Supporting Information

Shen et al. 10.1073/pnas.1112052108

SI Experimental Procedures

Animal Housing and Surgery. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Assessment and Accreditation of Laboratory Animal Care. Male Sprague–Dawley rats (250 g on arrival; Charles River Laboratories) were individually housed in a temperature- and humidity-controlled environment with a 12-h dark/light cycle (6:00 PM, lights on). Experiments were conducted during the rats' dark cycle. Rats received food ad libitum until 1 d before behavioral training, after which food restriction procedures (20 g of rat chow per day) were implemented and maintained throughout the duration of the experiment. Rats were allowed 1 wk to acclimate to the vivarium before inducing anesthesia and implanting jugular and intracranial cannula. The surgical details have been described in a previous study (1). Intracranial guide cannulas (30-guage) were stereotaxically implanted bilaterally into the core of nucleus accumbens (NAcore).

Heroin Self-Administration Procedures. All self-administration experiments occurred in standard operant chambers with two retractable levers, a house light, and a cue light and tone generator (Med Associates). During 3-h sessions on 14 consecutive days, rats were trained to press the active lever on a fixed ratio 1 schedule with 20-s timeout for an infusion of heroin-hydrochloride (100 μg per infusion for days 1–2; 50 μg/4-s infusion for days 3–4; 25 μg per infusion for days 5–14. Heroin was kindly provided by the National Institute on Drug Abuse, Rockville, MD). Concurrent with the drug infusion, a cue tone (2,900 Hz) and cue light immediately above the active lever turned on. After 14 d of self-administration, rats began extinction training. Active lever presses produced no drug infusion or light/tone cues. For heroin prime-induced reinstatement, rats received a single injection of heroin (0.25 mg/kg, s.c.) before beginning the 3-h session, and the session program was identical to the extinction program. For cue-induced reinstatement, no injection was given, but the session program was identical to the self-administration session program (i.e., cue tone and light turned on with an active lever press). Rats did not receive intravenous drug infusions during either mode of reinstatement trial.

Drug Treatment. For systemic treatment, ifenprodil (Ascent Scientific) was dissolved in sterile water with 3 mg/mL intraperitoneally, and was administered to rats 30 min before heroin or cue priming. For NAcore microinjection, ifenprodil was dissolved in artificial cerebrospinal fluid (aCSF) creating a final concentration of 10 μM ifenprodil, and was administered at a dose of with 8 ρmol per side via 33-gauge needle immediately before administering a heroin priming injection. After systemic or local ifenprodil, rats then underwent their appropriate reinstatement or locomotor testing.

Local Knockdown of NMDA2a/b Receptor Channel Using siRNA in NAcore. The sequence of siRNA silencing NMDA2a receptor channel (NR2A) was sense 5′-CAAGGACUGUAGCGAUGUUtt-3′ and antisense 5′-AACAUCGCUACAGUCCUUGgg-3′ (ID# S127808); and NR2B was sense 5′-GGAUGAGUCCUCCAUGUUCtt-3′ and antisense 5′-GAACAUGGAGGACUCAUCCtt-3′ (ID# 61879; Applied Biosystems). These siRNAs were preselected commercially (NR2A) or prevalidated (NR2B) (2), and reconfirmed by us using Western blotting that demonstrated nearly 40% knockdown in whole-cell lysate in vivo (Fig. 5*C*). To verify there is no off-target effect of these siRNAs, siRNA targeting NR2B was used as negative control for NR2A siRNA;vice versa (Fig. S6). To perform siRNA transfection in vivo, siRNAs were conjugated with a lipid-based siRNA transfection reagent (3) (X-tremeGENE; Roche Applied Science) at a volume ratio of 10:1 (siRNA final concentration 200 nM), and 2 μL of the mixture was microinjected into NAcore once daily for 3 consecutive days. The rats were used for reinstatement testing 24 h after the last injection. The microinjection procedure and coordinates were same as ifenprodil described above.

Diolistic Labeling. Rats were deeply anesthetized with ketamine HCl (87.5 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.), and perfused transcardially (35 mL/min) with 0.1 M sodium phosphate buffer (PB), followed by 300 mL 1.5% paraformaldehyde (PFA) in 0.1 M PB. Brains were removed and postfixed in the same fixative for 1 h, and then were coronally sectioned (150-μm thick) using a vibratome at room temperature. Tungsten particles (1.3-μm diameter, Bio-Rad) were coated with the lipophilic carbocyanine dye DiI (Invitrogen). DiI-coated particles were delivered diolistically into the tissue at 80 psi using a Helios Gene Gun system (Bio-Rad) fitted with a polycarbonate filter with 3.0 μm pore size (BD Biosciences). DiI was allowed to diffuse along neuron dendrites and axons in PBS containing 0.01% (wt/vol) thimerosal for 48 h at 4 °C, and then labeled sections were fixed again in 4% PFA for 1 h. After brief washing in PBS, tissues were mounted onto subbed slides in aqueous medium Prolong (Invitrogen).

Confocal Imaging and Quantification of Dendritic Spine. A confocal microscope (Zeiss LSM 510) was used to image the labeled sections. DiI was excited using the Helium/Neon 543-nm laser line. The micrograph of DiI-labeled dendrite was acquired via optical sectioning by a 63× oil immersion objective (Plan-Apochromat, Zeiss; NA = 1.4, WD = 90 μm) with pixel size 0.07 μm at *xy* plane and 0.1 μm intervals along the *z* axis. Before quantitative analysis, imaging data were deconvoluted by Autoquant (Media Cybernetics), and then a 3D perspective was rendered by the Surpass module of Imaris software package (Bitplane). Only cells localized to the NAcore were quantified, and spine quantification commenced on dendrites beginning at >75 μm distal to the soma, and after the first branch point. Measurements were made to a maximum of 200 μm from the soma, and the length of dendrite quantified was 40–55 μm. For each of three to five animals examined at each group, at least 12 neurons were analyzed. A protocol based on Filament module of IMARIS software was used that quantifies spine density and head diameter. The minimum end segment diameter (spine head) was set at ≥0.143 μm. The ratio of branch length to trunk radius was ≥1.5, and the branch length had to be >0.5 μm.

Biotinylation Assay. The NAcore was dissected and sliced into prism-shaped sections (200 μm) with a McIlwain tissue chopper (Vibratome). The tissue was incubated for 30 min in 300 μL PBS containing 1 mg/mL Sulfo-NHS-Biotin (Pierce) at 4 °C with gentle shaking. Reaction was quenched by adding ice-cold 100 mM glycine in PBS. The tissues were washed twice with ice-cold 100 mM glycine in PBS, and then homogenized by sonication in 300 μL 1% SDS in RIPA lysis buffer (Pierce) containing protease inhibitor mixture (Roche). After incubating 30–60 min on ice, the protein-contained solution was centrifuged at 10,000 × *g* for 10 min at 4 °C to remove insoluble material. The supernatant was added with 100 μL NeutrAvidin agarose resin and incubated for 2 h at 4 °C with gentle rotation. After washing with ice-cold

PBS twice, the biotinylated protein was eluted by 50 μL loading buffer (1% SDS and 50 mM DTT) with heating at 80 °C for 5 min. The concerned proteins in biotinylated fraction will be detected with immunoblotting. Antibodies used for immunoblotting include mouse monoclonal antibodies against the GluR2 (Millipore), NR1 (BD Biosciences), NR2A (BD Biosciences), and rabbit polyclonal antibodies against GluR1 (Abcam), NR2B (Millipore).

Electrophysiology in Vivo. The rats were anesthetized with urethane (1.5 g/kg, i.p.), and mounted in a stereotaxic apparatus (Narishige). Subcutaneous atropine methylbromide (0.3 mg/kg) was used to minimize secretions and improve ventilation as needed, and core temperature was maintained using a heating pad. Concentric bipolar stimulating electrodes (Rhodes Medical Instruments) were placed in the prelimbic medial prefrontal cortex [anteroposterior (AP), +3.0 mm; mediolateral (ML), +0.6 mm; dorsoventral (DV), –3.3 mm from brain surface]. Glass recording electrodes were pulled using a Narishige PE-2 puller (1–2 MΩ). Filling solution consisted of 0.5M sodium acetate and 2% pontamine sky blue. Recording electrodes were aimed at the dorsomedial region of the NAc (AP, +1.8 mm; ML, +1.3–1.5 mm; DV, –6.2 to –6.4 mm from the brain surface). The preparation was further stabilized with agar. Extracellular field potentials were amplified by a NPI Instruments SEC-05LX amplifier, and the data bandpass filtered at 300 Hz, then digitized by a National Instruments PCM-C1016E4 board feeding into a computer. Custom Labview Software (Lee Campbell, The Salk Institute, La Jolla, CA) was used for data collection and analysis. To ensure the accuracy and stability of the recordings, baseline measurements were obtained 1 h after surgery. Field potential amplitude was estimated as the difference between the mean of a 2- to 4-ms window before the stimulation artifact and the mean of a 1-ms window around 15 ms after the stimulation artifact (corresponding to the negative peak of the field potential). To record the field excitatory postsynaptic potential (fEPSP) response to heroin priming, data were collected every 60 s, at a 10-kHz sampling frequency, and then averaged every 3 min. Pulse width was set to 0.3 ms, and basal stimulation intensity corresponded to 40% of the minimum current intensity that evoked a maximum field response. To apply ifenprodil, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), or aCSF locally, a microinjection needle (33-guage) was inserted into the NAcore slowly (10 μm/30 s) to avoid maiming the local anatomical structure (AP, +2.0 mm; ML, ±1.5 mm; DV, −5.9 to −6.0 mm). Infusions were made by a microinjection pump (0.1 μL/min).

Whole-Cell Recordings in Acute Brain Slices. Rats were anesthetized with ketamine HCl (87.5 mg/kg, i.p.), and perfused transcardially with ice-cold aCSF containing: 210 mM sucrose, 1.25 mM NaH_2PO_4, 26 mM NaHCO3, 20 mM glucose, 6.0 mM MgCl2, 0.5 mM EGTA, 2.5 mM KCl, 3.0 mM NaPyruvate, 1.0 mM ascorbic acid, and 3.0 mM kynurenic acid bubbled with 95% O_2 and 5% CO_2 (pH 7.2–7.4). Brains were rapidly removed into ice-cold aCSF. Coronal slices (230-μm thick) containing the nucleus accumbens were cut in ice-cold aCSF with a vibratome, and incubated in 32 °C aCSF (126 mM NaCl, 2.5 mM KCl, 1.4 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose) for at least 45 min before returning to room temperature. Picrotoxin (50 μM) was added to block $GABA_A$ receptor-mediated inhibitory postsynaptic currents during recording. Cells were visualized using infrared-differential interference contrast optics. To assess excitatory synaptic transmission, neurons were voltage clamped at –80 mV using a Multiclamp 700A amplifier (Molecular Devices). Electrodes (1.5–2 MΩ) contained 117 mM cesium gluconate, 2.8 mM NaCl, 20 mM Hepes, 0.4 mM EGTA, 2 mM MgATP, and 0.3 mM MgGTP (285–295 mOsm, pH 7.2–7.4). Series resistance (<20 MΩ) and input resistance were monitored on-line with a 5-mV depolarizing step (100 ms) given with each stimulus. Afferents were stimulated at 0.05 Hz by a concentric bipolarectrode placed about 150 μm dorsomedial to the recorded neurons. The intensity of stimulation was chosen to evoke a 200–300 pA excitatory postsynaptic current (EPSC). To assess the contribution of NR2A/NR2B subunits in medium spiny neurons (MSNs), NMDAR-mediated EPSC was isolated by including CNQX (10 μM) and picrotoxin (50 μM) in aCSF when the voltage was clamped at −40 mV to relieve the voltage-dependent Mg^{2+} block of NMDA receptor channels. At the end of every experiment, D-AP5 was added in aCSF to verify this current was mediated by NMDAR exclusively. After recording the baseline of evoked NMDA EPSC (averaging 15 EPSCs, sampled at 0.033 Hz), the NR2A component was isolated by the bath application of selective NR2B antagonist Ro256981 (1 μM) with the same stimulus strength. Data were filtered at 2 kHz, digitized at 5 kHz, and collected and analyzed using AxoGraph X software (AxoGraph Scientific). AMPAR/NMDAR ratios were computed from evoked-EPSCs at +40 mV with and without 50 μM D-AP5. NMDA current decay was quantified by the decay time constant (τ) fitting to a single exponential (4, 5) and the time elapsed from the EPSC peak to 37% peak amplitude using AxoGraph X.

Statistics. All spine density and diameter data were statistically analyzed after averaging multiple dendrite segment measurement for each neuron. Protein data in the heroin groups were normalized to the values of saline-yoked groups. When two groups were compared, the data were statistically analyzed using a two-tailed unpaired *t* test. For comparing the multiple measurements in the same experiment the data were analyzed using a one-way or two-way ANOVA. The electrophysiological data were normalized to baseline field amplitude in each group and evaluated using a two-way ANOVA with repeated measures over time. Bonferroni tests were applied for multiple comparisons and $P < 0.05$ was considered statistically significant. All statistical tests are conducted using the SPSS or Prism (Graphpad) software.

1. LaLumiere RT, Kalivas PW (2008) Glutamate release in the nucleus accumbens core is necessary for heroin seeking. *J Neurosci* 28:3170−3177.
2. Mao LM, et al. (2009) Stability of surface NMDA receptors controls synaptic and behavioral adaptations to amphetamine. *Nat Neurosci* 12:602−610.
3. Salahpour A, Medvedev IO, Beaulieu JM, Gainetdinov RR, Caron MG (2007) Local knockdown of genes in the brain using small interfering RNA: A phenotypic comparison with knockout animals. *Biol Psychiatry* 61:65−69.
4. Popescu G, Robert A, Howe JR, Auerbach A (2004) Reaction mechanism determines NMDA receptor response to repetitive stimulation. *Nature* 430:790−793.
5. Barth AL, Malenka RC (2001) NMDAR EPSC kinetics do not regulate the critical period for LTP at thalamocortical synapses. *Nat Neurosci* 4:235−236.

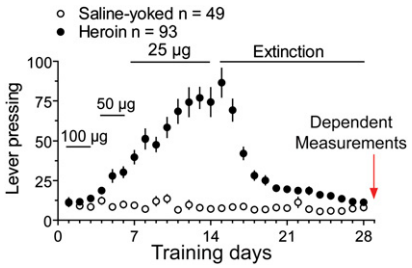

Fig. S1. Heroin treatment protocol used for data in all figures, showing the pattern of self-administration and extinction active lever pressing. The dose of heroin was decreased during self-administration training from 100 to 25 µg per infusion as shown.

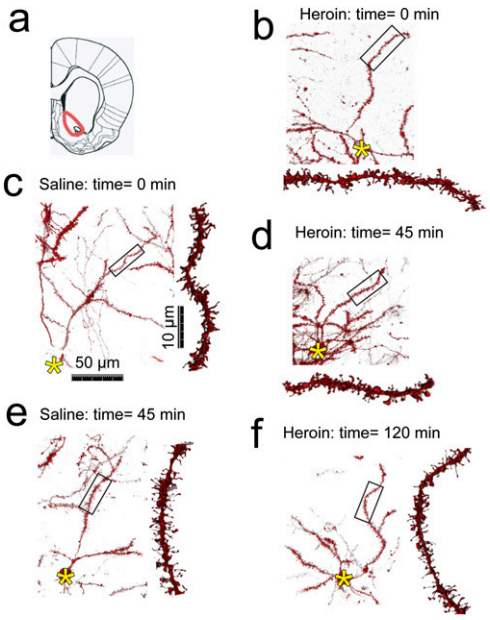

Fig. S2. (*A*) Illustration of the area in the NAcore where spine morphology was quantified. (*D–F*) Examples of neurons and dendritic segments quantified for spine morphology at different times after an acute injection of heroin (0.25 mg/kg, s.c.). Heroin and saline refer to self-administration of heroin or yoked saline controls, respectively; and the time refers to time after acute heroin. The rectangles in panels outline the dendritic segment shown in higher magnification. The yellow asterisk indicates the location of the cell soma. Quantification of spine head diameter and spine frequency for each dendritic segment is shown in Table S1.

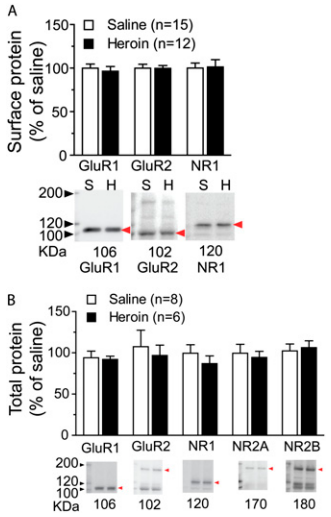

Fig. S3. (*A*) Examples of Western blots from a biotinylation assay showing no significant difference in the surface expression of the GluR1 and GluR2:00 AMPA receptor subunits between heroin-extinguished (H) and yoked-saline (S) animals. Lanes from left to right show protein size marker, saline, and heroin sample, respectively. Red arrowheads indicate the position of the proteins. Pooled data were normalized to the saline values and shown as mean ± SEM $*P < 0.05$, comparing heroin-extinguished to yoked-saline groups using unpaired *t* test. (*B*) Protein levels in whole cell lysates from the NAcore were not different between yoked saline (S) and heroin-trained (H) treatment groups. (*Upper*) Mean ± SEM; (*Lower*) example Western blots (first lane in each contains molecular markers). Black arrowhead indicates the position of the proteins. Red arrowheads indicate the position of the proteins.

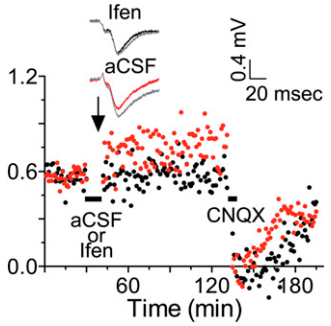

Fig. S4. Experiment examples showing that the long-term potentiation-like increase in fEPSP amplitude elicited by heroin in heroin-extinguished animals is prevented by pretreating the NAcore with the selective NR2B antagonist ifenprodil (Ifen). Bar indicates the aCSF or Ifen infusion, and arrow indicates acute heroin administration. (*Inset*) Sample traces of fEPSP (black or red before aCSF- or Ifen-infusion respectively, gray after infusion).

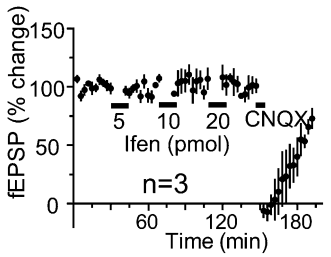

Fig. S5. Ifenprodil had no effect on fEPSP amplitude in control animals. Bar indicates the different dose of Ifen or CNQX infusion into NAcore.

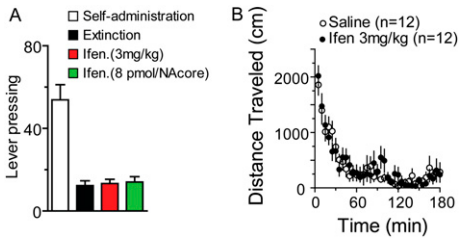

Fig. S6. (*A*) Ifenprodil alone did not change active lever pressing in cocaine-extinguished rats (3 mg/kg, i.p.; or 8 ρmol, microinjection in NAcore per side). (*B*) Locomotor activity is not altered by ifenprodil (3 mg/kg, i.p.). Ifenprodil or saline was administered 5 min before placing a drug-naive animal into an open field photocell chamber and locomotor activity monitored. The animal was not adapted to the chamber before drug administration. No difference in photocell counts was measured between ifenprodil and saline injections.

Table S1. Quantification of spine head diameter and spine frequency for each dendritic segment shown in the micrographs (Fig. S2 *B–F*)

	Spine head diameter frequency (%)					
Example	<0.20 μm	0.35 μm	0.5 μm	0.65 μm	>0.80 μm	Density (Spines/μm)
B	26.3	34.7	27.4	9.5	2.1	2.33
C	27.6	28.7	30.9	11.7	0.0	2.58
D	39.5	40.3	17.1	3.1	0.0	2.30
E	13.3	43.8	31.4	9.5	1.9	2.73
F	31.5	36.9	23.5	6.7	1.3	2.76

The spine head diameter is shown as a frequency distribution binned over 0.15-μm diameters.