Supporting Information

Ulrich-Lai et al. 10.1073/pnas.1007740107

SI Materials and Methods

Animals. The rat supplier varied depending on availability, and no differences were observed between rats from the two vendors. Rats were single-housed on a 12-h light/12-h dark cycle (lights typically on at 0600 hours) in a temperature- and humidity-controlled housing facility with ad libitum access to normal chow (LM-485; Harlan Teklad) and water. Upon arrival, rats acclimated to the housing facilities for at least 1 wk before the onset of experiments.

Palatable Drink Paradigm. A relatively small maximal volume of drink intake (4 mL per session) was selected to ensure that sucrose-fed and saccharin-fed rats would reliably drink the full volume, thereby minimizing variations in the amount consumed as a factor contributing to increased variability of effects. The relatively high concentration of sucrose (30%) was used to maximize the number of calories contained in the volume of drink permitted (8 mL/d with about 9 calories/d). Saccharin was included as a noncaloric sweet drink (1) to determine whether calories are necessary for the effects of sweet drink; the concentration of 0.1% was chosen based on literature reports (1, 2) and our own preliminary finding that the acquisition of training was optimal at this concentration.

Restraint Stress and Plasma Hormone Measurement. Rats were placed into well-ventilated restraint tubes for 20 min with tail-clip blood sampling (200 μL) into chilled tubes containing EDTA at 0, 20, 40, and 60 min after initiation of restraint. Poststress sampling time points were chosen to try to optimize assessment of the plasma corticosterone response (because this is the primary effector hormone of the axis), realizing that these time points may not be ideal for assessment of the plasma ACTH response. In some experiments, rats were then given an overdose of sodium pentobarbital followed by perfusion with saline and 4% paraformaldehyde for collection of brains. Blood samples were centrifuged (3,000 × *g*, 15 min, 4 °C), and plasma was stored at −20 °C until measurement of immunoreactive ACTH and corticosterone concentrations by RIA as described previously (3).

Sexual Activity Paradigm. The sexually receptive female rats were prepared by ovariectomy and implantation (s.c.) of silastic 17β-estradiol 3-benzoate (Sigma-Aldrich) implants (4) 8 d before the onset of the experiment. The female rats were then divided into three cohorts; each cohort received a priming dose of progesterone (500 μg in 100 μL of sesame oil, s.c., 4 h before placement with the males) every third day on a rotating schedule.

Telemetric Recording of Cardiovascular Parameters. The catheter tip of the telemetric device was inserted against the blood flow into the descending aorta and advanced so that the tip was posterior to the renal arteries. The catheter tube was then secured with a small cellulose patch (1 cm^2) and animal tissue adhesive such that circulation to the lower body was not occluded. The device capsule (containing the battery-powered sensor) was then sutured to the internal abdominal muscle wall. Rats were given pre-emptive analgesia (butorphanol, 0.4 mg/kg, s.c.) and antibiotic (gentamicin, 6 mg/kg, i.m.) before awakening from anesthesia. Rats recovered for at least 7 d before the onset of testing. Heart rate and blood pressure [mean arterial pressure (MAP)] were continuously monitored before and during a 20-min restraint stress test given on the morning of day 15 (after receiving limited, intermittent sucrose vs. water drink for the previous 14 d). Heart rate variability analysis in the frequency domain was performed on the pressure waveforms (Dataquest Analysis, Data Sciences International) to determine the high-frequency spectral power [0.8–4 Hz; considered an indirect index of the parasympathetic activity (5, 6)] and the low-frequency spectral power [0.25–0.8 Hz; considered an indirect index of primarily the sympathetic activity with perhaps some additional parasympathetic component (5, 6)]. For all cardiovascular variables, the restraint-induced response was determined by taking the peak response (defined as the mean of the first 10 min of restraint) minus the baseline (defined as the mean of the 40 min just before the experimenter entry to the animal room).

Behavioral Assessments. ***Social interaction.*** Immediately before social interaction testing on the morning of day 15, one-half of the rats in each drink group were given a 20-min restraint stress; social interaction is reduced after an acute stress challenge, thereby permitting measurement of sucrose effects in either basal (no restraint) or anxiogenic (postrestraint) conditions. The rats were then placed into a clean shoebox rat cage with an unfamiliar male conspecific of similar body weight for 10 min. The ensuing behavior was video-recorded, and the amount of time the experimental rat spent in social interaction (defined as touching, sniffing, and direct exploration of the interactor rat) was recorded by an observer unaware of treatment groups (7). As a positive control, a separate cohort of rats (n = 8–10 per group) was given the anxiolytic diazepam (1 mg/kg; Henry-Schein) or vehicle (2% Tween-20 in saline) 30 min before social interaction testing.

Open-field and elevated plus-maze tests. Rats (n = 10–11 per group) were given limited, intermittent sucrose drink (vs. water-drinking controls) as described above. Midday on day 12 of sucrose drink (starting ∼2.5 h after the morning drinking session), the rats were placed into a well-lit open field (∼1 m × ∼1 m) for 5 min. The ensuing behavior was video-recorded, and the time spent in stretch-attend postures and sniffing was recorded by an observer unaware of treatment groups. EthoVision (Noldus Information Technology) software was also used to determine total distance traveled by each rat.

Midday on day 15 of sucrose drink (starting at ∼2 h after the morning drinking session), these same rats were placed onto an elevated plus-maze (4 in. wide × 40 in. long, 0.125-in. lip on open arms, 14-in. height on closed arms) for 5 min in a dark room under red lights. The ensuing behavior was video-recorded, and an observer unaware of treatment groups assessed (*i*) the total time spent on open arms, (*ii*) the total time spent at the far end of the open arms, (*iii*) the time spent grooming, (*iv*) the number of head dips (exploratory looks out over edge of maze), and (*v*) the number of closed arm entries.

Basolateral Amygdala (BLA) Lesions. Rats were perfused with paraformaldehyde (see above), and immunolabeling for NeuN (1:1,000, MAB377, Chemicon), a marker of neuronal nuclei (8), was performed via a standard immunolabeling protocol (9) to determine the extent of neuronal loss after ibotenate lesions. Rats with ibotenate lesions were then classified as "hits" if the lesion sites included bilateral loss of the anterior and posterior subdivisions of the BLA at rostral-caudal level 29–30 of the Paxinos and Watson atlas (10). All other lesion rats (e.g., incomplete and/or unilateral lesions) were classified as "misses." BLA lesions (both hits and misses) often included structures lateral and ventral to the BLA (e.g., dorsal and ventral endopiriform nuclei), whereas more medial structures (e.g., central and medial amygdalar nuclei) were generally unaffected.

Microarray and Functional Clustering Analysis. Microarray analysis of gene expression was performed by the Cincinnati Children's Hospital Medical Center Microarray Core. Briefly, total RNA quality was verified (RNA 6000 Nano Assay, Agilent Bioanalyzer 2100; Hewlett-Packard), and cRNA was prepared (TargetAmp 1-Round Aminoallyl-aRNA Amplification Kit; Epicentre Biotechnologies) using both the TargetAmp t7-Oligo(dT) primer A (Epicentre Biotechnologies) and the Full Spectrum MultiStart Primers for T7 IVT (SBI System Biosciences) along with SuperScript III Reverse Transcriptase (Invitrogen). cRNA was biotin-labeled (Biotin-X-X-NHS; Epicentre Biotechnologies) and hybridized to Affymetrix microarray gene chips (Rat Genome 230 2.0 Array) according to manufacturer's instructions. Gene chips were visualized with R-phycoerythrin streptavidin (Invitrogen) and biotinylated anti-streptavidin antibody (Vector Laboratories) and scanned by using GeneChip operating software 1v4 on an Affymetrix GeneChip Scanner 3000. Expression data were normalized via global scaling, adjusting the target intensity value to 1,500. Hybridization controls included (*i*) comparison of 3′ and 5′ probe sets for internal control genes (GAPDH and β-actin) and (*ii*) prokaryotic spike controls to verify RNA and assay quality. Gene expression data were then analyzed by the Cincinnati Children's Hospital Medical Center Biomedical Informatics Core using GeneSpring software (Agilent Technologies). Filters were applied to remove genes with little-to-no expression in the BLA (signal <100) and those that were not affected by treatment (0.8- to 1.2-fold change), leaving 242 possible genes. The expression of these 242 possible genes was then assessed by two-tailed *t* test, leaving 145 genes whose expression was significantly altered by sucrose. Functional clustering analysis was performed on the list of 145 identified genes (Ingenuity Pathway Analysis) to identify canonical biological pathways that were significantly enriched in the identified genes.

BLA Immunolabeling. Paraformaldehyde-perfused brains from rats with a history of 14 d of limited intermittent sucrose (vs. water) were postfixed for ∼16 h at room temperature and then stored in sucrose (30% in PBS) at 4 °C. Brains were sectioned (25 μm) on a microtome, and the sections were stored in cryoprotectant (0.1 M PBS, 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol) at −20 °C. Sections were immunolabeled with rabbit primary antisera directed against phosphorylated (S133) cAMP response element-binding protein (pCREB; 1:500, 06-519; Millipore), phosphorylated (T286) calcium/calmodulin-dependent protein kinase IIα (pCamKIIα; 1:100, ab5683; Abcam), and synaptophysin (1:300, 18-0130; Invitrogen) in a standard immunolabeling protocol (11). Immunolabeling was not present when primary antibodies were omitted. pCREB immunolabeling was detected by use of biotin-conjugated goat anti-rabbit secondary antibody (Vector Laboratories) followed by incubation with avidin–biotin–peroxidase (Vectastain ABC Solution; Vector Laboratories) and reaction with 3,3′-diaminobenzidine. Synaptophysin and pCamKIIα immunolabeling were detected by use of cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories).

Image Analysis. NeuN (2.5× magnification) and pCREB (5× magnification) immunolabeling were imaged by using brightfield light microscopy (Zeiss Imager.Z1 microscope with Apotome, AxioCam camera, AxioVision Rel. 4.6 software; Carl Zeiss), and pCREB-positive cells were counted with Scion Image software (Scion). pCamKIIα (20× magnification) and synaptophysin (40× magnification) immunolabeling were imaged by using fluorescent microscopy (same imaging system as above). pCamKIIα-positive cells were manually counted. For synaptophysin, the percentage of area occupied was determined for four sets of projections compiled from an Apotome z-stack image (20 images of 0.5-μm thickness) for each side of the brain (for a total of eight projections); these values were then averaged to obtain the "percentage occupied" for each rat.

Statistical Analyses. For time-course data of the hormonal response to stress, the variance is often nonhomogenous; in these instances, ANOVA was performed after square-root transformation of the data. Differences in percentage change of integrated plasma corticosterone between water and sucrose were analyzed by one-tailed *t* test because they tested the a priori directional hypothesis of diminished responses by sucrose. Similarly, we hypothesized a priori that sucrose would be anxiolytic in assessments of behavioral anxiety, so these data were analyzed by one-tailed *t* test. Two-tailed *t* tests were used in all other instances where two groups were compared. Potential outliers were assessed with two different tests: (*i*) outliers were values that differed from the mean by more than 1.96 times the SD and (*ii*) outliers were values that were below the lower quartile or above the upper quartile by more than 1.5 times the interquartile range (12). A positive identification by both outlier tests was required before a value was removed from the analysis.

1. Collier G, Novell K (1967) Saccharin as a sugar surrogate. *J Comp Physiol Psychol* 64: 401–408.
2. Smith JC, Sclafani A (2002) Saccharin as a sugar surrogate revisited. *Appetite* 38: 155–160.
3. Ulrich-Lai YM, Engeland WC (2000) Hyperinnervation during adrenal regeneration influences the rate of functional recovery. *Neuroendocrinology* 71:107–123.
4. Robaire B, Ewing LL, Irby DC, Desjardins C (1979) Interactions of testosterone and estradiol-17β on the reproductive tract of the male rat. *Biol Reprod* 21: 455–463.
5. Malliani A, Pagani M, Lombardi F, Cerutti S (1991) Cardiovascular neural regulation explored in the frequency domain. *Circulation* 84:482–492.
6. Akselrod S, et al. (1981) Power spectrum analysis of heart rate fluctuation: A quantitative probe of beat-to-beat cardiovascular control. *Science* 213:220–222.
7. File SE, Seth P (2003) A review of 25 years of the social interaction test. *Eur J Pharmacol* 463:35–53.
8. Mullen RJ, Buck CR, Smith AM (1992) NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116:201–211.
9. Choi DC, et al. (2007) Bed nucleus of the stria terminalis subregions differentially regulate hypothalamic-pituitary-adrenal axis activity: implications for the integration of limbic inputs. *J Neurosci* 27:2025–2034.
10. Paxinos G, Watson C (1986) *The Rat Brain in Stereotaxic Coordinates* (Academic Press, New York).
11. Flak JN, Ostrander MM, Tasker JG, Herman JP (2009) Chronic stress-induced neurotransmitter plasticity in the PVN. *J Comp Neurol* 517:156–165.
12. McClave J, Dietrich F (1994) *Statistics* (Macmillan College Publishing Company, Inc., Englewood Cliffs, NJ).

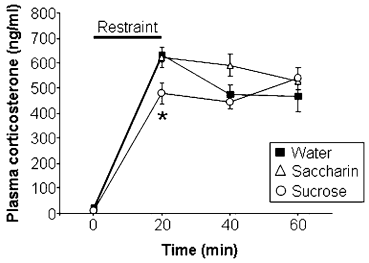

Fig. S1. Body weight was not altered by limited, intermittent consumption of sucrose or saccharin drink (drink presented twice daily over days 1–14).

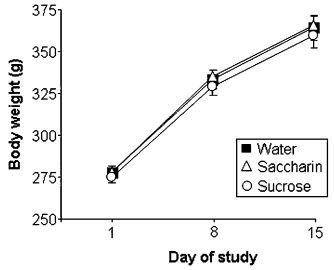

Fig. S2. A history of limited, intermittent consumption of palatable drink (sucrose) reduced the plasma corticosterone response to restraint. There was a significant sucrose × time interaction, whereas there were no main or interactive effects of saccharin when each were compared with water controls. *$P <$ 0.05 vs. water.

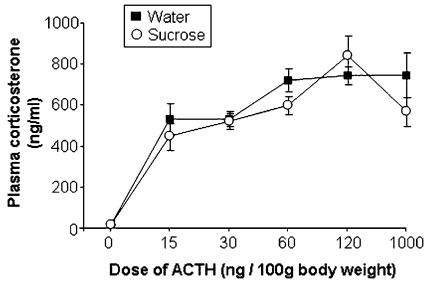

Fig. S3. A history of limited, intermittent consumption of palatable drink (sucrose) did not alter adrenal responsivity to ACTH. On the morning after completion of the 2-wk limited drink paradigm, rats were given dexamethasone phosphate (Sigma; 400 μg in 100 μL sterile saline, s.c.) to block the release of endogenous ACTH. At 2–4 h later, rats were given a dose of exogenous ACTH [Bachem; 0, 15, 30, 60, 120, or 1,000 ng per 100 g body weight in 100 μL of vehicle (0.5% protease-free BSA in PBS, pH 7.4), s.c.]. At 15 min after ACTH injection, rats (n = 6–7 per group) were decapitated with collection of trunk blood for measurement of plasma corticosterone. At the 0 ng ACTH dose, plasma corticosterone levels were low, demonstrating the effectiveness of the dexamethasone blockade. Plasma corticosterone levels increased significantly with increasing doses of ACTH, and this response was not affected by sucrose drink.

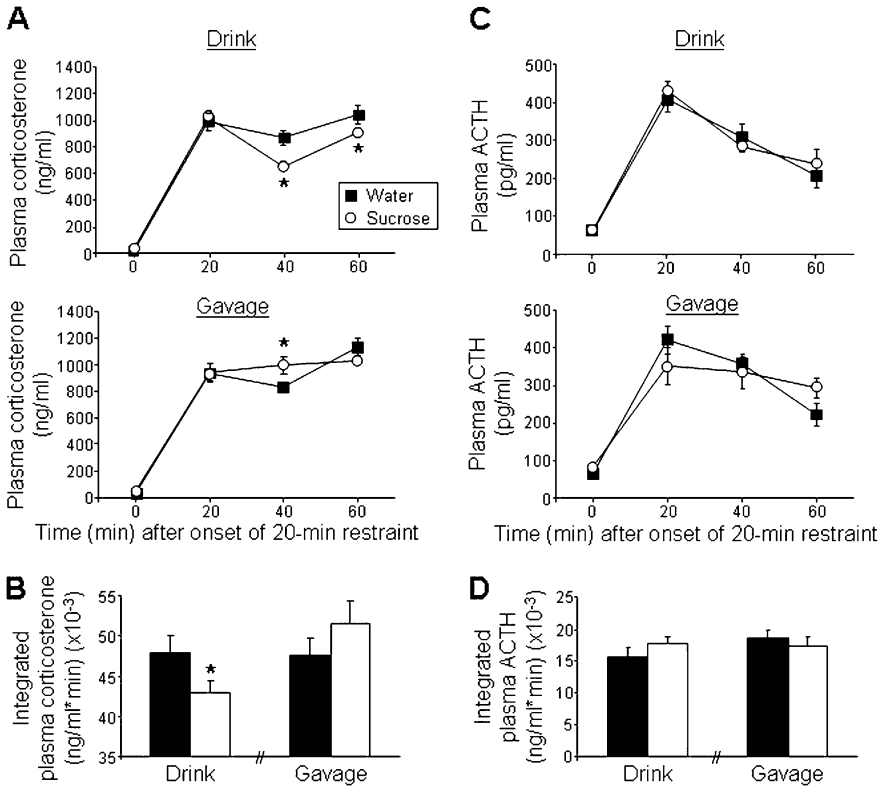

Fig. S4. A history of limited, intermittent consumption of palatable drink (sucrose) reduced the hypothalamic–pituitary–adrenocortical (HPA) axis response to restraint, whereas oral intragastric gavage of sucrose was without effect. (*A*) Time course of plasma corticosterone response to restraint (drink, *Upper*; gavage *Lower*). (*B*) Integrated plasma corticosterone response. (*C*) Time course of plasma ACTH response to restraint (drink, *Upper*; gavage *Lower*). (*D*) Integrated plasma ACTH response. Note that gavage of water did not increase the HPA response to restraint (relative to drinking water), suggesting that the gavage itself was not a chronic stressor. In contrast, gavage of sucrose increased the postrestraint plasma corticosterone at one time point (but not the integrated response), suggesting that gavage of this solution may have been a slight chronic stressor, perhaps because of the noncontingent administration of this high-osmolarity solution. *$P < 0.05$ vs. water.

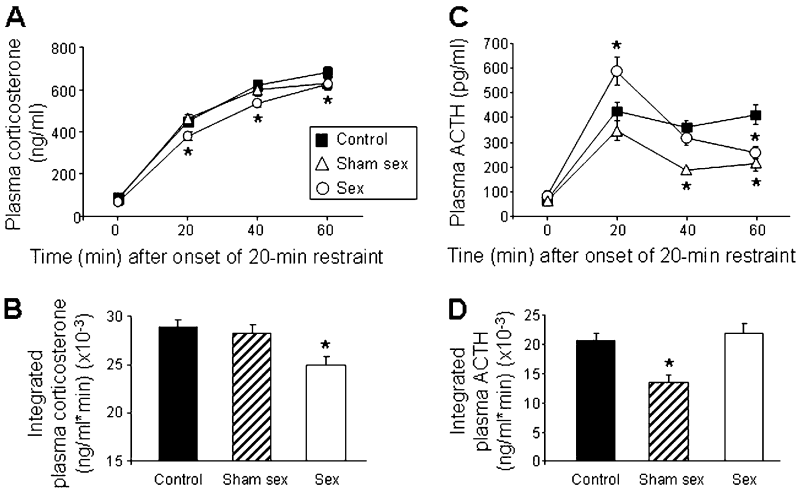

Fig. S5. A history of limited sexual activity reduced the HPA response to restraint. (*A*) Time course of plasma corticosterone response to restraint. (*B*) Integrated plasma corticosterone response. (*C*) Time course of plasma ACTH response to restraint. (*D*) Integrated plasma ACTH response. Note that sham sex (placement of a female rat into a small wire cage within the home cage of the male rats) did not increase the HPA response to restraint (and actually decreased the plasma ACTH response), suggesting that this was not an aversive experience for these sexually naive male rats. *$P < 0.05$ vs. control.

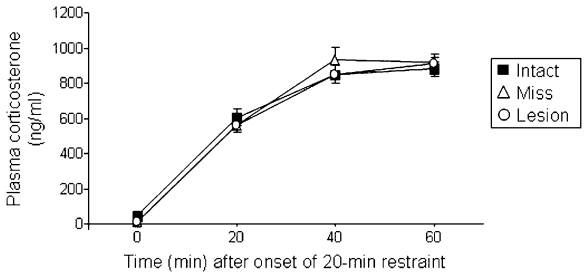

Fig. S6. Ibotenate lesions of the BLA did not alter the plasma corticosterone response to restraint in water-drinking rats. Note that these data are the same as that shown in Fig. 3 of the main text, regraphed to more easily compare the Lesion-Water, Miss-Water, and Intact-Water groups.

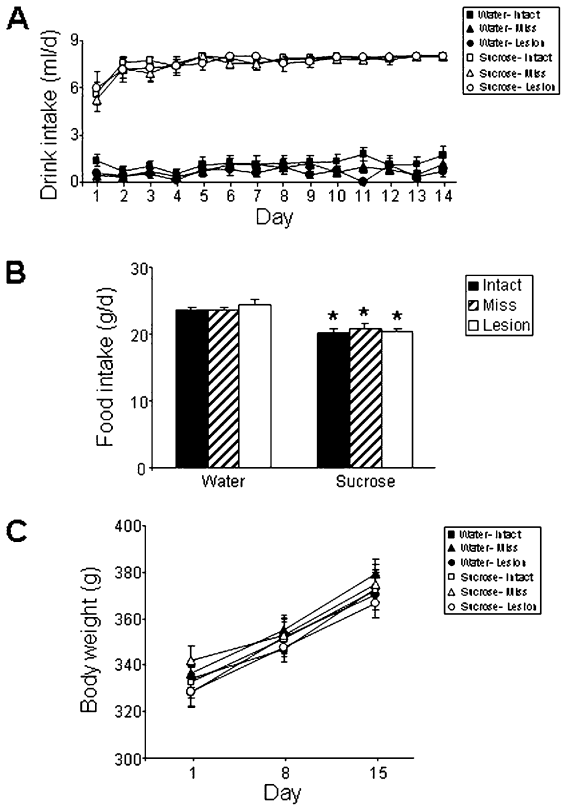

Fig. S7. Ibotenate lesions of the BLA did not alter sucrose drink intake, food intake, or body weight. (*A*) Rats quickly learned to drink sucrose in volumes approaching the maximum permitted (8 mL/d), whereas the water rats drank little, as expected because both groups also had free access to their normal water bottle. Moreover, neither lesions of the BLA nor lesions that missed the BLA altered drink intake. (*B*) Over the 2 wk of drink exposure, food intake was modestly reduced by sucrose drink and was not affected by BLA lesion or lesions that missed the BLA. $*P < 0.05$ vs. water. (*C*) Body weight was not altered by sucrose drink or BLA lesion (sucrose presented twice daily over days 1–14).

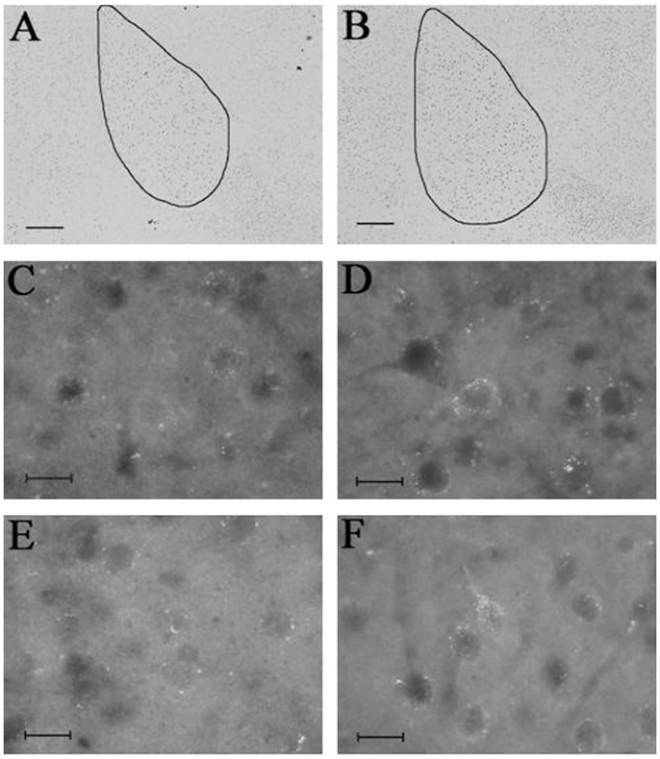

Fig. S8. Representative images of BLA immunolabeling: pCREB in rat with history of drinking water (*A*) or sucrose (*B*); pCamKIIα in rat with history of drinking water (*C*) or sucrose (*D*); and synaptophysin in rat with history of drinking water (*E*) or sucrose (*F*). (Scale bar = 200 μm for *A* and *B*; scale bar = 20 μm for *C*–*F*.)

Table S1. Functional clustering analysis on the set of identified genes detected 13 canonical pathways that were significantly enriched

Canonical pathway	Ratio	*P* value	Genes
Calcium signaling	7/206	0.00015	ATP2B2, CAMK2A, CREBBP, GRIA3, GRIN2A, RAP2B, RYR2
Synaptic long term potentiation	5/115	0.00099	CAMK2A, CREBBP, GRIA3, GRIN2A, PPP1R12A
Role of RIG1-like receptors in antiviral immunity	3/52	0.00101	CREBBP, DHX9, IRF7
Activation of IRF by cytosolic pattern recognition receptors	3/73	0.0054	CREBBP, DHX9, IRF7
NF-κB signaling	4/147	0.0087	BMPR1A, CREBBP, EGFR, GSK3B
Chemokine signaling	3/77	0.039	CAMK2A, GNAI2, PPP1R12A
PTEN signaling	3/101	0.020	BMPR1A, EGFR, GSK3B
cAMP-mediated signaling	4/162	0.024	ADRA2C, AKAP9, CAMK2A, GNAI2
Neuropathic pain signaling in dorsal horn neurons	3/104	0.031	CAMK2A, GRIA3, GRIN2A
Thrombin signaling	4/204	0.034	CAMK2A, EGFR, GNAI2, PPP1R12A
Integrin signaling	4/203	0.035	ARHGAP5, GSK3B, PPP1R12A, RAP2B
Huntington's disease signaling	4/234	0.047	CREBBP, EGFR, PACSIN1, SP1
Cardiac β-adrenergic signaling	3/136	0.049	AKAP9, PPP1R12A, RYR2

Ratio indicates the number of genes from the identified set that were members of a particular canonical pathway relative to the total number of genes in the canonical pathway. Note that many of the identified genes appear in multiple pathways (e.g., CAMK2A; CamKIIα).