Supplement 1

Materials and Methods

Subjects: Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN, USA) arrived in the laboratory at 21 days of age. All animals had *ad libitum* access to food and water, except during periods of restricted access to food as described below. The animal colony was on a 14:10 hr light cycle, with lights on at 6:00 am. All experiments were performed during the light phase of the cycle. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky or University of Texas Southwestern Medical Center and were in compliance with NIH's *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington D.C., USA, 1996).

Environmental Conditions: EC rats were housed in large cages (60 X 120 X 45 cm) with toys, while IC rats were housed alone in small cages (17 X 24 X 20 cm). With the exception of sucrose experiments and social behavior, group-housed rats (EC and SC) were not separated before the behavioral experiments. Multiple experiments were performed on rats; however, appetitive and anxiety tests preceded stress and drug tests. Further, stress and drug tests were not performed in the same animals. For EC and IC rats, a single group of rats was used for all operant tests. A separate group of rats was used for anxiety and depression tests (EPM followed by sucrose neophobia, then locomotor activity, sucrose preference, social behavior, anxiety-induced defecation, and finally

FST). A third group was used for CPP. A fourth group was used for protein analysis and a fifth group for mRNA.

Conditioned Place Preference Conditioning Apparatus: CPP was measured in a standard 3 chamber apparatus, with the two conditioning chambers measuring 15 cm X 25 cm X 35 cm. One chamber had gray walls while the other chamber had vertical alternating white and black stripes 2.5 cm wide. Floors were stainless steel wire mesh of differing mesh size to provide differential tactile cues. The center compartment measured 15 cm by 10 cm with white walls and a steel rod floor. Infrared photocells detected position and movement of the rats in the apparatus.

Conditioned Place Preference: On the day before conditioning, rats were allowed access to all three compartments for 20 min to test for initial bias. Animals with an initial bias over 300 sec were not used for the experiment. For conditioning sessions, rats were injected with cocaine or saline and confined to the appropriate chamber for 30 min. This occurred over 8 days, during which time drug was administered on alternating days with saline, for a total of four conditioning sessions each. For the final test session, rats were allowed free access to the three compartments and preference was determined as time spent in the drug compartment minus time spent in the saline compartment.

Operant Conditioning Chamber: Operant responding was tested in a standard two-lever operant conditioning chamber (ENV-007; Med Associates, St Albans, VT, USA). The chamber measured 28 X 21 X 21 cm with a metal rod floor. One panel of the chamber contained a response lever on each side of a recessed food tray. Cue lights above each lever were used to signal infusions. A personal computer using the Med-PC software package (Med Associates) recorded responses and delivered infusions via a Razel Model A infusion pump with a 3⅓ rpm motor.

Responding for Sucrose: Rats were maintained on restricted access to food over a period of 6 days to reach body weights of approximately 85% of their free-feed body weights. EC rats were separated from cohorts during the feeding period (60 min/day). On the fifth day of restricted food access, rats were exposed to sucrose pellets (Noyes; 45 mg) to alleviate neophobia during training. At 57 days of age, rats were placed in the operant conditioning chamber and given approximately 40 non-contingent sucrose pellets over a 20-min period to orient them to the food tray. Only one lever (the “active” lever) was available in the chamber during this training and each response on this lever was reinforced with a sucrose pellet. The position of the active lever was counterbalanced among rats and experimental conditions. On the next day, each rat was placed in the chamber, with only the active lever available, and shaped to respond on this lever for sucrose pellets under a continuous schedule of reinforcement until the rat earned 100 pellets.

Beginning on the next day, operant responding was assessed in EC and IC rats under an intermittent schedule of reinforcement. For each test session, both levers were available (the second lever had no programmed consequence) during a 15-min session where the rats could earn pellets. Rats were first assessed under a fixed ratio 2 (FR2) schedule of reinforcement; the FR schedule was incremented daily until an FR5 session was completed.

After regaining 100% of free-feed body weight (~10 days), rats were again assessed for responding for sucrose pellets. For three consecutive daily sessions, rats were allowed to respond for sucrose pellets for 15 min under an FR5 schedule of reinforcement.

Intravenous Catheter Implantation: After the sucrose-reinforcement phase of the experiment was completed, rats were anesthetized with ketamine (80 mg/kg, IP) and diazepam (5 mg/kg, IP), and implanted with an indwelling jugular catheter. The Silastic catheter (0.2 mm i.d.; Fisher Scientific, Pittsburgh, PA, USA) passed under the skin to exit from a piece of stainless-steel hypodermic tubing (22 ga) embedded in a cap of dental acrylic affixed to the top of the skull with four stainless-steel jeweler's screws. To prevent EC rats from chewing on the plastic tubing end-pieces of the catheters of cohorts in the home cage, a threaded aluminum standoff (Y-S6-6; Small Parts, Miami Lakes, FL, USA) was used to protect the plastic end-piece. The aluminum standoff was connected to the head-mount via a threaded nylon set screw (Y-SSN-632-6; Small Parts) with a hole drilled along the axis to allow for the exiting hypodermic tubing. Catheters were

flushed daily with 2 mg/ml heparinized saline (0.1 ml/day) to maintain patency. Rats were allowed 7 days to recover before commencement of cocaine self-administration.

Acquisition of Cocaine Self-Administration: Regardless of the unit dose, each infusion was delivered intravenously in a volume of 0.06 ml over 3.55 sec. The infusion was signaled with illumination of both cue lights for 20 sec. Responses within the 20-sec signaled timeout were not reinforced. The active lever for cocaine self-administration was the same as in the sucrose training sessions. Acquisition was assessed for 60 min daily for a total of 5 consecutive days.

Dose Response Functions for Cocaine Self-Administration: Initial infusions were delivered over 7.1 sec in a volume of 0.12 ml per infusion. A 60-sec timeout period, signaled by illumination of both cue lights, occurred with the onset of each infusion. The signaled timeout period was halved with each successive dose. However, after the fifth dose, the infusion duration was too short to deliver consistent infusions (0.22 sec); thus, the infusion syringe was replaced with another syringe containing a lower concentration of cocaine, such that a 3.55 sec infusion delivered the appropriate unit dose (0.015 mg/kg/infusion). Accordingly, both the infusion duration and signaled timeout were adjusted to 3.55 sec. Rats were tested in this manner for at least 7 sessions and until responding stabilized. Stable responding was achieved when rats exhibited peak responding at the

same unit dose across 3 consecutive sessions. Data from these 3 sessions were averaged to yield one value for each unit dose per rat.

Extinction of Cocaine Self-Administration: After the dose-response phase, all rats were allowed to stabilize responding for 0.5 mg/kg/infusion cocaine for a minimum of 3 sessions. Infusions during extinction sessions were administered in a volume of 0.12 ml over 7.1 sec. Once responding stabilized (less than 20% variability in the number of infusions, greater than a 2:1 ratio of active:inactive responses and at least 10 infusions per session across 3 consecutive sessions), extinction of cocaine self-administration was assessed for an additional 3 sessions. Similar to responding during the first 60 min, responding during the extinction period resulted in a 60-sec signaled timeout; however, the infusion pump did not deliver cocaine during the extinction period. Extinction was assessed separately with and without cue-light illumination.

Reinstatement of Cocaine Seeking: Infusions during the first 60 min were given across 7.1 sec, yielding a volume of 0.12 ml per infusion; a 60-sec timeout period was signaled by illumination of both cue lights. To ensure that responding was reliably extinguished for each rat, the reinstatement period was not initiated until at least 10 min had passed without a response. The reinstatement period began with an IP injection of cocaine (1.25, 2.5, 5, 10 or 20 mg/kg) or saline; the dose order was randomized among rats. During reinstatement, which was assessed

daily, a 30-sec signaled timeout period followed each response on the active lever.

Tests of Anxiety-Related Behavior: Anxiety-like behavior in the elevated plus maze was measured according to published procedures (1). The arms of the elevated plus maze measured 12 cm X 50 cm and the maze was 1 m from the floor.

Spontaneous Locomotor Activity: Activity was measured in a circular corridor (10 cm wide, 60 cm in diameter and 30 cm high; Med-Associates) via 4 photoelectric cells located every 90° along the circle as described previously (2). For spontaneous locomotor activity, rats were placed in circular corridor chambers and total activity was measured for 120 min.

Tests of Depression-Related Behavior: For sucrose preference testing, rats were first isolated at 5:00 pm with food but no water. When the lights went out at 7:00 pm, pre-weighed water and 1% sucrose bottles were placed on the home cage and rats were allowed to drink for 10 min.

For social interaction testing, rats were placed in novel square chambers (50 cm X 50 cm) and behavior was videotaped and later scored for 30 min. Pairs of rats were scored together using an observer-based scoring system (Noldus Observer, Netherlands). Durations of social grooming (one rat grooming the other), exploring, playing, and resting were coded as state variables. This

paradigm of short-term isolation before measuring social behavior has been shown to be sensitive to dopamine and opioid manipulations (3). To increase the fidelity of the data, EC rats were paired with EC rats and IC with IC.

The forced swim test was carried out according to published procedures (4). Rats were placed in 25° C water in tubs (60 cm tall filled to 45 cm) for 15 min for the initial session and 5 min for the subsequent test session.

Comparison of IC and SC Rats: The methods for these experiments were identical to those described above.

Analysis of pCREB in EC and IC Rats: Nucleus accumbens tissue was dissected from drug- and stress-naïve rats and placed into 250 μL of homogenization buffer containing 320 mM sucrose, 5 nM Hepes buffer, 50 mM NaF, 1% SDS, phosphatase inhibitor cocktails I and II (Sigma, P-2850; St. Louis, MO, USA) and protease inhibitors (Sigma, P-8340). Tissue was sonicated for 5 sec and boiled for 5 min to denature proteases. Protein concentrations were determined by using a Bio-Rad D_C protein assay and 50 μg protein was loaded onto a 10% Tris polyacrylamide gel. The protein was transferred to a PVDF membrane. Antibodies were dissolved in 5% powdered milk. Blots were exposed to Biomax film using a chemiluminescent detection system (Pierce Dura West, Rockford, IL, USA). A rabbit polyclonal anti-pCREB (Abcam cat. ab30651; Cambridge, MA, USA) was used at a concentration of 1:500. A Chemicon anti-CREB (ab3006; Millipore, Billerica, MA, USA) was used at 1:1000 to detect total CREB levels.

Subsequent BDNF blots utilized a Santa Cruz (sc546; Santa Cruz, CA, USA) rabbit polyclonal antibody at 1:1000.

Analysis of BDNF mRNA Levels: Rats were killed by rapid decapitation, nucleus accumbens was dissected, and mRNA was isolated using the RNA Stat-60 reagent (Teltest, Houston, TX, USA) according to manufacturer's directions. Contaminating DNA was removed with DNAse treatment (DNA-Free, cat# 1906; Ambion, Austin, TX, USA). Purified RNA was reverse-transcribed into cDNA (Superscript First Strand Synthesis, Invitrogen; cat# 12371-019; Carlsbad, CA, USA). BDNF was quantified using quantitative real-time PCR (SYBR Green; Applied Biosystems, Foster City, CA, USA) on a Stratagene Mx3000p 96-well thermocycler (La Jolla, CA, USA). PCR data were normalized to GAPDH levels. Primers for rat BDNF were: 5'-TCATACTTCGGTTGCATGAAGG-3' (forward) and 5'-AGACCTCTCGAACCTGCCC-3' (reverse).

Construction and Packaging of AAV (adeno-associated virus)-shRNA to CREB: The pACP plasmid contains AAV2 inverted terminal repeats flanking a CMV promoter, a multiple cloning site, and an intron and polyadenylation signal derived from SV40. The enhanced green fluorescent protein (eGFP) cDNA was ligated into pACP to generate EGFP-pACP. To obtain each AAV-shRNA, synthetic oligos encoding the shRNA and its respective complement (Integrated DNA Technologies, Coralville, IA) were annealed and ligated into unique *Bbs*I and *XbaI* sites in the mU6pro plasmid (a gift from D. L. Turner). Each shRNA

encoded a sense, loop, antisense and adenosine terminator. The U6-shRNA cassette was then excised from the mU6pro vector with *Sal*I and *Xba*I and ligated into EGFP-pACP downstream of the SV40 sequence. Packaging of the AAV was carried out using a standard triple transfection protocol to generate helper virus-free pseudotyped AAV2/10 virus (5). An AAV2/10 rep/cap plasmid provided AAV2 replicase and AAV10 capsid functions (6,7), while adenoviral helper functions were supplied by pHelper (Stratagene). Briefly, AAV-293 cells were transfected with 1.33 pmol of pHelper, and 1.15 pmol each of AAV2/10 and one of the AAV vector plasmids, via calcium phosphate precipitation. The cells were harvested 48 hours later and the pellets resuspended in DMEM, freeze-thawed three times and centrifuged to produce clarified viral lysate. The vector stocks were titered by real-time PCR using the ABI Prism 7700 Sequence Detection System from Perkin-Elmer Applied Biosystems as previously described (8). The average titer of the preparations was approximately 1 x 10^{12} DRP/ml.

Immunohistochemistry: Viral expression and CREB protein knockdown were verified using immunohistochemistry for GFP and CREB. Brains were perfused with saline and 4% paraformaldehyde. Brains were post fixed overnight before being cryoprotected with glycerol. Brains were sliced at 40 um. Sections were washed with phosphate-buffered saline and then blocked with normal donkey serum. GFP was detected using a chicken anti-GFP polyclonal antibody (Aves #gfp1020; Tigard, OR, USA), whereas CREB was detected with a rabbit anti-

CREB (Chemicon #ab3006). GFP was resolved with a CY2-conjugated donkey anti-chicken and CREB with a CY3 donkey anti-rabbit.

Stereotaxic Surgery: AAV vectors were injected bilaterally (0.7 μl/side in 7 min) into the nucleus accumbens shell using coordinates from Paxinos and Watson (9; AP = 1.7, L = 2.4, D = -6.7 mm from bregma, 10° lateral angle) according to published procedures (4, 10). AAV expression does not reach its peak until 2 – 4 weeks after surgery. Thus, rats were allowed 21 days before behavioral testing to allow for vector expression. Accuracy of all stereotaxic injections was determined after the behavioral experiments and animals with injection sites outside the nucleus accumbens shell (<5% of all animals studied) were not used for analysis. Control rats were injected with a scrambled shRNA-expressing vector where the sequence did not match any known mammalian gene. Rats were approximately 275 g at time of surgery.

RNAi Behavioral Experiments: The purpose of these studies was to examine the ability of decreased CREB activity in the nucleus accumbens to mimic the EC phenotype independent of environment. Thus, all RNAi experiments were performed in pair-housed rats. Rats were tested in several behavioral paradigms including locomotor activity, cold-stress induced defecation, sucrose neophobia, EPM, sucrose preference and social behavior. Rats were subjected to multiple different behavioral paradigms according to a standard algorithm where anxiety and appetitive tests preceded stress tests.

Statistical Analyses: Data for all experiments were studied with analyses of variance (ANOVAs). For environmental experiments, the Environment factor was a between-subject factor, while the Session or Dose factors (where appropriate) were within-subject repeated-measures factors. Planned comparisons were used to compare individual sessions or doses among environmental conditions when appropriate. Bonferroni corrections were applied to multiple testing. Data were considered significant at $P < 0.05$. Occasionally, previously published research provided directional hypotheses allowing a one-tailed test. These conditions are described for each case.

Results

Conditioned Place Preference in EC and IC Rats: A two factor ANOVA revealed a significant main effect of Drug on conditioned place preference [$F(1,38) = 9.9$, $P < 0.005$] and the planned comparison revealed that EC rats showed greater cocaine conditioned place preference than IC rats [$F(1,25) = 4.6$, $P < 0.05$; Fig. 1 A].

Responding for Sucrose Pellets in EC and IC Rats: A 3-factor ANOVA (Environment X Session X Squad) for responding by EC and IC rats at 85% of free-feed body weight for sucrose pellets under FR2 – FR5 schedules of reinforcement revealed that EC rats responded more for sucrose than IC rats [main effect of Environment; $F(1,20) = 3.0$, $P < 0.05$; one-tailed test; data not shown]. Planned comparisons revealed that EC rats emitted significantly more responses than IC rats during the FR2 and FR5 sessions (data not shown).

Dose Response Functions for Cocaine Self-Administration in EC and IC Rats: In addition to the significant interaction, the 3-factor ANOVA (Environment X Dose X Squad) performed on the dose response data from EC and IC rats revealed a significant Dose main effect [$F(7,112) = 17.9$, $P < 0.001$]. Planned comparisons confirmed that EC rats responded significantly less than IC rats for the 0.03, 0.015 and 0.0075 mg/kg/infusion doses.

When assessing total drug intake, an ANOVA revealed main effects of Dose [$F(7,112) = 419.6$, $P < 0.001$] and Enrichment [$F(1, 18) = 8.53$, $P < 0.01$] in addition to the significant interaction.

Extinction of Cocaine Self-Administration in EC and IC Rats: In addition to the main effect of Environment, the 3-factor ANOVA (Environment X Session X Squad) of extinction with light cue also revealed a main effect of Session, meaning that both EC and IC rats decreased responding across extinction sessions [$F(2,36) = 27.5$, $P < 0.001$]. There was also a significant Environment X Session interaction [$F(2,36) = 4.6$, $P < 0.05$], which showed that the difference between EC and IC rats decreased across sessions. However, planned comparisons showed that EC rats had lower response rates than IC rats during all 3 extinction sessions (Fig. 1E). In contrast, there were no significant differences in responding during extinction when no cue light was present (data not shown).

Cocaine-Induced Reinstatement of Cocaine Seeking in EC and IC Rats: A 3-factor ANOVA (Environment X Dose X Squad) revealed that IP cocaine produced a dose-dependent reinstatement of cocaine responding [$F(5,75) = 20.0$, $P < 0.001$] in addition to the Environment main effect.

Spontaneous Locomotor Activity in EC and IC Rats: In addition to the main effect of Environment, the two-factor ANOVA also revealed a main effect of Time

[F(8,176) = 127.4, P < 0.001] and a Time X Environment interaction [F(8,176) = 5.4, P < 0.001].

CREB RNAi Spontaneous Locomotor Activity: A two-factor ANOVA revealed only a main effect of Time [F(8,176) = 140.1, P < 0.001; Fig. 7 A], suggesting that the CREB RNAi alone was insufficient to alter spontaneous locomotor activity.

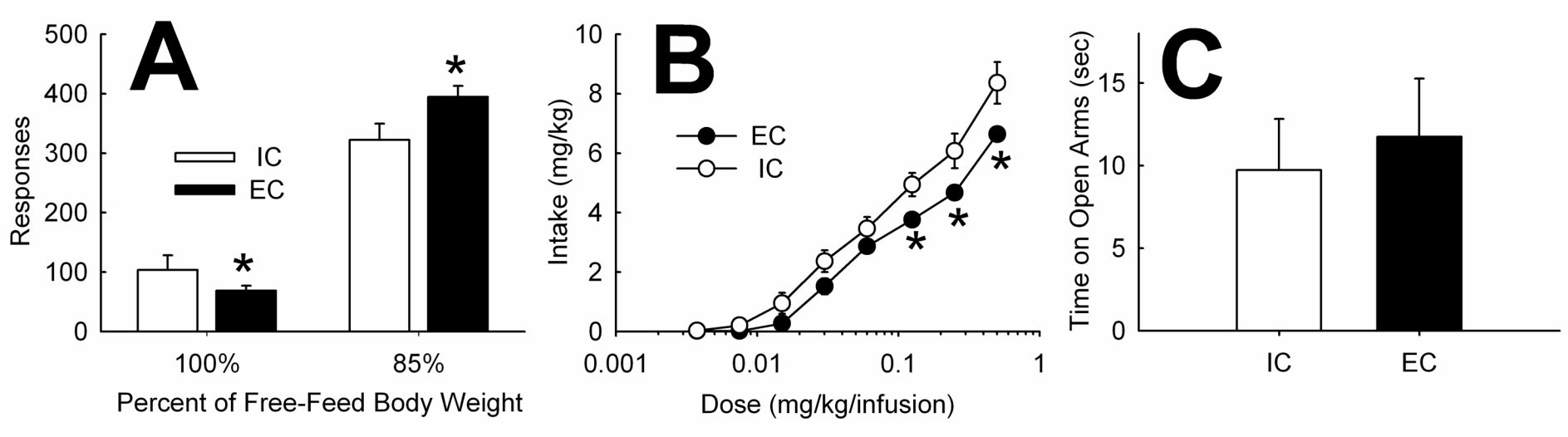

Figure S1: Effects of environmental enrichment on behavior. (**A**) Effect of enrichment on operant sucrose responding under conditions of low (100% free feed body weight) or high (85% free feed body weight) hunger motivation. Points represent mean (± SEM) number of responses by EC and IC rats for sucrose pellets under an FR5 schedule of reinforcement. * significant difference from IC of same feeding condition. (**B**) Effect of enrichment on cocaine self-administration dose-response function. Points represent mean (± SEM) cocaine intake by EC (n=11) and IC (n=9) rats. * significant difference in responding from IC rats at the same unit dose ($P<0.05$). (**C**) Effect of enrichment on behavior in the EPM. Points represent mean (± SEM) seconds on the open arm.

1. Barrot M, Olivier JD, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ, *et al.* (2002): CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc Natl Acad Sci USA* 99:11435-11440.
2. Rahman Z, Schwarz J, Zachariou V, Gold SJ, Wein M, Choi KH, *et al.* (2003): RGS9 modulates dopamine signaling in striatum. *Neuron* 38:941-952.
3. Niesink RJ, Van Ree JM (1989): Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. *Neuropharmacology* 28:411-418.
4. Green TA, Alibhai IN, Unterberg S, Ghose S, Tamminga CA, Neve RL, Nestler EJ (2008): Induction of activating transcription factor 2 (ATF2), ATF3 and ATF4 in the nucleus accumbens and effects on behavior related to addiction, anxiety and depression. *J Neurosci* 28:2025-2032.
5. Xiao X, Li J, Samulski RJ (1998): Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* 72: 2224–2232.
6. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM (2002): Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci USA* 99:11854-9.
7. De BP, Heguy A, Hackett NR, Ferris B, Leopold PL, Lee J, *et al.* (2006): High levels of persistent expression of alpha1-antitrypsin mediated by the nonhuman primate serotype rh.10 adeno-associated virus despite preexisting immunity to common human adeno-associated viruses. *Mol Ther* 13:67-76.

8. Clark KR, Liu X, McGrath JP, Johnson PR (1999): Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. *Hum Gene Ther* 10:1031–1039.
9. Paxinos G, Watson C (1997): *The rat brain in stereotaxic coordinates, Compact 3rd ed.* San Diego, CA: Academic Press.
10. Green TA, Alibhai IN, Hommel JD, DiLeone RJ, Kumar A, Theobald DE, *et al.* (2006): Induction of inducible cAMP early repressor (ICER) in nucleus accumbens by stress or amphetamine increases behavioral responses to emotional stimuli. *J Neurosci* 26:8235-42.