Supplementary Materials and Methods

Subjects

Seventy-five adult male Sprague-Dawley rats (Harlan Laboratories, Inc., St. Louis, MO), approximately 90 days old (325 g) were used for the study. Rats were housed individually in a temperature- and humidity-controlled, AAALAC-accredited animal facility under a 12h/12h reversed light/dark cycle (lights on at 6:00 pm) and had access to food at all times, except when in the experimental chambers. Water was available at all times during the study, including in the experimental chambers. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH.

Catheterization surgery

Rats were implanted with chronic indwelling catheters under ketamine HCl (100 mg/kg, ip, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (2 mg/kg, ip, Lloyd Laboratories, Shenandoah, IA) anesthesia. A silicon tubing catheter (Silastic®; Dow Corning Co., Midland, MI; 0.64 mm i.d.; 1.19 mm o.d.) was inserted into the right posterior facial vein and down into the jugular vein so that it terminated at the right atrium. The catheter was sutured to the vein and continued subcutaneously to the animal's back where it exited 2 cm posterior to the scapula via a back-mounted 22-gauge guide cannula (Plastics One Inc., Roanoke, VA) attached using dental acrylic to a piece of polypropylene monofilament surgical mesh (Atrium Medical, Co., Hudson, NH) to permit connection of a polyethylene delivery line (0.58 mm i.d. x 1.27 mm o.d.; Plastics One Inc., Roanoke, VA) encased in a stainless steel spring leash (Plastics One). The delivery line was connected to a 30-ml syringe in a motor-driven pump (Razel, Stamford, CT) via a leak-proof fluid swivel (Instech Lab. Inc., Plymouth Meeting, PA) suspended above the chamber to

allow drug delivery. The swivel and leash assembly was counter-balanced to permit relatively unrestrained movement. Rats were allowed to recover for at least three days prior to SA testing during which time they were provided acetaminophen (480 mg/L) in their drinking water. After implantation, rats were injected with a sterile cefazolin antibiotic solution (15 mg, iv; West-Ward Pharmaceutical Co., Eatontown, NJ) each day. Catheters were filled daily with a heparin solution (83 i.u./ml) and capped whenever the leash/delivery line assembly was disconnected.

Self-administration apparatus

Twenty operant conditioning chambers encased in sound attenuating cubicles (Med-Associates Inc., St Albans, VT) were used. One retractable lever and stimulus light were mounted on the front wall of the chamber. A second lever and light were located on the back wall. Exhaust fans in the cabinets provided ventilation and white noise to mask extraneous sound.

Self-administration training and testing

Following recovery from surgery, rats were trained to self-administer cocaine (1.0 mg/kg/inf, iv, NIDA, Bethesda, MD) or provided access to saline solution by pressing a lever under a FR1 schedule during daily 2-h sessions, within which the active (i.e., front) lever was extended into the chamber and the corresponding stimulus light was illuminated. Pressing the lever resulted in an iv infusion of drug or saline solution (200µl over 5 s) followed by a 25-s time-out period during which the stimulus light was extinguished but the lever remained extended. Responding on a second, inactive (i.e., back) lever was recorded but had no programmed consequences. Once a stable response pattern was observed during the 2-h sessions (total responding < 10% variation from the mean over 3 consecutive sessions), rats were ready for SA testing. During SA

testing, the duration of daily cocaine/saline access was increased to 6 hrs. Rats were permitted to self-administer under these conditions for 14 days prior to exposure to extinction (Ext), home-cage (Home), or SA box (Box) conditions after self-administration period.

Withdrawal conditions following cocaine self-administration (Post-SA)

Cocaine and saline SA rats were exposed to one of three conditions during post-SA (Extinction, Home, Box). For Extinction rats, the cocaine solution was replaced with saline for ten consecutive 6-h extinction sessions conducted over a 12-14 day period. Aside from saline rather than cocaine availability, these sessions were otherwise identical to the SA sessions. Saline Extinction rats continued to have access to saline during these ten sessions. For the other two conditions rats did not undergo extinction during the 12-14 day post-SA period, but rather remained in their home cages (Home rats) or were introduced into the SA boxes for six hours each day with the levers retracted and stimulus lights extinguished (Box rats).

Cocaine-seeking behavior

Cocaine-seeking behavior was recorded in six Home rats, eight Box rats, and ten Extinction rats. For the Home and Box rats, cocaine seeking was measured during a 6-h session within which lever pressing resulted in saline infusions conducted after a 12-14 day post-SA period during which they remained in their home cages (Home rats) or were introduced into the SA boxes for six hrs each day with the levers retracted and stimulus lights extinguished (Box rats). For Extinction rats, data collected during the final 6-h extinction session (i.e., extinction day 10) were used for comparison. The Home and Box rats in which cocaine seeking was measured were not used for protein determination.

Anatomical dissection and biochemical subcellular fractionation

At the end of the post-SA period, Home, Box, and Extinction rats were decapitated and brains quickly removed from the skull and placed in ice-cold saline for one minute. The tissue was blocked and coronal slices containing areas of interest were cut using a rat brain matrix (ASI, Warren, MI). The medial prefrontal cortex (mPFC) was blocked between plates 6 and 11 (Bregma 5.16-3.00 mm) (paxinos and Watson, 2005). The dorsal and ventral PFC border was set at the middle of prelimbic cortex based on previous anatomical and functional studies (Heidbreder and Groenewegen, 2003) and was hand dissected. The ventral tegmental area was blocked between plates 77-85 (Bregma -5.28-6.24 mm) and was hand dissected. All tissue samples were immediately frozen on dry ice and stored at -80 °C. The membrane preparation and biochemical fractionation were based on methods by Dunah and Standaert (2001) and Toda et al, (2003). A schematic of the membrane purification procedure, adopted from Dunah and Standaert (2001), is presented in Supplementary figure 1. The tissue was homogenized in ice-cold TEVP buffer containing (in mM) 10 Tris-HCl, pH 7.4, 5 NaF, 1 Na_3VO4, 1 EDTA, 1 EGTA, 320 sucrose, using a Dounce glass homogenizer. An aliquot of the whole tissue Dounce homogenate (H fraction) was collected, and the remainder was centrifuged at 1000 g to remove nuclei and large debris (P1). The supernatant (S1) was centrifuged at 10,000 g to obtain a crude synaptosomal fraction (P2) and subsequently was lysed hypo-osmotically for 30 min at 4 °C and centrifuged at 25,000 g to pellet a synaptosomal membrane fraction (LP1), which is enriched in postsynaptic density. All centrifugations were carried out at 4 °C. LP1 pellets were resuspended in TEVP. The H and LP1 fractions were solubilized with the addition of SDS to 1% (v/v) and

used for protein concentration measurement and Western Blot Analysis. All fractions were saved and stored at -80°C.

Western blot analysis

Two independent protein analyses were performed. In the first analysis, all saline-treated animals (n=10 per post-SA treatment group) were compared across post-SA conditions (i.e., 8 saline samples from Post-SA treatment conditions (2-3 samples from each Post-SA treatment) were loaded onto each gel and the mean percent changes compared to Home samples on the same gel were determined). This was done to identify any effects of post-SA conditions that were independent of cocaine exposure. The second analysis compared cocaine-treated animals (n=10) to their corresponding saline control group (n=10) for each post-SA treatment (i.e., 3-4 samples each from saline and cocaine treated animals were loaded onto each gel and the mean percent change resulting from cocaine self-administration was determined based on values for saline samples on the same gel). Due to the large number of samples, each of the three comparisons (10 saline and 10 cocaine for each post-SA treatment) was done separately.

Protein concentration was determined by the Lowry based DC protein assay (Bio-Rad, Hercules, CA). Protein samples were resolved by SDS-PAGE according to the method described by Ghasemzadeh et al., 2003. Briefly, proteins were transferred from the gel to a polyvinyldine fluoride (PVDF) membrane using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Membranes were blocked for 2 hour at room temperature (Tris-buffered saline containing 3% non-fat dry milk) and incubated with commercially obtained primary antibody in antibody buffer (blocking buffer containing Tween-20, 50 μl/100 ml) overnight at 4 °C, washed with antibody buffer, and incubated with HRP-conjugated secondary antibody for 90 minutes at room

temperature. Membranes were washed and immunolabeling was visualized with enhanced chemiluminescence (ECL method). Chemiluminescent images were captured using a Kodak Image Station 4000MM. Band density was measured using Kodak Molecular Imaging Software v4.0. Commercially available antibodies were used to probe for the presence of proteins. The following primary antibodies were used in this study: mouse NMDAR1 (Millipore, Cat. # 05-432, 1:3000 dilution), rabbit GluR1 (Millipore, Cat. # 07-660, 1:10,000 dilution), rabbit mGluR5 (Millipore, Cat. # 06-451, 1:10,000 dilution), mouse PSD95 (Antibodies Inc., Cat. # 75-028, 1:10,000 dilution), mouse PICK1 (Antibodies Inc., Cat. # 75-040, 1:3000 dilution), rabbit Homer1b/c (Santa Cruz Biotech, Cat. # sc-20807, 1:3000 dilution), goat Actin (Santa Cruz Biotech, Cat. # sc-1615, 1:10,000). The following HRP-conjugated secondary antibodies were used: goat anti-rabbit IgG (Millipore, Cat. # 12-348, 1:20,000 dilution), goat anti-mouse IgG (Millipore, Cat. # 12-349, 1:20,000 dilution).

Statistics

The self-administration data (infusions/session) were evaluated using two-way ANOVA with repeated measures over sessions. Responding on test day (infusions/session) was compared using one-way ANOVA comparing the three post-SA treatment groups. In both cases, differences between groups were determined using *post hoc* Fisher's PLSD. For analysis of cocaine self-administration induced alterations in protein levels under each of the three post-SA conditions, immunoreactivity of protein samples from cocaine and saline rats was normalized as percent change relative to the respective mean saline control values for samples on the same gel. Normalized saline and cocaine values were then compared separately for each post-SA condition using Student’s t-tests. To permit comparison of cocaine-induced changes across post-SA

treatment conditions, saline self-administration effects on protein levels determined from a separate protein analysis were initially compared across post-SA conditions using one-way ANOVA followed by the Fisher's PLSD test to identify any effects of post-SA conditions that were independent of cocaine exposure. For this analysis saline self-administration induced protein alterations under each of the post-SA conditions were normalized as percent change relative to the respective mean Home sample saline values determined from the same gel. In cases where there were no differences following saline exposure across post-SA conditions, percent changes in protein following cocaine self-administration were compared across post-SA conditions using one-way ANOVA, followed by *post-hoc* testing using the Fisher's PLSD test. Statistical significance was set at $p < 0.05$.