Withdrawal From Cocaine Self-administration Alters the Regulation of Protein Translation in the Nucleus Accumbens

Supplemental Information

Subjects and drug self-administration

All experimental procedures were approved by the Rosalind Franklin University Institutional Animal Care and Use Committee in accordance with the USPHS Guide for Care and Use of Laboratory Animals. A total of 93 male Sprague-Dawley rats were used (250-275g on arrival; Envigo, Indianapolis, IN). Rats were group housed upon arrival (3/cage) in temperature- and humidity-controlled conditions with a 12-hour reverse light/dark cycle (lights off at 8:00 A.M.) until surgery and then single-housed for the remainder of the experiment. Food and water were available ad libitum. Rats were acclimated to the vivarium for at least 1 week prior to surgeries to implant intra-jugular catheters. As described previously (1), animals were anesthetized using a cocktail of ketamine HCl (80mg/kg) and Xylazine (10mg/kg) (i.p.). A Silastic catheter (PlasticsOne, Roanoke, VA) was inserted and secured in the right jugular vein, and connected to a subcutaneous back mount, exiting the back in the mid-scapular region. Intravenous catheters were flushed daily with cefazolin (0.2mL of 0.1 g/mL in sterile 0.9% saline) to prevent infection and maintain catheter patency. Rats recovered for one week before self-administration training. Rats were trained to nose-poke to self-administer saline or cocaine (FR1 schedule, 0.5 mg/kg/infusion, 6 h/day for 10 days). Infusions were paired with a light cue. Following the final self-administration session, rats were returned to home cages for either 1 or >40 days before conducting experiments. All training occurred during the dark cycle.

Metabolic labeling

A schematic of the experimental timeline is depicted in Supplemental Figure S1. Details of tissue processing are provided in the Methods and Materials section of the main text. Previous

work in hippocampal tissue has demonstrated that several hours of post-slicing recovery is necessary to achieve stable basal levels of protein translation (2). As shown in Supplemental Figure S2, we found that optimal conditions for the measurement of protein translation in NAc tissue required at least 3 hours of post-dissection recovery, in line with this previous work (2). Following recovery, tissue was incubated with ^{35}S-Met/Cys in the presence or absence of test drugs. Based on related studies (2-4), the following drug concentrations were used: cycloheximide (Sigma-Aldrich, St. Louis, MO), 60 µM; LY367385 (Tocris; Bristol, U.K.), 50 µM; 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride (MTEP; Tocris), 25 µM; and APV (Tocris), 50 µM. The vehicle for test drugs was always <1% DMSO.

Autoradiography

Tissue labeled with ^{35}S-Met/Cys was homogenized in ice-cold homogenization buffer containing 1% Triton X-100 and proteins were TCA-precipitated, as previously described (2). A portion of the sample was processed for SDS-PAGE, transferred to nitrocellulose, and exposed to a phosphorimager screen for 24 h before reading with a phosphorimager (Typhoon; GE Healthcare, Little Chalfont, UK). The same membrane was then stained with Ponceau S to visualize total protein in each lane. Optical density was analyzed using TotalLab Quant software; a ratio of ^{35}S-incorporation to total protein was calculated for each lane and used as a measure of overall translation in each sample.

Synaptoneurosome preparation

After 55 days of withdrawal from saline or cocaine self-administration, rats were decapitated and the NAc was dissected from 2 mm coronal slices prepared with a brain matrix. Immediately following dissection, synaptoneurosomes were prepared as previously described (5), with slight modifications. Briefly, NAc punches were homogenized in 500 µL of homogenization buffer [HB; 20 mM HEPES, 0.5 mM EGTA, 1X Preasome Inhibitor Cocktail Set 1 (Calbiochem; Temecula,

CA)]. Homogenates were passed through a 100 µm-pore filter and then through a 5 µm filter (Millipore). After homogenates were passed through each filter, the filters were washed with 50 µL of HB and the washes were added to the homogenates to maximize yield. Homogenates were centrifuged at 14,000 x g for 20 minutes at 4^{o}C. The pellet containing the synaptoneurosomes was frozen on dry ice, stored at -80^{o}C, and finally lysed in a lysis buffer [0.605 g Tris-HCl, 0.25 g sodium deoxycholate, 0.876 g NaCl, 1 µg/mL PMSF, 5 mL 20% SDS, 1X Protease Inhibitor Cocktail Set 1 (Calbiochem) in 100 mL dH_2O] for immunoblotting.

Whole cell RNA extraction, reverse transcription, and quantitative (q)PCR

RNA was extracted using TRI Reagent and the corresponding protocol (Invitrogen, Waltham, MA). Briefly, freshly dissected NAc tissue was homogenized in 50 µl TRI Reagent, brought up to 400 µL with TRI Reagent and incubated for 5 min at room temperature (RT). Homogenates were then centrifuged at 12,000 x g for 10 min at 4°C and aqueous supernatants transferred to new tubes. Next, 50 µL of bromochloropropane (BCP) was added. Samples were shaken vigorously, incubated for 5 min at RT and centrifuged at 12,000 x g for 15 min at 4^{o}C. About 75% of the clear, aqueous RNA phase was transferred into a clean tube. Then, 150 µL of nuclease free water and 50 µL BCP were added. Samples were shaken vigorously, incubated for 10 min at RT and centrifuged at 12,000 x g for 15 min at 4°C. About 75% of the aqueous RNA layer was transferred to a new tube and 3M sodium acetate (1:10) and 1 µL glycogen were added and mixed. For RNA precipitation, isopropanol (1:1) was then added and samples were incubated overnight at -20°C. The next day, samples were centrifuged at 12,000 x g for 10 min at 4°C. After discarding the supernatant, the RNA pellet was washed with 70% ethanol and centrifuged at 7,500 x g for 5 min at 4°C. Ethanol was removed and pellets were allowed to air-dry for 5 min. RNA pellets were then dissolved in 50 µL nuclease free water and stored at -80°C. Reverse transcription of RNA was completed using the commercially available SuperScript III Reverse Transcriptase kit (Invitrogen). Manufacturer instructions were followed.

qPCR assays were performed using Fam-labeled probes for target genes and a Vic-labeled probe for the housekeeping control gene (GAPDH), along with TaqMan Gene Expression Master Mix (Applied Biosystems). qPCR reactions were run in a ViiA7 instrument (Applied Biosystems). Samples were run in duplicate and relative expression was calculated using the ΔΔCt with GAPDH as the control. See Supplemental Table S1 for mRNA primer sequences.

Synaptoneurosome RNA extraction and qPCR

Synaptoneurosome RNA was extracted and precipitated as above but smaller volumes were used for processing. Reverse transcription of synaptoneurosome RNA was completed using the commercially available SuperScript III Reverse Transcriptase kit (Invitrogen). Manufacturer instructions were followed.

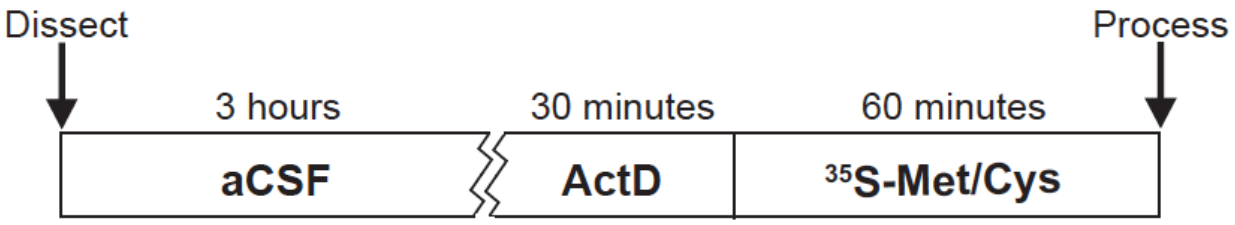
TaqMan PreAmp Master Mix Kit (Applied Biosystems, Waltham, MA) was used for cDNA pre-amplification. The PreAmp Reaction was made up as follows: synaptoneurosome cDNA (6 µL), primer sets (1.5 µL each), and PreAmp Mix (15 µL). The final volume was brought to 30 µL with nuclease free water. cDNA was pre-amplified in a Mastercycler Gradient Thermal Cycler (Ependorf, Hauppauge, NY) using the following program settings: 95ºC hold for 10 min, 14 denaturation cycles at 90ºC for 15 sec, and annealing and extension at 60ºC for 4 min. The pre-amplification products were then diluted 5X with nuclease free water and stored at -20ºC. Uniformity of the pre-amplification was verified by comparing cDNA templates from the pre-amplified and unamplified NAc samples. ΔΔCt values were within the range of ±1.5 for all target genes between the pre-amplified and unamplified samples. qPCR assays were performed as described in the previous section but using actin as the housekeeping control gene.

Immunoprecipitation

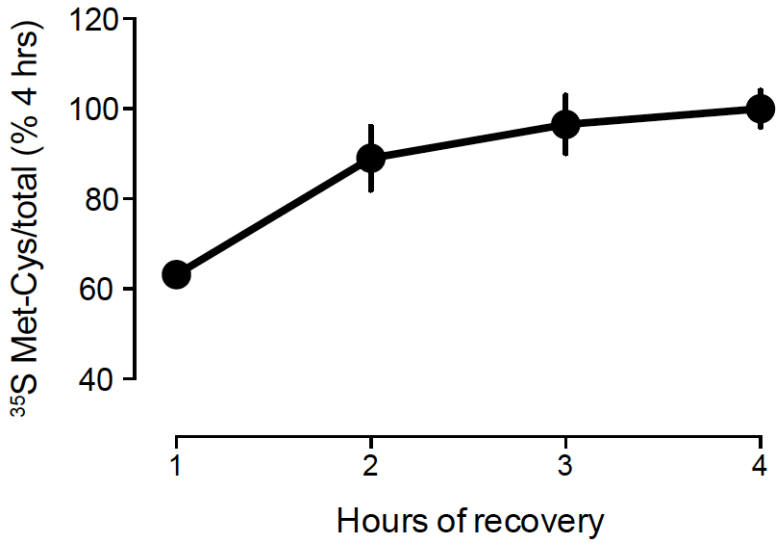
Using immunoprecipitation methods previously described (1, 6), we quantified levels of GluA1 and GluA2 AMPAR subunits that had incorporated either ^{35}S-Met/Cys or puromycin. Briefly, rats were decapitated, brains rapidly removed, and NAc (mainly core) dissected on ice from a 2 mm coronal section obtained using a brain matrix. Membranes were lysed by sonication and sedimented by centrifugation at 23,000 x g for 2 min at 4°C. The pellet was solubilized with 1% Triton X-100 in 10 mM HEPES pH 7.4 containing 2 mM EDTA, 2 mM EGTA, protease and phosphatase inhibitors I and II (Calbiochem; Temecula, CA) for 45 minutes at 37°C. Insoluble material was removed by centrifugation at 23,000 x g for 2 min at 4°C. The supernatant was stored at -80°C until use. For immunoprecipitation, 3 µg of antibody (GluA1, Abcam, #ab109450 or Millipore, #MAB2263; GluA2, NeuroMab, #75-002; puromycin, Millipore, #MABE343 or Kerafast, #3RH11) or an equal amount of control IgG was incubated with 20 µL of A/G agarose slurry (Pierce, Rockford, IL) for 4 h at 4°C. The pellet was collected by centrifugation at 3000 x g for 1 min and washed 3 times with TBS plus 0.1% Tween 20. 100 µL of membrane prep was incubated with the washed pellet overnight at 4°C. The agarose bound antibody was pelleted by centrifugation at 3000 x g for 1 min. 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. After the final immunoprecipitation, the unbound fraction was mixed with an equal volume of sample treatment buffer (Invitrogen, Carlesbad, CA) and heated to 100°C for 4 min. For Western analysis, samples were run on a 4-12% Bis-Tris gel (Invitrogen) and transferred to PVDF membranes for GluA1 and GluA2 immunoblotting. Band densities were determined using ImageQuant software.

Statistical analysis

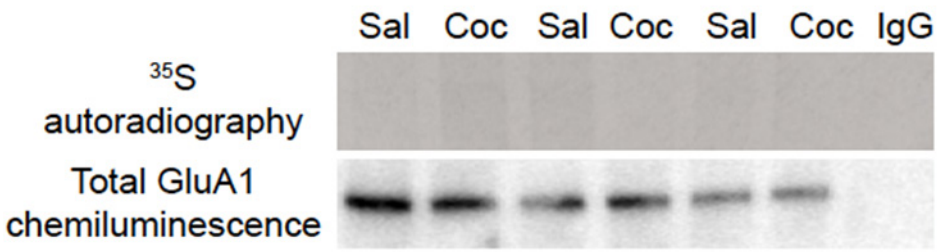
For rats in metabolic labeling and puromycin experiments (Figures 1-3), the NAc from one hemisphere was used for the control group, the NAc from the other hemisphere was used for the experimental group, and data were analyzed using a paired t-test. For puromycin immunoprecipitation experiments and qPCR experiments (Figures 4-6), both hemispheres were pooled in order to yield enough sample to immunoprecipitate or quantify mRNA for GluA1 and GluA2. These samples were analyzed either with an unpaired t-test or ANOVA with Tukey's post-hoc test, as appropriate.



Supplementary Figure S1. Schematic of the experimental timeline for ^{35}S-Met/Cys labeling. Freshly dissected NAc tissue was allowed 3 hours of post-dissection recovery in net-wells containing oxygenated aCSF, based on data shown in Supplemental Figure 2. Tissue was then transferred to a different net-well containing ^{35}S-Met/Cys for 60 minutes. The transcription inhibitor actinomycin D (25µM) was present during the last 30 min of the aCSF incubation and throughout the ^{35}S-Met/Cys incubation. Tissue was then processed for SDS-PAGE, immunoblotting and autoradiography as described in the Methods and Materials section of the main text and in Supplemental Methods (above).



Supplementary Figure S2. Three hours of recovery after NAc tissue preparation yields maximal and stable ^{35}S-Met/Cys labeling. Freshly dissected NAc tissue was incubated for varying times (1-4 hours) in oxygenated aCSF, with addition of the transcription inhibitor actinomycin D for the last 30 min of the incubation. Tissue was then processed for SDS-PAGE, immunoblotting and autoradiography as described in the Methods and Materials section of the main text and in Supplemental Methods (above). Samples were run in triplicate. Optical density of the entire lane was measured for each sample, and results normalized to total protein in the lane measured with Ponceau S staining. Data are presented as mean ± SEM, with the 4 hour value set as 100%.



Supplementary Figure S3. Incubation of NAc tissue with ^{35}S-Met/Cys does not result in sufficient incorporation of isotope to quantify newly translated GluA1 protein. NAc tissue was dissected from rats that self-administered saline or cocaine (6 h/day for 10 days) and then underwent >40 days of withdrawal. Tissue was incubated with ^{35}S-Met/Cys (10 µCi/mL) as described in the Methods and Materials section of the main text in order to tag newly translated proteins. Then, GluA1 was immunoprecipitated as described in Supplemental Methods. Near quantitative immunoprecipitation of GluA1 was achieved, i.e., GluA1 levels in the bound fraction were ~95% of those detected in an equal amount of starting material (data not shown). Immunoprecipitated material was processed for SDS-PAGE and immunoblotted for GluA1 (bottom images, labeled "Total GluA1 chemiluminescence"). Specificity of the immunoprecipitation was demonstrated by comparing tissue from saline and cocaine rats immunoprecipitated with GluA1 antibody (6 lanes on the left) to tissue immunoprecipitated with control IgG (right-hand lane). The same immunoblot was exposed to a phosphorimager screen for >3 weeks, with the goal of detecting newly translated GluA1 (top images, labeled "^{35}S autoradiography"). Despite robust GluA1 levels in all lanes as revealed by immunoblotting, autoradiography failed to detect a quantifiable band at the molecular weight where GluA1 should migrate, even after exposure to a phosphorimager screen for >3 weeks, although by eye we could observe an extremely faint band at ~100kD. These results indicate that only a very small portion of the GluA1 pool is newly translated, as expected, and that ^{35}S-Met/Cys labeling therefore does not result in sufficient isotope incorporation into GluA1 to compare rates of GluA1 translation between experimental groups. In parallel experiments not shown, NAc tissue was labeled with puromycin, GluA1 was immunoprecipitated, and the immunoprecipitated material was immunoblotted with puromycin antibody to detect newly translated GluA1. Here we failed to detect any signal in the immunoblot. We speculate that a faint band was detectable after ^{35}S-labeling but not after puromycin labeling because puromycin labeling likely reduces the efficiency of the GluA1 pull-down by modifying the protein whereas incorporation of ^{35}S into the GluA1 protein does not. Regardless of the labeling method, however, results demonstrate that pull-down with GluA1 antibody followed by autoradiography (^{35}S) or immunoblotting (puromycin)

does not yield a quantifiable measure of newly translated GluA1 protein. Therefore, for experiments shown in Fig. 4 of the main text, we took the different strategy of labeling with puromycin, immunoprecipitating all newly translated proteins with puromycin, and then immunoblotting for GluA1 (or GluA2).

Supplementary Table S1: List of qPCR probes and primers

Protein	Gene	TaqMan probe[a]
GluA1	gria1	Rn00709588_m1
GluA2	gria2	Rn00568514_m1
GluA3[b]	gria3	Rn00583547_m1
GAPDH	Gapdh	Rn01775763_g1
Actin	Actb	Rn00667869_m1

[a]TaqMan catalog number for ordering from Applied Biosystems. [b]Similar to results described in the main text for *Gria2*, *Gria3* mRNA levels in homogenates prepared from NAc core or shell did not differ between saline controls and cocaine "incubated" rats (core: t_{16}=0.06, p=0.95; shell: t_{18}=0.14, p=0.89), whereas a trend towards a reduction in the cocaine group was found in synaptoneurosomes prepared from the entire NAc (t_{20}=1.60, p=0.13).

Supplemental References

1. Loweth JA, Scheyer AF, Milovanovic M, LaCrosse AL, Flores-Barrera E, Werner CT, et al. (2014): Synaptic depression via mGluR1 positive allosteric modulation suppresses cue-induced cocaine craving. *Nature neuroscience*. 17:73-80.
2. Osterweil EK, Krueger DD, Reinhold K, Bear MF (2010): Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 30:15616-15627.
3. Sutton MA, Ito HT, Cressy P, Kempf C, Woo JC, Schuman EM (2006): Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell*. 125:785-799.
4. Scheyer AF, Wolf ME, Tseng KY (2014): A protein synthesis-dependent mechanism sustains calcium-permeable AMPA receptor transmission in nucleus accumbens synapses during withdrawal from cocaine self-administration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 34:3095-3100.
5. Most D, Leiter C, Blednov YA, Harris RA, Mayfield RD (2016): Synaptic microRNAs Coordinately Regulate Synaptic mRNAs: Perturbation by Chronic Alcohol Consumption. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 41:538-548.
6. Ferrario CR, Loweth JA, Milovanovic M, Wang X, Wolf ME (2011): Distribution of AMPA receptor subunits and TARPs in synaptic and extrasynaptic membranes of the adult rat nucleus accumbens. *Neuroscience letters*. 490:180-184.