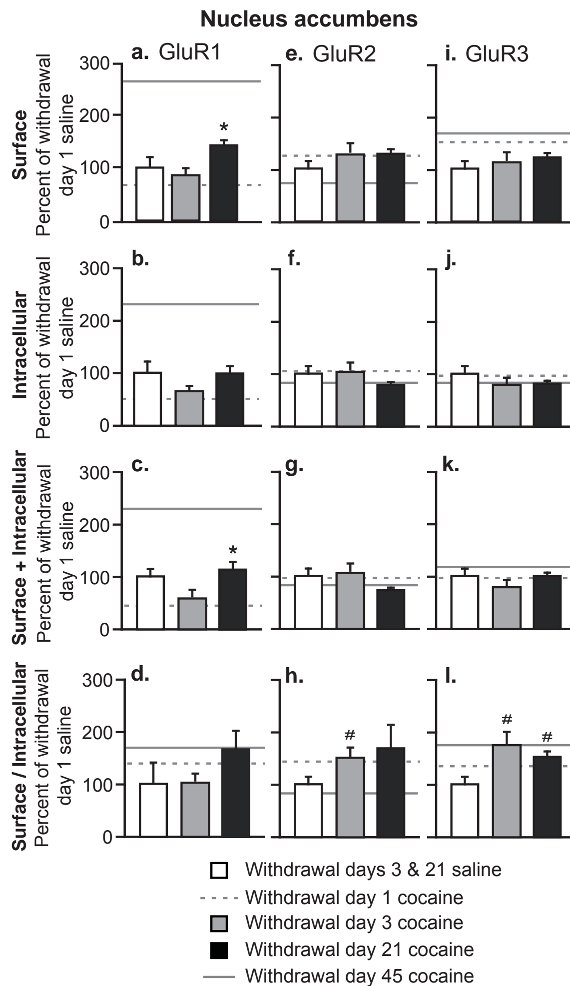
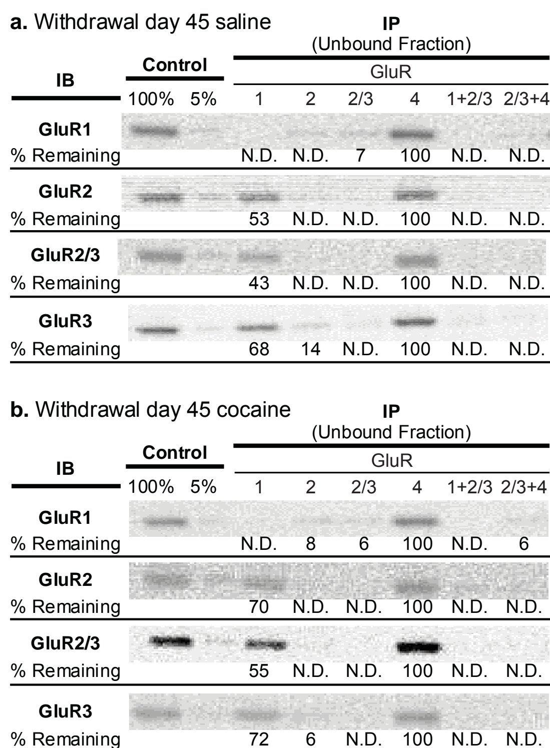
SUPPLEMENTARY FIGURES AND LEGENDS



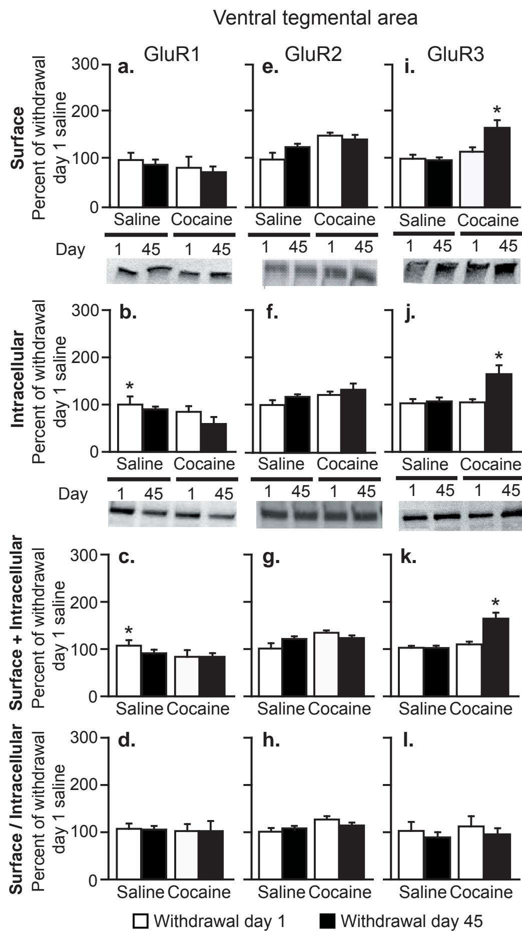
Supplementary Figure 1. *Cocaine-exposed rats at intermediate withdrawal times (3 and 21 days) show intermediate changes in GluR1 expression and distribution.* Rats were trained to self-administer saline or cocaine and killed after 3 or 21 days of withdrawal. Saline-exposed rats did not differ at the two withdrawal times and were therefore pooled. Protein crosslinking analysis was performed using a dissection that included the entire accumbens. To enable comparison of AMPA receptor parameters on withdrawal days 3 and 21 with results from withdrawal days 1 and 45 (Fig. 2), mean values for cocaine-exposed rats on withdrawal days 1 and 45 (expressed as percent of saline rats on withdrawal day 1) are shown by dashed and solid lines, respectively, in each graph. The one-way ANOVA results reported below are based on the pooled saline group, cocaine withdrawal day 3,

and cocaine withdrawal day 21; significant results were followed by post-hoc tests. GluR1: Cell surface (**a**) and total (**c**) GluR1 levels were increased in cocaine-exposed rats on withdrawal day 21 compared to withdrawal day 3 and saline-exposed group ($F_{2,26}$=22.4, and $F_{2,26}$=3.6, respectively, p values<0.05). Note that day 3 and day 21 values for these parameters were higher than day 1 values (dotted lines) and lower than day 45 values (solid lines). These results, together with Fig. 2, indicate that GluR1 levels increase gradually over 45 days of withdrawal, but much of the increase occurs between withdrawal days 21 and 45. There were no group differences in intracellular GluR1 (**b**) or the GluR1 surface/intracellular ratio (**d**). GluR2: There were no group differences in cell surface (**e**), intracellular (**f**), or total (**g**) GluR2 levels. The GluR2 surface/intracellular ratio (**h**) was greater in cocaine-exposed rats on withdrawal day 3 compared with pooled saline-exposed rats ($F_{2,26}$=7.4, p<0.05). Together with Fig. 2, these results indicate that there are no substantial changes in GluR2 expression or distribution over 45 days of withdrawal. GluR3: The GluR3 surface/intracellular ratio (**l**) was increased in cocaine-exposed rats on withdrawal days 3 and 21 compared with saline-exposed rats ($F_{2,26}$=4.0, p<0.05). Similarly, the GluR3 surface/intracellular ratio was increased in cocaine-exposed rats on withdrawal day 45 compared with the saline-exposed group and there was a trend towards an increase on withdrawal day 1 (Fig. 2). Values on days 3 and 21 were within the same range as values for days 1 and 45 (dotted and sold lines in panel **l**, respectively). Together, these results indicate that the GluR3 surface/intracellular ratio is increased after withdrawal from cocaine in a time-independent manner. Surface GluR3 (**i**) did not increase significantly on days 3 and 21 compared with saline-exposed group, whereas time-independent increases in this parameter were observed in the cocaine-exposed group on withdrawal days 1 and 45 (dotted and sold lines in panel **i** and Fig. 2). Intracellular (**j**) and total (**k**) GluR3 did not change significantly on withdrawal days 3 and 21, consistent no changes on withdrawal days 1 and 45 (Fig. 2). Surface, intracellular and total GluR1-3 values were normalized to total protein in the lane determined using Ponceau S. The surface/intracellular ratio is independent of total protein loaded on the gel. Data (mean±S.E.M, n=5-15 per group) are expressed as a percentage of the pooled saline group. * Different from the other conditions, p<0.05; # Different from pooled saline, p<0.05.

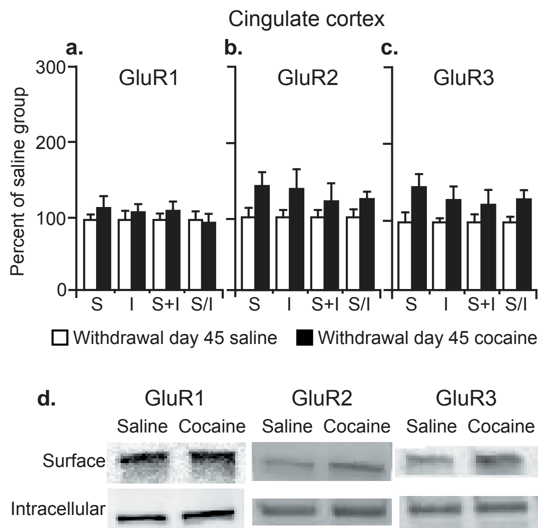


Supplementary Figure 2. *Quantitative co-immunoprecipitation of AMPA receptor subunits in the nucleus accumbens after prolonged withdrawal from cocaine self-administration*. AMPA receptor subunit composition was compared in accumbens tissue from (**a**) saline- and (**b**) cocaine-exposed rats that were killed on withdrawal day 45, using the quantitative co-immunoprecipitation method described in the Supplementary Methods. Immunoblots (IB) show the percentage of AMPA receptor subunits remaining (unbound fraction) after immunoprecipitation (IP) of solubilized accumbens tissue. The AMPA receptor subunit antibodies used to IP are indicated at the top of **a** and **b** panels. The antibodies used to IB are indicated on the left of **a** and **b** panels. The left two lanes in each row show immunoblotting of IgG control IP'ed tissue and indicate the range of immunoreactivity detected. The percent remaining is calculated from the standard curve generated by controls of 5% (shown), 25%, 50%, 75% and 100% (shown) run on each blot. In saline-exposed rats, unbound GluR1 was below the limit of detection (not detectable; N.D.) after IP with either GluR2 or GluR2/3 antibodies, indicating that nearly all GluR1 is associated with GluR2. In contrast, three sets of results indicated

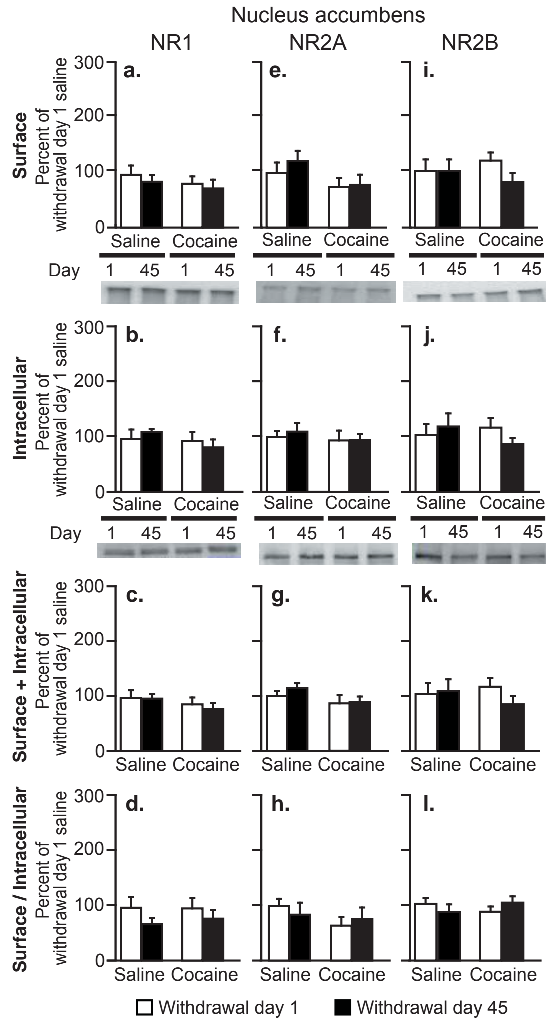
decreased association between GluR2 and GluR1 after prolonged withdrawal from cocaine self-administration: 1) After GluR1 IP, cocaine-exposed rats show an increase in GluR2 and GluR2/3 remaining in the unbound fraction (53 and 43% in control rats and 70 and 55% in cocaine rats, respectively), indicating an increase in GluR2 and GluR3 not associated with GluR1. 2) After GluR2 IP, cocaine-exposed rats show an increase in GluR1 remaining in the unbound fraction (N.D. in controls and 8% in cocaine rats), indicating an increase in GluR1 not associated with GluR2 (GluR1/3 or homomeric GluR1; GluR4 is not present in medium spiny neurons[22,23]). 3) After GluR2/3+4 IP, cocaine-exposed rats show an increase in GluR1 in the unbound fraction (N.D. in controls and 6% in cocaine rats), indicating an increase in GluR1 not associated with any other subunit (homomeric GluR1). These data do not permit conclusions about the magnitude of the increase in GluR2-lacking AMPA receptors, because the absolute amount of GluR1 protein increased after 45 days of withdrawal from cocaine (Fig. 2). One result was inconsistent with our other findings. After IP for GluR2/3, cocaine-exposed rats did not show an increase in unbound GluR1 (7% for controls, 6% for cocaine). Also notable is a decrease in unbound GluR3 in cocaine-exposed rats after IP for GluR2 (14% for controls, 6% for cocaine), suggesting increased association between GluR2 and GluR3 in cocaine rats on withdrawal day 45. This may seem inconsistent with data in Fig. 2 indicating no increase in GluR2 expression after prolonged withdrawal from cocaine. It is possible, however, that the same overall level of GluR2 expression is maintained by substituting some GluR2/3 receptors for GluR1/2 receptors. This would explain the IP results, as well as the lack of change in GluR2 expression and the increase in GluR3 surface expression (although some new GluR3 on the surface is likely GluR1/3) in Fig. 2.



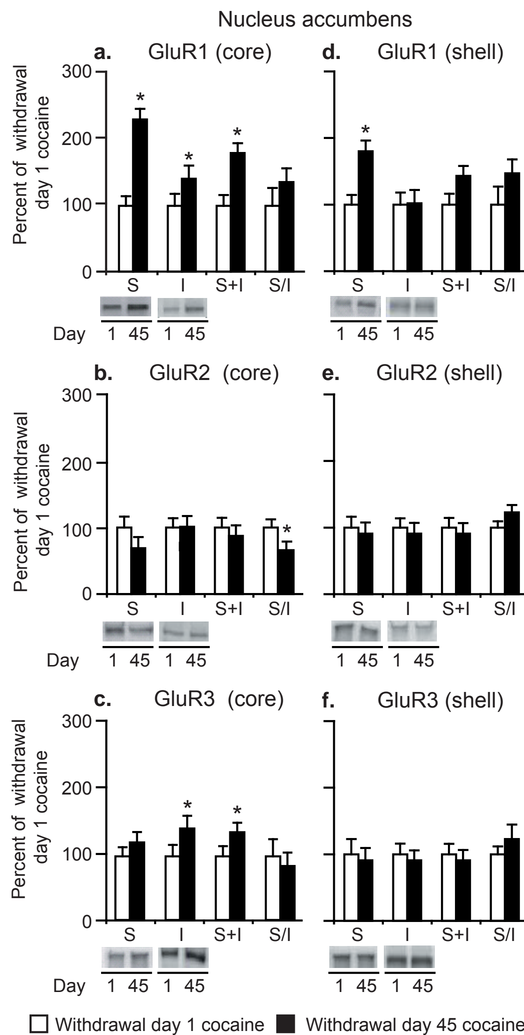
Supplementary Figure 3. *Effect of cocaine self-administration and subsequent withdrawal on AMPA receptor subunit expression and distribution in the ventral tegmental area (VTA).* Saline- and cocaine-exposed rats were compared on withdrawal days 1 and 45. No significant drug exposure by withdrawal day interactions were observed for GluR1 (**a-d**) or GluR2 (**e-h**). Cell surface (**i**), intracellular (**j**), and total (**k**) GluR3 levels were increased 45 days after withdrawal from cocaine ($F_{1,31}$=4.9, $F_{1,31}$=4.9, and $F_{1,31}$=5.0, respectively, p values<0.05 for drug exposure by withdrawal day interaction) but the surface/intracellular ratio (**l**) was not changed. Data (mean±S.E.M) are expressed as a percentage of the saline-exposed group on withdrawal day 1; n=6-9 per group. Surface, intracellular and total GluR1-3 values were normalized to total protein in the lane determined using Ponceau S. The VTA was dissected from a 2 mm slice obtained with a brain matrix (approximately -4.50 to -6.50 mm from Bregma[1]). * Different from the other groups, p<0.05.



Supplementary Figure 4. *Cocaine self-administration and subsequent prolonged withdrawal does not significantly alter AMPA receptor subunit expression and distribution in the cingulate cortex.* Saline- and cocaine-exposed rats were compared on withdrawal day 45. There were no significant differences with respect to surface (S), intracellular (I), and total (S+I) protein levels or the surface/intracellular ratio (S/I) for GluR1 (**a**), GluR2 (**b**), or GluR3 (**c**). Data (mean±S.E.M) are expressed as a percentage of saline-exposed groups on withdrawal day 45; n=7 per group. Surface, intracellular and total GluR1-3 values were normalized to total protein in the lane determined using Ponceau S. The cingulate cortex was dissected from a 2 mm slice obtained with a brain matrix (approximately 2.5 to 4.5 mm from Bregma[1]) by harvesting cortical tissue dorsal to the prelimbic cortex. (**d**) Representative Western blots for GluR1, GluR2, and GluR3.

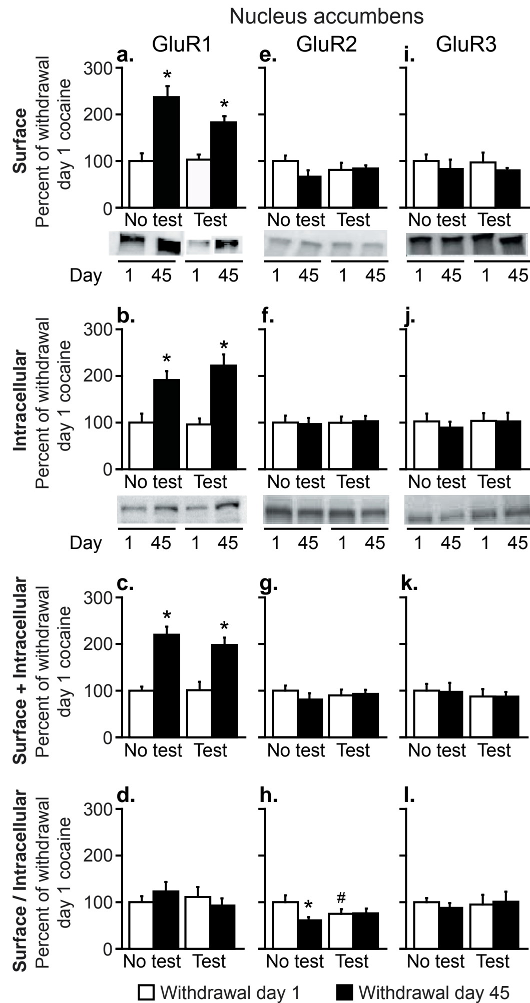


Supplementary Figure 5. *Cocaine self-administration and subsequent prolonged withdrawal does not significantly alter NMDA receptor subunit expression and distribution in the nucleus accumbens.* Saline- and cocaine-exposed rats were compared on withdrawal days 1 and 45. There were no significant differences with respect to surface, intracellular and total (surface + intracellular) protein levels or surface/intracellular ratios for NR1 (**a-d**), NR2A (**e-h**), or NR2B (**i-l**). Data (mean±S.E.M) are expressed as a percentage of saline-exposed group on withdrawal day 1; n=7-11 per group. Surface, intracellular and total values were normalized to total protein in the lane determined using Ponceau S.



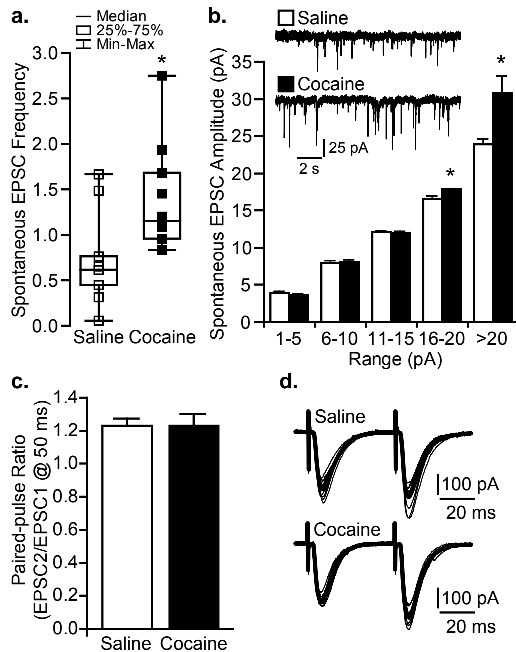
Supplementary Figure 6. *AMPA receptor adaptations after withdrawal from cocaine self-administration occur primarily in the nucleus accumbens core subregion.* Cocaine-exposed rats were compared on withdrawal days 1 and 45. AMPA receptor adaptations in the core paralleled those observed when the entire accumbens was dissected (data for entire accumbens are shown in Fig. 2). (**a**) In core, surface (S), intracellular (I), and total (S+I) GluR1 levels were increased on withdrawal day 45 compared to withdrawal day 1 ($F_{1,14}$=36.5, $F_{1,14}$=12.5, and $F_{1,14}$=72.6, p<0.01, respectively, p values<0.01). (**b**) In core, the GluR2 surface/intracellular ratio (S/I) was decreased after 45 days of withdrawal from cocaine ($F_{1,14}$=8.2, p<0.05). (**c**) In core, GluR3 intracellular and total levels were increased after 45 days of withdrawal from cocaine ($F_{1,14}$=8.1 and $F_{1,16}$=5.9, respectively, p

values<0.05). (**d**) In shell, GluR1 surface levels were increased after 45 days of withdrawal from cocaine ($F_{1,13}=3.3$, $p<0.05$). (**e, f**) In shell, there were no significant changes in expression or distribution of GluR2 or GluR3. GluR3 surface levels in the entire accumbens were increased in cocaine-exposed rats on withdrawal days 1 and 45 compared with the saline-exposed group (Fig. 2) but this effect cannot be evaluated in the present data set, which strictly compares cocaine-exposed rats on withdrawal days 1 and 45. Data (mean±S.E.M) are expressed as a percentage of cocaine-exposed rats on withdrawal day 1; n=7-9 per group. Surface, intracellular and total GluR1-3 values were normalized to total protein in the lane determined using Ponceau S. * Different from withdrawal day 1, $p<0.05$.



Supplementary Figure 7. *A 30 min cue-induced cocaine-seeking test has little effect on AMPA receptor expression and redistribution in the nucleus accumbens after withdrawal from cocaine self-administration*. All rats shown were trained to self-administer cocaine. Surface (**a**), intracellular (**b**), and total (**c**) levels of GluR1 were increased on withdrawal day 45 regardless of whether rats received an extinction test for cue-induced cocaine seeking on withdrawal day 1 or 45 (test) or were killed on these days without a test (no test) (main effects of withdrawal day: $F_{1,25}=9.9$, $F_{1,25}=11.0$ and $F_{1,25}=10.7$, respectively, p values<0.01). Surface (**e**), intracellular (**f**), and total (**g**) levels of GluR2 did not differ between test and no test groups. The GluR2 surface/intracellular ratio (**h**) changed as indicated by a significant test condition (test, no test) by withdrawal day interaction ($F_{1,25}=4.6$, $p<0.05$). The GluR2 surface/intracellular ratio was lower on withdrawal day 45 than on withdrawal day 1 in the no test group (*p<0.05) and lower on withdrawal day 1 in the test group compared to

withdrawal day 1 in the no test group ($^{\#}p<0.05$). (**i-l**) No significant differences were observed for GluR3 between test and no test groups. Data (mean±S.E.M) are expressed as a percentage of cocaine-exposed rats on withdrawal day 1 in the no test group; n=7-8 per group. Surface, intracellular, and total GluR1-3 levels were normalized to total protein in the lane determined using Ponceau S.



Supplementary Figure 8. *Nucleus accumbens medium spiny neurons recorded after prolonged withdrawal from cocaine self-administration exhibit increased frequency and amplitude of spontaneous EPSCs (sEPSC) compared with the saline-exposed group but no change in the paired-pulse facilitation ratio.* Recordings were performed from medium spiny neurons of the nucleus accumbens after 42-47 days of withdrawal from saline or cocaine self-administration. (**a**) Medium spiny neurons recorded from cocaine-exposed rats (n=11 from 4 rats) exhibit a significant increase in the frequency of AMPA receptor-mediated sEPSCs when compared with the saline-exposed group (n=9 from 3 rats) (expressed as number of events/s, box-plot, $t_{18=}$=2.51, *p<0.05). (**b**) Bar graph summarizing the mean amplitude by range of sEPSC size obtained from the same recordings used to compute the frequency data shown in (**a**). Medium spiny neurons recorded from saline- and cocaine-exposed rats showed similar sEPSC amplitude at sEPSC size ranges <15 pA. However, amplitude analysis of sEPSC size >15 pA revealed a significant increase in the cocaine group compared with the saline group (t_{18}=2.25, p<0.05 at 15-20 pA, and t_{18}=2.34, p<0.05 at >20 pA). Inset shows two traces of sEPSCs recorded from nucleus accumbens neurons after 45 days of withdrawal from saline or cocaine self-administration. (**c**) Bar graph summarizing the presence of paired-pulse facilitation in both groups of neurons. The paired-pulse facilitation ratio was calculated as the ratio of

the amplitude of evoked $EPSC_2/EPSC_1$. Accumbens medium spiny neurons recorded from rats in the cocaine-exposed (n=11) and saline-exposed (n=9) groups showed similar paired-pulse facilitation ratios of ~1.2 (i.e., amplitude of $EPSC_2$ is 20% larger than $EPSC_1$). (**d**) Traces of evoked EPSCs recorded from cocaine- and saline-exposed rats showing the presence of paired-pulse facilitation. * Different from saline, $p<0.05$.

SUPPLEMENTARY METHODS WITH ADDITIONAL REFERENCES

Subjects

The subjects were male Sprague Dawley rats (Harlan, Indianapolis, IN; biochemical experiments) and Long Evans rats (Charles River, Raleigh, NC; Naspm experiment performed at the IRP/NIDA) weighing 250-275g upon arrival. The rats were housed individually on a reverse 12h/12h light-dark cycle (lights out at 0900 hours). Rats had access to water and food ad libitum at all times unless specified. All procedures followed the "Principles of Laboratory Animal Care" (NIH publication no. 86-23, 1996) and were approved by the local Animal Care and Use Committees. The cocaine- and saline-trained rats were implanted with either intravenous catheters or intravenous catheters plus bilateral cannulae aimed at the nucleus accumbens (see below). The inclusion of saline-exposed control rats that are drug-free but still exposed to the same experimental conditions was used to control for effects of ageing on our molecular measures.

Surgical procedures

The rats were anesthetized using isoflurane gas (Henry Schein, Melville, NY) and flunixin meglumine was administered before surgery (2 mg/kg, i.p.) as an analgesic. A silastic catheter was inserted into the right auricle through the external jugular vein, passed under the skin and fixed in the mid-scapular region. The rats recovered from surgery for at least seven days prior to beginning self-administration training sessions. During this time, catheters were flushed every 24-48 h with sterile 0.9% saline. The rats undergoing intravenous self-administration experiments together with intracranial infusions (Naspm accumbens injection experiment) were anesthetized with sodium pentobarbital and chloral hydrate (60 and 25 mg/kg, i.p.), and permanent guide cannulae (23-gauge, Plastics One, Roanoke, VA) were implanted bilaterally 1 mm above the nucleus accumbens and were aimed at the core sub-region (coordinates: 6° angle aimed medially, AP +1.7, ML ±2.5, and DV −6.0)[1]. Following cannulae implantation, silastic catheters were inserted into the jugular vein, attached to a modified 22-gauge cannula and mounted to the rat's skull with dental cement (see ref. [2,3]). Buprenorphine (0.1 mg/kg,

s.c.) was given after surgery as an analgesic and the rats recovered for 7-10 days before behavioral testing began. During the recovery and training phases for these rats, catheters were flushed every 24-48 h with sterile 0.9% saline + the antibiotic Gentamicin (0.08 mg/mL).

Intracranial injections

The intracranial injection methods were based on our previous studies[2,3]. 1-Naphthylacetyl spermine trihydrochloride (Naspm; Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate buffered saline (PBS). Injections of vehicle or Naspm (10, 20 and 40 µg/side) were made with Hamilton syringes (Hamilton, Reno, NV) that were connected to 30-gauge injectors (Plastics One, Roanoke, VA). A volume of 0.5 µL was infused into each side over 1 min and the injector was left in place for 1 min after the injections. The rats were tested within 15 min after intracranial injections. The Naspm doses were based on previous reports[4,5] and on an initial study with sucrose-trained rats (see below). At the end of the experiments, the rats were deeply anaesthetized, their brains were removed, and coronal sections (40 µm) were sliced on a cryostat and stained with Cresyl Violet (ICN Biomedicals Inc., Aurora, OH). Cannulae placements were verified under a microscope and their anatomical location is depicted in Fig. 4c.

Cocaine self-administration training

Following recovery from surgery, the rats were allowed to self-administer for 6 h/day cocaine or saline for 10 days (biochemical and electrophysiological experiments) or for 10-12 days (Naspm accumbens injections experiment). The self-administration chambers (MED Associates, St. Albans, VT) were located in sound-attenuating cabinets. Rats were either housed chronically in these chambers (for the Naspm experiments), or they were placed daily in these chambers; sessions began approximately at the start of the dark cycle. For the Naspm experiments, the self-administration chambers were equipped with two levers. Presses on one (active, retractable) activated the infusion pump and delivered an infusion of cocaine (0.75 mg/kg); presses on the other (inactive, stationary) had no effects. A fixed-ratio-1 reinforcement schedule was used, with a 40-s

timeout period after each infusion; cocaine infusions were accompanied by a 5-s tone-light cue. Each session began with the insertion of the active lever and the illumination of a houselight that remained on for the entire session. At the end of each session, the houselight was turned off and the active lever retracted. To facilitate the acquisition of cocaine self-administration, food was removed from the chambers during the 6-h sessions of the first 3 training days. The number of cocaine infusions was limited to 20/h to prevent overdose. For all other experiments, the self-administration chambers were equipped with 2 holes located 2 cm above the floor. Nose-poking in the active hole activated the infusion pump and delivered an infusion of saline or cocaine (0.5 mg/kg); nose-poking in the inactive hole had no consequences. In addition to activating the infusion pump, nose-poking in the active hole was paired with a 5-s discrete light cue, located inside the nose hole. A time-out period of 10 s was used during the first hour or for the first 10 infusions (whichever occurred first) and then the time-out period was extended to 30 s for the remaining hours, to prevent cocaine overdose. Food and water were present at all times. For sucrose self-administration (results shown in Fig. 4b, n=10), procedures were the same as those described above for cocaine self-administration for the Naspm experiment, except that active lever presses led to the delivery of 0.75 mL of 10% sucrose solution into receptacles located near the lever. After stable sucrose self-administration behavior was achieved, the rats were injected every other day with vehicle or Naspm (10, 20 or 40 µg/side) into the accumbens 15 min before the test sessions, which were separated by regular training days. The order of the injections of the vehicle and the different Naspm doses was counterbalanced. Naspm (40 µg/side) or its vehicle was also injected during cocaine self-administration in a sub-group of rats (n=5), as described above for sucrose.

Tests for cue-induced cocaine seeking

At the end of the training phase, the rats were returned to the animal facility where they remained for 1 or 45 days (the rats in the late withdrawal period were handled several times per week). After this time, they were brought back to the self-administration chambers, where they were tested for cue-induced cocaine-seeking under extinction conditions; that is, all conditions were the same as during

training, with the exception that responding on the active device was not reinforced with drug. During the extinction tests, lever or nose-poke responding led to contingent presentations of the tone-light or light cue previously paired with cocaine infusions. The number of responses in the previously active lever or hole was used as a measure of cocaine-seeking.

Protein crosslinking

Each experimental group consisted of 7-18 rats, with the exception of cocaine withdrawal day 21 (n=5). The rats were decapitated, their brains were rapidly removed, and the nucleus accumbens (or other region of interest) was dissected on ice from a 2 mm coronal section obtained using a brain matrix. Tissue was immediately chopped into 400 µm slices using a McIllwain tissue chopper (Vibratome, St. Louis, MO). Slices were added to Eppendorf tubes containing ice-cold artificial CSF which was spiked with 2 mM bis(sulfosuccinymidal)suberate (BS^3; Pierce Biotechnology, Rockford, IL) immediately after addition of the tissue. Slices were crosslinked for 30 min at 4°C with gentle agitation. Crosslinking was terminated by addition of 100mM glycine (10 min at 4°C). Slices were pelleted by brief centrifugation, re-suspended in ice-cold lysis buffer containing protease and phosphatase inhibitors, sonciated for 5 sec to disrupt tissue, and centrifuged (20,800 x g, 2 min) as described previously[6,7]. The supernatant fraction was aliquoted and stored at -80°C. BS^3 is a membrane impermeant crosslinking agent. Therefore, it selectively crosslinks cell surface proteins, forming high molecular weight aggregates. Intracellular proteins are not modified and thus retain their normal molecular weight. This enables surface and intracellular pools of a particular protein to be distinguished by SDS-PAGE and Western blotting. Variants of this assay have been used previously to measure glutamate receptor surface expression in dissociated cells and brain slices[8-16]. We adapted the assay to detect receptor redistribution produced after in vivo treatments[6]. We and others have shown that incubation of brain slices or dissociated cultures with BS^3 does not crosslink intracellular proteins (e.g., actin, tubulin, synapsin, tyrosine hydroxylase, and protein kinases) unless BS^3 crosslinking is performed in a lysed preparation [6-12,14,15].

Western blot analysis of glutamate receptor subunits in crosslinked tissue

Samples were run on 4-15% gradient Tris-HCl gels (Bio-Rad, Hercules, CA) under reducing conditions, proteins were transferred to PVDF membranes, and membranes were washed in Tris buffered saline (TBS) and blocked with 1% goat serum/5% nonfat dry milk in TBS-Tween-20 (TBS-T). Membranes were incubated overnight at 4°C with the following 1° antibodies: GluR1 (1:500; Millipore, Billerica, MA), GluR2 (1:1000, Millipore), GluR3 (1:500; Millipore), NR1 (1:500; Millipore), NR2A (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA), and NR2B (1:1000; Millipore). Membranes were washed with TBS-T solution, incubated for 60 min with HRP-conjugated anti-rabbit IgG or anti-mouse IgG (1:10,000; Upstate Biotechnology, Lake Placid, NY), washed with TBS-T, rinsed with ddH_2O, and immersed in chemiluminescence (ECL) detecting substrate (Amersham GE, Piscataway, NJ). Images were captured with Versa Doc Imaging Software (Bio-Rad). Diffuse densities of surface and intracellular bands were determined with Quantity One software (Bio-Rad). Values for surface, intracellular and total (surface + intracellular) protein levels were normalized to total lane protein determined using Ponceau S (Sigma-Aldrich) and analyzed with TotalLab (Nonlinear Dynamics, Newcastle, UK). The surface/intracellular ratio did not require normalization, because both values are determined in the same lane.

Quantitative co-immunoprecipitation

Using the methods developed by Wenthold and colleagues[17,18] and with the help of advice from the Wenthold laboratory, we quantitatively determined AMPA receptor subunit composition in the accumbens after 45 days of withdrawal from cocaine or saline self-administration. Briefly, the rats were decapitated, their brains were rapidly removed, and the accumbens was dissected on ice from a 2 mm coronal section obtained using a brain matrix. Tissue from 3 rats from each experimental group was combined and homogenized in 50mM Tris-HCl pH7.4 containing protease inhibitor cocktail (Calbiochem, San Diego, CA) (40-60mg wet weight/mL). The membranes were sedimented by centrifugation at 100,000 x g for 30 min at 4°C. The pellet was then solubilized with 1% Triton X-100 in 50mM Tris-HCl pH 7.4 containing 1mM EDTA for 45 min at 37°C. Insoluble material was

removed by centrifugation at 100,000 x g for 30 min at 4°C. The supernatant was stored at -80°C until use. For co-immunoprecipitation, 3-5μg of antibody (GluR1, GluR2, GluR2/3, or GluR4) or an equal amount of control IgG was incubated with 10-20μL of 50% protein A agarose slurry (Pierce, Rockford, IL) for 4 h at 4°C. The pellet was collected by centrifugation at 1000 x g for 30 s and washed 3 times with TBS 0.1% Triton X-100. 100μL of membrane prep was incubated with the washed pellet overnight at 4°C. The agarose bound antibody was pelleted by centrifugation at 1000 x g for 30 sec. This created two fractions, the bound (pellet) and unbound (supernatant). The unbound fraction was then subjected to another round of immunoprecipitation. Two rounds of immunoprecipitation pulled down >95% of the target AMPA receptor subunit (e.g., in Supplemental Fig. 2, after IP for GluR1, no GluR1 is detected by immunoblotting). After the final immunoprecipitation, the unbound fraction was mixed with an equal volume of sample treatment buffer (Invitrogen, Carlsbad, CA) and heated to 70°C for 10 min. For Western analysis, samples were run on 4-12% Bis-Tris gels (Invitrogen) and transferred to PVDF membranes for immunoblotting. Membranes were washed in dH_2O and blocked with 1% goat serum with 5% Carnation milk in 0.05% Tween-20 in TBS, pH 7.4 for 1 h at room temperature. Membranes were then incubated with subunit-specific antibodies (Millipore: GluR1, 1:500; GluR2/3, 1:2000; GluR2, 1:1000; GluR3, 1:500) overnight at 4°C. Membranes were then washed with TBS-Tween solution, incubated for 60 min with HRP-conjugated anti-rabbit IgG or anti-mouse IgG (1:10,000; Upstate Biotechnology, Lake Placid, NY), and washed again with TBS-Tween, followed by TBS. Membranes were then rinsed with dH_2O, immersed in chemiluminescence (ECL) detecting substrate (Amersham GE) for 1 min, and visualized with VersaDoc imaging software (Bio-Rad) (between 5 and 60 s, depending on the antibody). Diffuse densities of bands were determined using Quantity One software (Bio-Rad). The percent of total AMPA receptor subunit remaining in the unbound fraction was calculated based on the standard curve created from control IgG immunoprecipitated tissue, as described in the legend to Supplemental Fig. 2.

Electrophysiology

As previously reported[19] the rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) before being decapitated. Brains were rapidly removed into ice-cold artificial cerebral spinal fluid (aCSF) containing (in mM): 125 NaCl, 25 $NaHCO_3$, 12.5 glucose, 3.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 3 $MgCl_2$, 0.05 APV, and 0.05 picrotoxin (pH 7.45, 295-305 mOsm). Coronal slices (300 μm thick) containing the nucleus accumbens were cut in ice-cold aCSF with a Vibratome, and incubated in warm (~35°C) aCSF solution constantly oxygenated with 95% O_2-5% CO_2 for at least 60 min before recording. In the recording aCSF (delivered at 2 ml/min), $CaCl_2$ was increased to 2 mM and $MgCl_2$ was decreased to 1 mM. Patch pipettes (6-9 MΩ) were pulled from 1.5 mm borosilicate glass capillaries (WPI, Sarasota, FL) with a horizontal puller (Model P97, Sutter Instrument, Novato, CA), and filled with a solution containing 0.125 % Neurobiotin and (in mM): 140 Cs-gluconate, 10 HEPES, 2 $MgCl_2$, 3 Na_2-ATP, 0.3 GTP, 0.1 spermine, 1 QX-314 (pH 7.3, 280-285 mOsm). All chemicals and drugs were purchased from Sigma-Aldrich.

Nucleus accumbens medium spiny neurons from the core region were identified under visual guidance using infrared-differential interference contrast (IR-DIC) video microscopy with a 40x water-immersion objective (Olympus BX51-WI). The image was detected with an IR-sensitive CCD camera and displayed on a monitor. Whole-cell patch-clamp recordings were performed with a computer-controlled amplifier (MultiClamp 700B; Axon Instruments, Union City, CA), digitized (Digidata 1440; Axon Instruments), and acquired with Axoscope 10.1 (Axon Instruments) at a sampling rate of 10 KHz. The liquid junction potential was not corrected and electrode potentials were adjusted to zero before obtaining the whole-cell configuration. Nucleus accumbens medium spiny neuron synaptic responses were elicited by local electrical stimulation (0.05 to 0.30 mA square pulses of 0.3 ms duration delivered every 20 s) of excitatory inputs using a bipolar electrode made from a pair of twisted Teflon-coated nichrome wires (tips separated by approximately 200 μm) and placed ~300 μm lateral to the recorded neurons. The intensity of stimulation was chosen from the minimum amount of current necessary to elicit a synaptic response with <15% variability in amplitude during baseline

recording[20]. Only neurons that retained such synaptic response reliability during the subsequent 20 min of baseline recording were included in the present study. If the current intensity required was >0.3 mA, the neuron was discarded.

All recordings were conducted in voltage clamp configuration at 33-35°C in the absence of TTX. Control and drug-containing aCSF were continuously oxygenated throughout the experiments. After 20-30 min of baseline recording, a solution containing the GluR2-lacking AMPA receptor antagonist Naspm (100-200 μM) was perfused for 10 min followed by a 20-30 min washout period. Changes in input resistance, spontaneous EPSC (frequency and amplitude), evoked EPSC amplitude and paired-pulse ratio (at 50 ms interval) were analyzed before and after drug application. In addition, we collected several points of the current-voltage relationship (holding Vm at -70 mV, -50 mV, -30 mV, +20 mV, +40 mV and +60 mV) of the evoked AMPA-mediated EPSC during baseline to compute the rectification index. The rectification index was calculated by correcting any potential shifts in the reversal potential values (E_{rev})[21] and computed using the following equation: $RI = (I_{-70}/(70 - E_{rev}))/(I_{+40}/(40 - E_{rev}))$. Thus, RI is expressed as a ratio that will increase when rectification increases. I_{-70} and I_{+40} are the EPSC current amplitudes recorded by holding the membrane potential at −70 mV and +40 mV, respectively. The E_{rev} values were obtained from the *I-V* relationship. Finally, we performed frequency and amplitude analyses of spontaneous AMPA receptor-mediated events using Clampfit 10 (Axon Instruments). All comparisons were performed from 3 min segments of baseline recordings acquired at 10 KHz. For each neuron, we assessed cumulative histograms and conducted Kolmogorov Smirnov tests. All measures are expressed as mean±S.E.M. All neurons included in the present study were labeled with Neurobiotin. Their location and morphology were further confirmed as medium spiny neurons in the core region of the nucleus accumbens.

Statistical analyses

Data from self-administration experiments were analyzed with the statistical program SPSS (GLM procedure). The nose-poke or lever-press data from the extinction tests for cue-induced cocaine-seeking were analyzed with Analysis of Variance (ANOVA) with Withdrawal Day (1, 45) as the

between-subjects factor, and Hole or Lever (previously active, inactive) as the within-subject factor. For the Naspm accumbens injection experiment, the statistical analyses also included the within-subjects factor of Session Hour. For biochemical studies, group differences in protein levels were analyzed by ANOVA using Drug exposure (saline, cocaine) or Extinction test (yes, no) and Withdrawal Day (1, 45) as the between-subjects factors, followed by a post hoc Tukey test. For experiments on the effect of Naspm on cocaine or sucrose self-administration, the ANOVA included the within-subjects factors of Naspm Dose (Vehicle, 40 μg) and Session Hour (1-6). For electrophysiological studies, drug effects were compared using Student's t-test or repeated-measures ANOVA. Differences between experimental conditions were considered statistically significant when $p<0.05$.

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