Cocaine Use Reverses Striatal Plasticity Produced During Cocaine-Seeking

Supplemental Information

Supplemental Experimental Procedures

Subjects. Male Sprague–Dawley rats (250–300 g, Charles River Laboratories) were maintained on a 12–12 hour reverse light-dark cycle with *ad-libitum* access to food and water prior to beginning operant training. Food was restricted to 25 g/day standard chow 7 days after surgery. All experimental procedures were approved by the Animal Care and Use Committee of the Medical University of South Carolina and performed in accordance with guidelines of the National Institutes of Health.

Surgery and Self-administration. Rats were surgically implanted with intravenous silastic catheters in the right jugular vein under anesthesia with ketamine (87.5 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.). Ketorolac (3 mg/kg, i.p.) was administered prior to surgery and as needed postoperatively to provide analgesia. Prophylactic antibiotic (Cefazolin 10 mg/0.1 ml, i.v.) was administered during surgery. The catheter was secured to the vein with silk sutures and was passed subcutaneously to the middle of the back where it terminated in a connector consisting of a modified 22-gauge cannula (Plastics One, Roanoke, VA) embedded in dental cement attached to surgical mesh (Atrium, Hudson, NH). Rats used in the *in vivo* zymography experiments additionally underwent stereotaxic surgery to deliver cannula to the nucleus accumbens core (Kopf Instruments, Tujunga, CA). Cannulas (23 ga) were implanted bilaterally above the NAcore [anteroposterior (AP), + 1.5 mm; mediolateral (ML), ±2.0 mm; dorsoventral (DV), -5.5 mm] (1). Obdurators were maintained in all cannulas throughout behavioral training to prevent contamination with debris and removed only for experimental procedures.

Catheters were flushed daily with heparin (0.1 mL of 100 IU) until the end of self-administration, and catheter patency was confirmed at the end of each study. Rats were trained to acquire operant responding for food pellets in a single 2-hour session prior to beginning 2-hour daily cocaine self-administration sessions. Rats self-administered cocaine (National Institute of Drug Abuse) for 2 weeks until reaching a criterion of at least 10 days with greater than 10 infusions, followed by 1-2 weeks of extinction training as described previously (2). Training was performed in standard operant chambers containing a house and cue light, tone, and two identical retractable levers (Med Associates, St. Albans, VT). Subjects were trained on a fixed-ratio 1 schedule of reinforcement wherein each active lever press resulted in a cocaine infusion (0.2 mg/0.05 ml) paired with a light and tone (78 dB, 4.5 kHz) and followed by a 20 s timeout period with drug unavailability signaled by the absence of the house light. During extinction training, active lever presses no longer resulted in delivery of cocaine or cues. Extinction was carried out for 7-14 days or until criteria were met wherein the animals averaged fewer than 25 lever presses for the 2 days prior to each reinstatement test. A subset of animals served as yoked-saline controls and received a noncontingent saline infusion paired with light and tone cues according to a pre-programmed pattern of responding based on the cocaine average, but were otherwise treated identically throughout. All behavioral sessions lasted 2 hours and were performed at the same time each day, 6 days/week.

Reinstatement (Drug-seeking/Drug-using). During the first 10 minutes of the session, responses on the active lever resulted in contingent delivery of previously drug-paired light and tone cues without drug delivery akin to a standard cue-induced reinstatement session. After the initial cue period, response-contingent access to cocaine through the catheter was restored for up to 45 minutes under typical self-administration conditions. In the comparison group, intravenous saline was made available. Finally, during the last 65 minutes of the trial both drug infusion and cue delivery ceased and lever pressing resulted in no programmed consequences (Fig 1b).

Calculating Brain Cocaine Concentrations. To estimate the concentration of cocaine in brain, infusions of cocaine were converted to brain concentrations of cocaine using the following equation:

$$c = \frac{\mathrm{d}k}{v(\alpha - \beta)} \left(e^{-\beta t} - e^{-\alpha t}\right)$$

Where d is the dose of cocaine per infusion, *k* the rate constant of transfer between blood and brain (0.223), and *v* the apparent volume of distribution in the brain (0.044). Constants α (0.642) and β (0.097) represent the rate of distribution between blood and brain and the rate of clearance from the body, respectively (3).

Diolostic Labeling. All dendritic spine quantification procedures were similar to those previously described (4). Rats were deeply anesthetized with ketamine HCl (87.5 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) before being transcardially perfused with phosphate buffer (PB) followed by 1.5% paraformaldehyde (PFA) in PB. Brains were removed and post-fixed for 1 hour, then coronally sectioned at 200 μm on a vibratome in phosphate buffered saline (PBS). Tungsten particles (1.3 μm diameter; Bio-Rad, Hercules, CA) were coated with the lipophilic carbocyanine dye DiI (Life Technologies, Grand Island, NY), and these 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 a 3.0 μm pore size (BD Biosciences, San Jose, CA). DiI was allowed to diffuse along neuronal axons and dendrites in PBS for 24 hours at 4° C, and then fixed again in 4% PFA in PBS for 1 hour at room temperature. Tissue was mounted onto slides in aqueous medium Prolong Gold (Life Technologies).

Confocal Imaging and 3D Reconstruction for Dendritic Spine Analysis. DiI-labeled sections were imaged on a confocal microscope (LEICA) using a Helium/Neon 543 nm laser line. Images of labeled neurons and segments were acquired via optical sectioning using a 63x oil immersion objective with pixel size 0.01 μm in the XY-plane and 0.13 μm intervals along the Z-axis. Images

were deconvolved using AutoQuant (Media Cybernetics, Rockville, MD) prior to analysis and a 3D perspective was rendered by the Surpass module of the Imaris software package (Bitplane, Concord, MA). Only spines on dendrites beginning >75 μm from the soma and after the first branch point were quantified on cells localized to the NAcore. The length of quantified segments was 45-55 μm. Three to twelve segments were analyzed from each animal (mean = 7.86 segments per rat) and typically one segment from each neuron. The minimum spine head diameter was set at 0.14 μm.

Slice Preparation for Electrophysiology. Rats were anesthetized with ketamine HCl (87.5 mg/kg, i.p.) and decapitated. 220μm thick coronal sections including NA core were sliced using a VT1200S vibratome (Leica Microsystems, Wetzlar, Germany). Slices were incubated at room temperature in artificial cerebrospinal fluid (aCSF), (in mM: 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.4 NaH_2PO_4, 2.4 $CaCl_2$, 11 glucose, 25 $NaHCO_3$, 0.4 ascorbic acid, 2.0 NaPyruvate, bubbled with 95% O_2 and 5% CO_2). The incubation solution also contained 5 mM kynurenic acid and 50 μM D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) to decrease excitotoxicity.

Whole Cell Patch Clamp Recordings. All recordings were collected at 32°C (controlled by TC-344B, Warner Instrument Corporation, Hamden, Connecticut). Cells were chosen from the dorsomedial NAcore, where the prefrontal inputs are most dense (5). Inhibitory synaptic transmission was blocked with picrotoxin (100 μM). Multiclamp 700B (Axon Instruments, Union City, CA) was used to record excitatory postsynaptic currents (EPSCs) in whole cell patch-clamp configuration. Data were acquired at 10 kHz, and filtered at 2 kHz using AxoGraph X software (AxoGraph Scientific, Sydney, Australia). Glass microelectrodes (1.5-2.5 MΩ) were filled with cesium-based internal solution (in mM: 128 cesium methanesulfonate, 10 HEPES potassium, 1 EGTA, 1 MgCl2, 10 NaCl, 2.0 MgATP, and 0.3 NaGTP, 5 QX-314, pH 7.4). To evoke postsynaptic currents, we placed a bipolar stimulating electrode (FHC, Bowdoin, Maine) ~300 μm dorsomedial

of the recorded cell to maximize chances of stimulating PL afferents. The stimulation intensity was chosen to evoke EPSCs of 200-400 pA while the cell was voltage clamped at -80 mV, which typically represented 30-70% of the maximum possible eEPSC amplitude. Recordings were collected every 20 s and begun >10 min after the cell membrane was ruptured to allow diffusion of the internal solution into the cell. AMPA currents were first measured at −80 mV to ensure stability of response. Then the membrane potential was gradually increased until +40 mV. Recording of currents was resumed 5 min after reaching +40 mV to allow stabilization of cell parameters. Currents composed of both AMPA and NMDA components were then obtained. Then D-AP5 was bath applied (50 μM) to block NMDA currents and recording of AMPA currents at +40 mV was started after 2 min. NMDA currents were obtained by subtracting the AMPA currents from the total current at +40 mV. For all recordings series resistance was measured with a 2 mV hyperpolarizing step (10 ms) given with each stimulus and holding current were monitored online. Recordings with unstable holding current or series resistance, as well as recordings in which series resistance exceeded 20 MΩ were aborted.

***In Vivo* Zymography.** MMP activity was measured using an *in vivo* zymography assay as described previously (6). Rats were microinjected with intramolecularly dye-quenched fluorescein conjugated gelatin (Life Technologies) reconstituted in PBS to 1 mg/ml pH 7.2-7.4 in the NAcore (1.5 μl/hemisphere at a rate of 0.5 μl/min) with a 20 min incubation time in the home cage or the operant environment depending on the experimental group. Proteolytic cleavage by the gelatinases (MMP-2 and MMP-9) results in an activity-dependent increase in green fluorescence that increases linearly with time. After gelatin incubation, rats were overdosed with sodium pentobarbital (100 mg/kg, i.p.) before being transcardially perfused with phosphate buffer (PB) followed by 4.0% paraformaldehyde (PFA) in PB. Brains were removed and post-fixed for 1 hour, then coronally sectioned at 50 μm on a vibratome in phosphate buffered saline (PBS). Sections were mounted in Prolong Gold medium and stored at 4 C until imaged. Images were obtained on a

confocal microscope (LEICA) using a 488 nm Argon laser line, emissions filtered to 515-535 nm through a 10x objective with 0.3 numerical aperture. Only slices in which the injection site and anterior commissure (AC) could be visualized in the same frame were used for quantification. ImageJ (NIH) was used to quantify images. The anterior commissure provided a landmark for the NAcore but was masked to prevent being quantified. In addition the microinjector tract was masked out from the quantification as MMP activity is induced as part of the acute inflammatory response to tissue damage from the introduction of the injector. Fluorescence was quantified as integrated density from 4-6 sections per rat, and the integrated densities were averaged within each rat and normalized to extinction values.

Statistics. Statistics were performed using SAS or Prism (GraphPad Software, La Jolla, CA). Behavioral data were analyzed by a one- or two-way ANOVA as appropriate followed by Tukey *post hoc* tests for multiple comparisons. Spine density and diameter data were analyzed with a two-level nested ANOVA with a Satterthwaite approximation (7). If the nested ANOVA was significant, post hoc evaluations were made over each experiment (time point of analysis) using a one-way ANOVA followed by Dunnett's post hoc for comparison to extinction values. For electrophysiological measurements data were analyzed as individual cells from >4 animals per group using a one-way ANOVA. For zymography measurements fluorescence was averaged from 4-6 coronal sections (100 μm thick) per animal and data were analyzed as animal number using a one-way ANOVA. Significance was set at $p \leq 0.05$ and data are presented as mean ± SEM.

Supplemental Figure

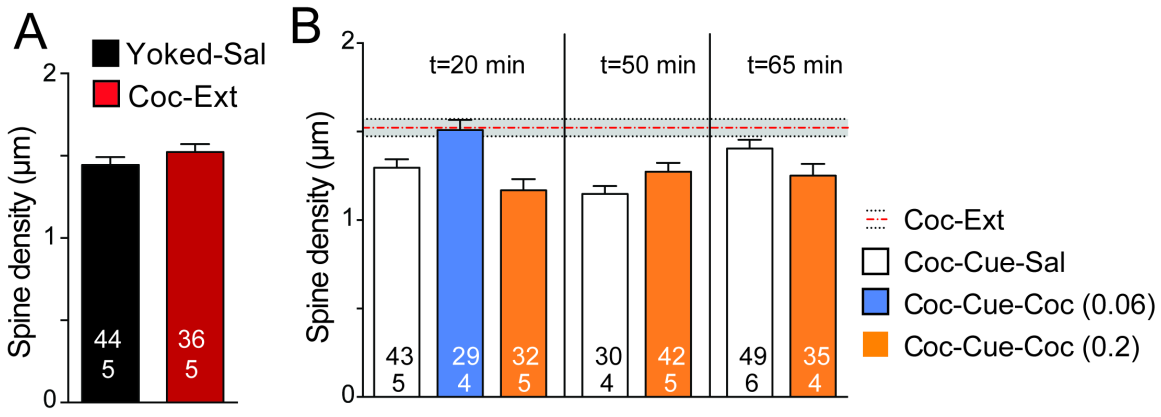

Figure S1. Changes in spine density measured from the same rats where dh was quantified as shown in figure 2. (A) Spine density (mean ± SEM) comparison between yoked saline and extinguished rats shows no effect of earlier cocaine self-administration and extinction training. **(B)** Spine density comparison (mean ± SEM) between all reinstatement treatment groups compared to extinction baseline (dotted red line) demonstrated no overall significant difference between groups (One-way ANOVA $F_{(7,30)}$= 0.864, p= 0.053; Nested ANOVA $F_{(7,29)}$= 1.844, p= 0.117). The number in each bar indicates the number of dendritic segments quantified over the number of animals in each group.

Supplemental References

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