Supporting Information

Crews et al. 10.1073/pnas.1118514109

SI Materials and Methods

Animal Housing Protocol. F3 generation vinclozolin-lineage (V-L) and DMSO control-lineage (C-L) male rats were selected out of litters from untreated F2 generation mothers in M.K.S.'s laboratory at Washington State University (WSU) according to established protocols (1). Briefly, approximately four different F0 generation females were used to generate different lineages for the F1–F3 generations with no sibling or cousin breeding to avoid inbreeding artifacts. Gestating female F0 generation Sprague–Dawley rats were injected with the fungicide vinclozolin (100 mg/kg) daily during fetal gonadal sex determination (embryonic days 8–14), and the F1 generation were bred to generate the F2 generation, and then the F2 were bred to generate the F3 generation (1). At approximately postnatal day (PND) 10 (before weaning), each animal was injected with a small microchip (AVID) s.c. between the shoulder blades. The animals were then shipped to D.C.'s laboratory at the University of Texas at Austin (UTA) from WSU on PND 22, 1 d after weaning. Upon arrival at the UTA, one animal from each lineage (control and vinclozolin) was pair-housed (one control and one vinclozolin animal) and remained in these dyads throughout the duration of the study. Because of the natural variation in dates of breeding, there was a 4-d spread of birth date of animals in the first cohort, but all animals were born on the same day in the second cohort. However, all pair-housed animals were no more than 1 d apart in birth age and were paired randomly to prevent an age effect on cage mates.

Each dyad of animals was randomly placed in a six-wide, five-high metal housing rack in standard translucent polycarbonate rat cages (46 cm × 24 cm × 20.5 cm) with ad libitum access to tap water and standard rat chow (rodent chow 5LL2 Prolab RMH 1800 diet; Purina). The animal room was on a 14-h/10-h light/dark schedule. For environmental enrichment, a 7-cm-diameter PVC pipe was placed in each cage.

Uterine and litter sex ratios can influence adult phenotype (2–4), and so the sex ratio of each litter was determined at birth and weaning. The litters were then divided according to criteria described in de Medeiros et al. (4). Equal (E) litters (n = 5 litters totaling 20 animals) containing equal numbers of males and females (or no more than 1 individual more of either sex). Male-biased (Mb) litters (n = 5; 23 animals) contained ≥50% males. Female-biased (Fb) litters (n = 9; 42 animals) contained ≥50% females. All of the litters that provided animals in the study could be divided into one of these three groups.

Chronic Restraint Stress (CRS). The day after receipt at UTA, half of the dyads were randomly chosen to be administered a CRS treatment. This paradigm entailed 6 h of daily CRS consecutively for 21 d and duplicated the methodology of ref. 5. The restraint apparatus consisted of a 25.4-cm^2 metal wire mesh folded in half and bound with a plastic mold that allowed for two openings and prevented any rough edges. The front and rear openings of the restraint cage were clipped shut with binder clips with an additional binder clip on one side of the animal to allow for adjustment of the size of the restraint as the animals grew. Animals to be stressed were removed from the animal housing room at 9:00 AM and transported to a procedural room separate from the housing room. Each animal was hand-placed in a restraint at 1 h after lights off (9:30 AM) by either allowing the animal to enter the restraint on its own will or gently coaxing the animal into the restraint. Extra care was taken to not forcefully place the animals in the restraint as to prevent any unnecessary stress. Stressing was performed between 9:30 AM and 3:30 PM, with lights off and no red light. At 3:30 PM, animals were immediately removed from the restraint cages by removing the binder clip closest to the animal's nose, allowing them to crawl out of the restraint into their home cage at will. As each animal was placed back in its home cage, it was scanned to confirm identity and correct cage mate placement. The animals were then transported back to the housing room and placed back in their original housing rack. Dyads that were not to be stressed were left in the housing room, untouched. Restraint cages and binder clips were washed after stressing each day with soap and water and left to dry for the next day's stressing.

Twice a week (Tuesday and Friday), after the animals had been stressed, animals were weighed, beginning at 4:00 PM. While each dyad was being weighed, the animals were handled for about 3 min each to allow the animal to become accustomed with the experimenter and to prevent any unnecessary stress while the animals had to be handled for stressing or behavioral testing. After the initial 21 d of CRS, all animals were left in the housing room constantly and were only removed for handling, weighing, and behavioral testing on scheduled days.

Animal Groups and Numbers. There were four treatment groups: V-L stress (VS), V-L nonstress (VNS), C-L stress (CS), and C-L nonstress (CNS). A subset of each group was not tested (4, 2, 4, and 2, respectively); all remaining dyads were behaviorally tested. Two cohorts of animals were run, the first in January and the second in May. Cohort sample sizes are shown in the following table:

Cohort	CNS	VNS	CS	VS
1	8	8	11	10
2	8	8	9	9
Total	16	16	20	19

Body Weight (BW). Animals were weighed every 3–8 d from PND 21 (at weaning) to PND 108. Analysis focused on two questions: how do the groups differ through time or at any one point in time and how does weight change within a group as a function of time and experience. The first question was addressed by using a three-way ANOVA with repeated measures.

The second question was addressed in the following manner. Differences in BW between a stress group and a nonstress group and between V-L and C-L were compared at each time point (PNDs 21, 29, 32, 37, 40, 46, 52, 57, 61, 67, 71, 79, 81, 88, 96, 102, and 108). The effect of CRS on BW was compared between and within lineages at each time point.

The nonparametric Wilcoxon rank-sum test was used to analyze the association between a lineage and stress treatment because of the asymmetric distribution of BW in each group. All statistical comparison was performed with JMP 8.0 (SAS Institute); P values < 0.05 were considered to be statistically significant.

Behavioral Testing. Beginning at PND 114, each individual received four different behavioral tests with behavior quantified by using the automated Stoelting ANY-maze video-tracking system. Each test was separated by 48 h. All of the dyads (V-L and its yoked C-L cage mate) were tested in open-field (OF), forced-swim (FS), and two social-affiliation tests. The order of the tests was counterbalanced to treatment, although the OF test was obligatory to the last day of

testing because it was performed during the light phase to control for the time between tests. Animals were then killed 42 h later. After each trial, the apparatus was cleaned with 70% (vol) ethanol and allowed to dry before the next test.

The FS and sociability tests were performed during the dark cycle, beginning at 1 h after lights off (9:30 AM). The test arenas were softly illuminated with red lights, positioned as to not affect ANY-maze tracking. The OF was performed during the light cycle under illumination, beginning at 1 h after lights on (7:30 PM).

To prevent ambiguities in testing each animal, a scanning system was used that automatically scanned the identity of the animal, via an AVID identification microchip, that was automatically entered into ANY-maze and began each test.

FS test. This test was based on the methods of Porsolt et al. (6). However, because there was no intermittent treatment to administer, a single 15-min paradigm was used. Animals were placed in a 19 cm × 50 cm Plexiglas cylinder with 40 cm of water. Each animal was allowed 15 min in the cylinder with no possibility of escape (a piece of clear glass was placed over each apparatus to prevent escape). The ANY-maze system was then instructed to measure the time that each animal was immobile, defined as no more movement than necessary to keep the head above the water to breathe. The depth of the water was such that an animal would not be able to balance itself on the bottom of the tank with its tail. If an animal sank to the bottom of the tank and exhaled their breath under water, they were immediately removed from the tank and scored as a "did not finish." Water for the FS was tap water that had been placed in containers overnight to allow the temperature of the water to come up to room temperature (23–25 °C). After each trial, the water was poured out and the cylinder was rinsed with water, rinsed with 70% ethanol, rinsed with water again, and then filled with room temperature water. After each trial, each animal was hand-dried with a towel and placed back into its cage with a red light directly above the cage for warming the animal from hypothermia.

OF test. Each animal was tested in a 100 cm × 100 cm × 40 cm apparatus partitioned into four equivalent arenas of 50 cm × 50 cm each). A soft white light bulb (60 W) was placed directly over the enclosure. No other ambient lights were on during testing. Each animal was placed at the corner where the partition met (i.e., in the center of the 100 cm × 100 cm arena) to rule out initial placement of the animal in the arena as a factor affecting behavior. The animal was then allowed a 15-min period to roam the arena with no interruption. A 10-cm perimeter border around the edges of the arena was drawn digitally in ANY-maze and defined as an "outer region," and anything inside of that 10-cm border was defined as the "inner region." ANY-maze was instructed to calculate the time in the inner region versus the time in the outer region as well as the following measures: total distance traveled (m), average speed (m/s), number of line crossings, time in inside zone (s), number of entries into the outside zone, and time in outside zone (s). To obtain a measure of time spent in corners, the OF was divided into 25 equal segments, and the four corner segments were summed. After the 15-min trial period, each animal was removed and returned to its home cage. The arena floor and walls were cleaned with 70% ethanol and left to air-dry before the beginning of the next trial.

Tests for sociability. Two tests for sociability were modeled after protocols in Moy et al. (7). A 100 cm × 100 cm × 40 cm Plexiglas enclosure (Stoelting) was partitioned into three arenas by 40-cm high walls with a 10 cm × 11 cm opening to allow movement of the rat between arenas. At the beginning of the test, the animal was allowed to become accustomed to its environment for 5 min. The center arena was used for this, and the entrances to the two side arenas were blocked by standard poster board taped to the wall of the arena. A 5-min habituation period preceded each test. All stimulus animals were PND 120 intact Sprague–Dawley male rats from Harlan. The stimulus animal cage was a 15 cm × 30 cm enclosure with vertical rods spaced at 1 cm apart to prevent fighting and biting but allowing for facial recognition and investigation.

For sociability test 1 (Soc 1), the blockades to the two side chambers were removed after the habituation period, allowing the experimental rat to explore any of the three chambers. The position (left or right) of the stimulus animal was systematically rotated between trials. The experimental rat was given 10 min to become familiar with the stimulus animal. ANY-maze was instructed to record the time spent in the chamber with animal 1 versus the center and other chambers (containing an empty cage).

In Soc 2, a second stimulus animal (novel animal), pair-housed with the first animal (familiar animal) was placed in the empty cage in the opposite chamber. The experimental rat then had the choice of associating with the familiar rat or with the novel rat or not associating with either. ANY-maze was instructed to record the time spent with the novel animal versus the time spent with the familiar animal versus the time spent in the center arena (no animal). After this second 10-min period, the rat being tested was returned to its home cage, and the arena and metal enclosures were wiped down with 70% ethanol and allowed to air-dry.

Hormone Assays. The hormone RIAs were performed by the Hormone Assay Core Laboratory at the Center for Reproductive Biology, WSU. Circulating concentrations of corticosterone (CORT), testosterone (TESTO), and leptin were determined by commercial assays (TESTO and CORT from Siemens Medical Solutions with coefficients of variation at less than 6.18% and 6.7%, respectively). Each cohort was analyzed in separate assays for TESTO and CORT, with the latter hormone conducted in a single assay. The leptin commercial assay was from Millipore with a coefficient of variance less than 7.3%.

Brain Processing. For each dyad, the brain of each male was cut in the sagittal plane at the midline (Fig. S8*A*, *A–D*). Each half was combined with the complementary half of the other individual (alternating the right and left halves) such that each "brain" consisted of symmetrical halves of the V-L and a C-L male pair. One "combined" brain was prepared for cryostat sections (40 μm) in three alternating sets: the first set was for cytochrome oxidase histochemistry (8) and the second and third sets were kept for future studies, such as in situ hybridization and immunohistochemistry of target genes for better resolution of gene expression. The other combined brain was prepared for punches of discrete nuclei by first slicing in 2-mm sections with a Zivic brain matrix (Fig. S8*A*, *E–G*. The following coordinates are relative to bregma based on the Paxinos and Watson (9) rat brain atlas: the beginning of each blocked slice was +0.36, −1.64, −3.64, and −5.64. The following areas were taken using a 1-mm punch (Stoelting): primary and secondary motor cortex (CRTX) at the level of the basolateral amygdala (BLA), BLA, and CA1 and CA3 of the hippocampus. Tissue punches were placed singly in 1-mL Eppendorf tubes containing 200 μL of TRIzol. All brain analyses (microarray and cytochrome oxidase histochemistry) were conducted after all tissues had been collected so that material from all individuals could be done in a single run for each procedure.

Cytochrome Oxidase and Brain Nuclei. Behavior is a function of brain activity, which obtains ∼90% of its energy from the aerobic metabolism of glucose (8). Cytochrome oxidase (COX) is a rate-limiting enzyme in oxidative phosphorylation. COX activity reflects the metabolic history of brain areas, and COX levels and/or activity constrain the activity of a neuron, thereby limiting the likelihood of a behavior (10). Thus, factors that constrain brain metabolism also constrain behavior. Considering that behavior results from coordinated activity in networks of specific brain nuclei (11), information on the metabolic activity in neural networks is of interest.

Metabolism is assessed by cytochrome oxidase histochemistry in 14 discrete brain regions (Tables S1 and S2): All regions but the CRTX have been implicated in the neural and behavioral consequences of response to CRS.

The primary disadvantage of the split-brain method is that midline nuclei such as the paraventricular nucleus (PVN) and anteroventral periventricular nucleus (AvPv) are damaged and in most instances cannot be imaged with confidence. For example, the PVN is integral to the stress response (12). Although we were able to obtain total metabolic measurements for the PVN, the sample sizes were limited (two or more per group). The PVN in C-L males had substantially higher activity (>35%) than V-L males in the nonstress condition ($P = 0.02$); this effect was reduced to ~10% difference in the stress condition. Only the C-L males showed a response to CRS (32% decrease; $P < 0.01$), whereas the V-L males showed an approximately 10% change in the stress condition.

Landscape Analysis. The functional landscape analysis developed involves principal component analysis (PCA) followed by discriminant function analysis (DFA) and permutation analysis to determine whether the targeted traits at each level of biological organization (physiology, behavior, and brain nuclei metabolism) are different between groups (11, 13). Performing a traditional PCA requires either omitting individuals with missing data points or imputing the missing data. We opted for an alternative method for PCA termed probabilistic PCA (PPCA), capable of accommodating missing data (13). Before the analysis, individuals with greater than 50% of their data missing were removed ($n = 5$ individuals), and the data were recentered to mean 0 and scaled via vector normalization. The remaining missing data were imputed during PPCA ($n = 36$ data points) (14). Unlike traditional PCA, PPCA can handle missing data (14). In PPCA, an expectation-maximization algorithm is used to fit a Gaussian latent-variable model (14). The maximum-likelihood estimate for the missing data was determined from the observed conditional distributions for individuals with complete data (13, 14). All data-preprocessing and PCA were carried out using the MultiDimBio (13) R package (v0.0.2) and bioconductor (release 2.8) R package pcaMethods (v1.32.0) (15). The imputed data account for 6% of the total dataset.

A multivariate ANOVA (MANOVA) was conducted on the results of the PPCA. Lineage, stress, their interaction, and housing dyad were included in the model. The interaction between dyad and the other group variables was not modeled because the effect of dyad should be random with respect to lineage and stress. The number of unique housing dyads also preclude this analysis because there are not enough degrees of freedom. Post hoc analysis was carried out with four ANOVAs, one for each principal component axis. Significance was determined by using a false discovery rate correction as implemented in the R package fdrtools (16). All analyses were performed with R v2.12.2 (17).

The effects of lineage and stress on the physiological, behavioral, brain metabolism, and essential phenotypes were analyzed (Fig. 2 *A–D*, respectively).

PCA was performed on six measures of body phenotype: leptin level, TESTO level, CORT level, adrenal weight, testes weight, and animal BW. The first four principal components were maintained and account for 81% of the variance (Fig. S2*A*). The first and second principal components (PC1 and PC2) were organ/animal weight and circulating hormones, respectively. The third principal component (PC3) was dominated by leptin levels, and the fourth principal component (PC4) related adrenal size to CORT levels. A MANOVA of the principal component scores for effects of lineage, stress, and dyad revealed a significant effect of lineage ($F_{1, 32} = 4.49, P = 0.006$), stress ($F_{1, 32} = 4.00, P = 0.011$), and dyad ($F_{34, 32} = 4.49$, $P = 0.006$) but no significant interaction. The individual importance of each axis was explored post hoc by using four separate ANOVAs. There was a significant effect of lineage in PC1 and PC3 ($F_{1, 32} = 7.54$, qFDR = 0.02; $F_{1, 32} = 7.31$, qFDR = 0.021), stress in PC1 ($F_{1, 32} = 17.88$, qFDR = 0.002), and dyad in PC2 and PC4 ($F_{34, 32} = 2.59$, qFDR = 0.013; $F_{34, 32} = 7.31$, qFDR = 0.008), where qFDR is the false discovery rate q value.

PCA of the combined behavioral tests revealed that the first three principal components account for 93% of the variance (Fig. S2*B*). A MANOVA of the principal component scores for effects of lineage, stress, and dyad revealed no significant effects variation. The individual importance of each axis was explored post hoc by using four separate ANOVAs, again with no statistically significant effects of lineage, stress, dyad, or their interactions. PCA of the essential phenotype captured 69% of the variance observed among and between the groups.

PCA revealed that nine brain nuclei capture 87% of the variance: BLA, medial amygdala (MeAmy), central amygdala (CeAmy), anterior cortical amygdala (CoAmy), posteromedial cortical amygdala (PMCo), medial posterior dorsal amygdala (MePD), stria terminalis (ST), and CA1 and CA3 of the hippocampus. The first four principal component axes account for >92% of the variation. The first principal component likely represents a measure of general activity in the sampled brain regions, with all nuclei except ST strongly loading onto the axis (Fig. S2*C*). The second axis is dominated by CA1 and CA2, and the third axis is almost entirely determined by ST. The fourth axis accounts for slightly more than 5% of the variation and is strongly determined by PMCo and, to a lesser extent, CA1, CA3, and CoAmy. To test for the effect of lineage, stress, and their interaction, we used the principal component scores as response variables in a MANOVA. Housing dyad (dyad) is also included as a covariate. There is a significant effect of lineage ($F_{1, 29} = 8.99$, $P < 0.001$) and dyad ($F_{1, 29} = 3.14$, $P < 0.001$), a trend toward a significant interaction ($F_{1, 29} = 2.56$, $P = 0.063$), and a nonsignificant effect of CRS. The individual importance of each axis is explored post hoc by using four ANOVAs. The overall result is a strong effect of vinclozolin on the response to CRS. The first and fourth principal component axes contribute to lineage ($F_{1, 29} = 6.55, P = 0.028$; $F_{1, 29} = 8.60, P = 0.021$) and dyad effects ($F_{1, 29} = 4.04$, $P = 0.001$; $F_{1, 29} = 2.35$, $P = 0.024$). The third principal component accounts for the interaction between lineage and CRS ($F_{1, 29} = 4.64$, qFDR = 0.055), and the second principal component only contributes to the effect of dyad ($F_{1, 29} = 2.48$, qFDR = 0.022). There is a significant effect of dyad in the MANOVA and all ANOVAs, except for the PC3 ANOVA.

RNA Preparation. From each individual, 1-mm punches were taken from the CA1 and CA3 of the hippocampus, the BLA, and the CRTX. After RNA isolation, three different pools, each containing three different males, were generated in each of the four conditions. Microarray analysis was performed on each pool in triplicate, involving three different experiments and aged animal pools. The sample histograms for raw data for all 48 arrays are shown in Fig. S3 and after preprocessing (,*SI Materials and Methods*) all arrays corresponded well with no outlying arrays. Individual rats were homogenized in 200 μL of TRIzol, and then 600 μL of TRIzol was added to final volume of 800 μL. Samples were stored at −80 °C or −20 °C until RNA extraction. Then, 100 μL of the above TRIzol homogenate from four randomly chosen individuals within the same tissue/treatment group were pooled together for one RNA sample (one microarray biological replica). Samples from the same set of four dyads were pooled for one vinclozolin or control replica for all four brain areas, stress or nonstress. For microarray analysis, three biological replicas were prepared as above for each brain area/treatment group. A total of 48 samples/chips were analyzed: 4 (brain areas) X 2 (control or vinclozolin) X 2 (stress or nonstress) X 3 (biological replicas). RNA was extracted from pooled TRIzol samples according to

standard TRIzol extraction protocols (Invitrogen) and stored in aqueous solution at −80 °C until microarray analysis.

Microarray Analysis. The microarray analysis was performed by the Genomics Core Laboratory at the Center for Reproductive Biology, WSU, by using standard Affymetrix reagents and protocol. Briefly, mRNA was transcribed into cDNA with random primers, cRNA was transcribed, and single-stranded sense DNA was synthesized, fragmented, and labeled with biotin. Biotin-labeled ssDNA was then hybridized to the Rat Gene 1.0 ST microarrays containing more than 29,000 transcripts (Affymetrix). Hybridized chips were scanned on an Affymetrix Scanner 3000. CEL files containing raw data were then preprocessed and analyzed with Partek Genomic Suite 6.5 software using an RMA GC content-adjusted algorithm. Raw data preprocessing was performed in four groups, one for each of four brain areas (BLA, CRTX, CA1, and CA3; Fig. S4 *B*, *C*, *E*, and *F*). Comparison of raw data array histogram graphs for each brain tissue demonstrated the data for all 12 chips belonging to one of four tissues were similar and appropriate for further preprocessing and analysis as a whole group (Fig. S4 *A* and *D*). Preprocessing of raw data involved multiple testing corrections and false discovery rate corrections (18).

The microarray quantitative data involves hybridization signals from an average of 28 different oligonucleotides arrayed for each gene, and the hybridization must be consistent to allow a statistically significant quantitative measure of gene expression and regulation. In contrast, a quantitative PCR (qPCR) procedure only uses two oligonucleotides, and primer bias is a major factor in this type of analysis. Therefore, we did not attempt to use PCR-based approaches for gene expression measurement because we feel the microarray analysis is more accurate and reproducible without the primer bias found in PCR-based approaches, as previously described (18).

All microarray CEL files from this study have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE26737), all arrays combined with one accession number. For gene annotation, Affymetrix annotation file RaGene1_0stv1.na31.rn4.transcript.csv was used unless otherwise specified.

Gene Network and Pathways. Global literature analysis of differentially expressed gene lists for each tissue or treatment group was performed by using Pathway Studio software (Ariadne Genomics), which performs an interaction analysis and builds subnetworks of genes and cell processes connected to each other. Resulting lists of differentially expressed genes for each treatment and each tissue were analyzed for KEGG (Kyoto Encyclopedia of Genes and Genomes, Kyoto University, Japan) pathway enrichment with Pathway-Express, a web-based tool freely available as part of the Onto-Tools (http://vortex.cs.wayne.edu) (19), and the Search Pathway tool at http://www.genome.jp/kegg/pathway.html.

Other Statistical Analysis. All measures for behavioral analysis were acquired automatically from the ANY-maze behavioral tracking program (Stoelting). Visual confirmation of the system was performed as each animal was tested to ensure accuracy. All behavioral measures were formatted for use in SPSS to perform statistical analyses in two basic variations, parametric and nonparametric. Several parametric statistics were used.

For the behavioral tests, a two-way ANOVA (stress × lineage) was performed on each of the measures obtained from ANY-maze looking for main effects of stress, main effects of lineage, and interactions as well as for each Hochberg's GT2 post hoc analysis applied. Significance was determined at $\alpha < 0.05$. Additionally, *t* tests were performed on a 2 × 2 design looking for stress effects within lineage (i.e., C-L nonstress condition versus C-L stress condition or V-L nonstress condition versus V-L stress condition) or lineage effects within stress (i.e., V-L stress condition versus C-L stress condition or V-L nonstress condition versus C-L nonstress condition). *t* test statistics are indicated as being either one- or two-tailed analyses depending on a priori hypotheses of each of the measures. That is, if directionality was hypothesized, statistics are displayed as one-tailed analyses with a significance $\alpha = 0.05$.

To clarify social interactions and rule out the confounding effects of differences between cage mates (social context), statistics were performed on a matched-pair basis. As previously described, animals were pair-housed with an animal of the other lineage (C-L with V-L). The value of each measure for each C-L animal was subtracted from the measure for its V-L cage mate, creating a difference score for each measure. Because the resulting scores were not normally distributed, based on the Kolmogorov–Smirnov test for normality, they were compared with nonparametric statistics. To compare the effects of CRS between dyads, the same difference scores described above were compared with a Kruskal–Wallis one-way ANOVA. To determine an effect of lineage within each dyad, a pair-matched signed-rank Wilcoxon test was performed. In all these tests, statistical significance was $P < 0.05$.

In addition to traditional comparisons, a combined *Z* score of multiple measures was composed for each behavioral test based on the work of Bellani et al. (20). Each measure was prechosen as a measure of anxiety or sociability whereupon it was transformed to a *Z* value. These *Z* values were then summed for each animal within each behavioral test. These sum scores of *Z* values were then compared in a two-way ANOVA and by *t* tests, as described above. The combined and averaged *Z* scores for each group were plotted onto a surface map to create a behavioral phenotype of that group. To determine the change in a phenotype relative to a stress or lineage condition, surface plots were subtracted across lineage or stress, but not both, to elucidate those effects (Z2–Z1). All significance levels were restricted to $\alpha = 0.05$.

At death, each animal's adrenals and testes were removed and weighed for analysis. The weights of these organs were compared as whole weights and as an index to BW, thus controlling for total size of the animal. As with the statistical analyses on behavioral measures, organ weight data were parsed into their respective groups and compared via a two-way ANOVA and *t* tests. The same interdyad comparison described above was used to determine any effects of social context. All significance levels were restricted to $\alpha = 0.05$.

Hormone assays were performed at WSU, and subsequent statistical analyses were performed at UTA. CORT, TESTO, and leptin were measured by RIA and were compared both directly and as an index of organ weight (e.g., TESTO to testes weight). As with the statistical analyses on behavioral measures, hormone data were parsed into their respective groups and compared via a two-way ANOVA and *t* tests. The same interdyad comparison described above was used to determine any effects of social context. All significance levels were restricted to $\alpha = 0.05$. In the instance of CORT levels, there appeared to be an effect of litter sex ratio. Although the litter sex ratio of the V-L and C-L does not differ in the colony at large, post hoc analysis of the data revealed that males from equal and female-biased litters exhibit a significant decline (averaging 39%) in CORT levels as a consequence of CRS in both C-L and V-L groups, whereas males from male-biased litters exhibit a significant elevation (31%) in CORT levels. There is no effect of litter sex ratio for either TESTO or leptin.

1. Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* 308:1466–1469.

2. Crews D, Fuller T, Mirasol EG, Pfaff DW, Ogawa S (2004) Postnatal environment affects behavior of adult transgenic mice. *Exp Biol Med (Maywood)* 229:935–939.

3. Crews D, Rushworth D, Gonzalez-Lima F, Ogawa S (2009) Litter environment affects agonistic behavior and brain metabolic activity of adult estrogen receptor α knockout mice. *Frontiers in Behavioral Neuroscience* 3:12.
4. de Medeiros CB, Rees SL, Llinas M, Fleming AS, Crews D (2010) Deconstructing early life experiences: Distinguishing the contributions of prenatal and postnatal factors to adult male sexual behavior in the rat. *Psychol Sci* 21:1494–1501.
5. Romeo RD, Bellani R, McEwen BS (2005) Stress-induced progesterone secretion and progesterone receptor immunoreactivity in the paraventricular nucleus are modulated by pubertal development in male rats. *Stress* 8(4):265–271.
6. Porsolt RD, Le Pichon M, Jalfre M (1977) Depression: A new animal model sensitive to antidepressant treatments. *Nature* 266:730–732.
7. Moy SS, et al. (2004) Sociability and preference for social novelty in five inbred strains: An approach to assess autistic-like behavior in mice. *Genes Brain Behav* 3(5):287–302.
8. Gonzalez-Lima F, Cada A (1998) Quantitative histochemistry of cytochrome oxidase activity. *Cytochrome Oxidase in Neuronal Metabolism and Alzheimer's Disease*, ed Lima FG (Plenum, New York), pp 55–90.
9. Paxinos G, Watson C (2007) *The Rat Brain in Stereotaxic Coordinates* (Academic, New York).
10. Sakata JT, Crews D, Gonzalez-Lima F (2005) Behavioral correlates of differences in neural metabolic capacity. *Brain Res Brain Res Rev* 48(1):1–15.
11. Crews D, Lou W, Fleming A, Ogawa S (2006) From gene networks underlying sex determination and gonadal differentiation to the development of neural networks regulating sociosexual behavior. *Brain Res* 1126(1):109–121.
12. Herman JP, Flak J, Jankord R (2008) Chronic stress plasticity in the hypothalamic paraventricular nucleus. *Prog Brain Res* 170:353–364.
13. Scarpino S, Gillette R, Crews D (2012) MultiDimBio: An R package for the functional landscape analysis of multivariate data. *J Stat Softw*, in press.
14. Tipping ME, Bishop CM (1999) Probabilistic principal component analysis. *J R Statist Soc B* 61:611–622.
15. Stacklies W, Redestig H, Scholz M, Walther D, Selbig J (2007) pcaMethods—a bioconductor package providing PCA methods for incomplete data. *Bioinformatics* 23:1164–1167.
16. Strimmer K (2008) fdrtool: A versatile R package for estimating local and tail area-based false discovery rates. *Bioinformatics* 24:1461–1462.
17. R Development Core Team (2011) *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, Vienna, Austria).
18. Nilsson EE, et al. (2010) Gene bionetwork analysis of ovarian primordial follicle development. *PLoS ONE* 5:e11637.
19. Draghici S, et al. (2007) A systems biology approach for pathway level analysis. *Genome Res* 17:1537–1545.
20. Bellani R, Luecken LJ, Conrad CD, Conrad CD (2006) Peripubertal anxiety profile can predict predisposition to spatial memory impairments following chronic stress. *Behav Brain Res* 166(2):263–270.

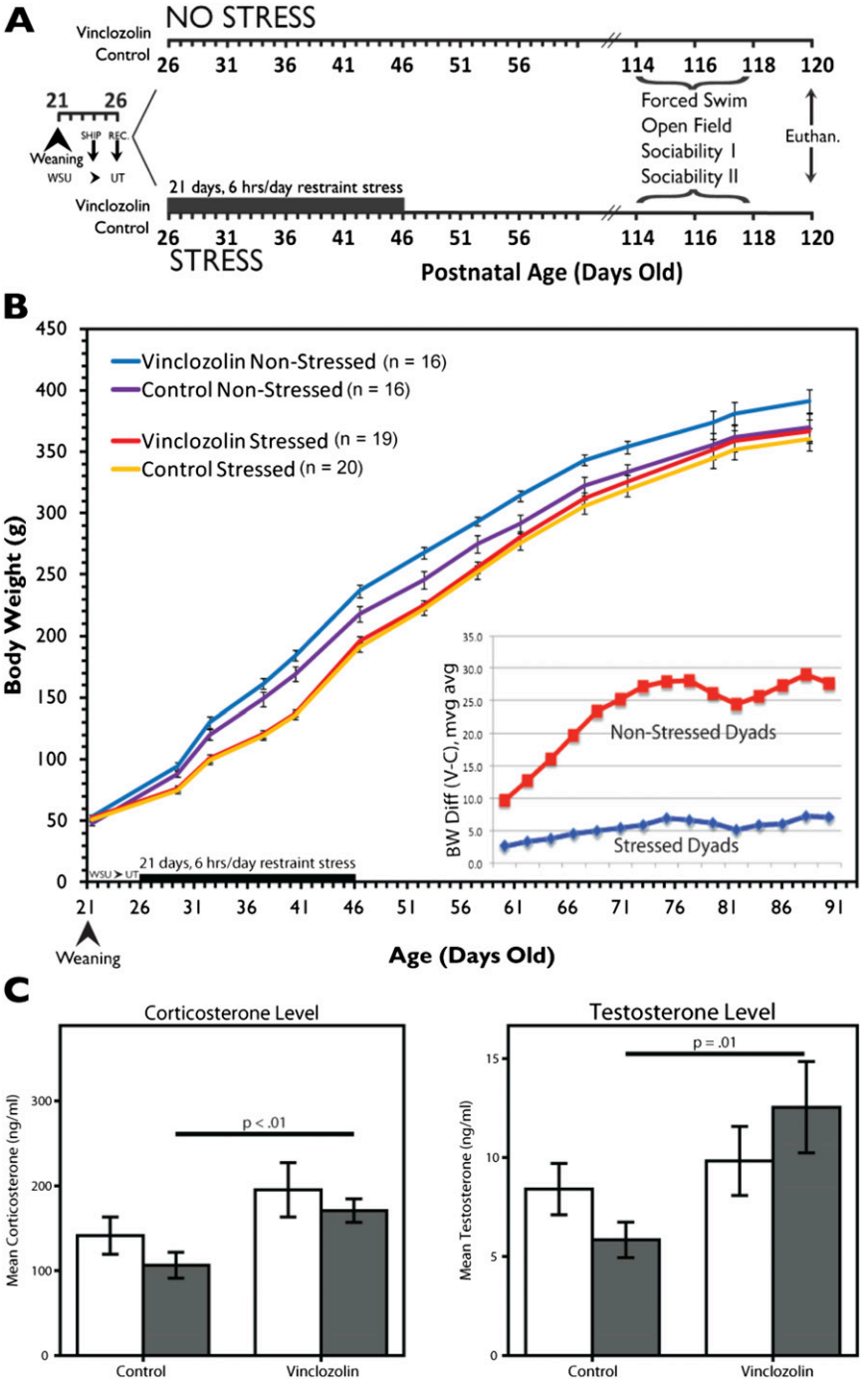


Fig. S1. Experimental design and phenotypic analysis. (*A*) Paradigm to test the interaction of germ line-dependent (V-L) and context-dependent (CRS during adolescence) epigenetic effects on morphology, physiology, behavior, metabolism, and gene networks in the brain. Individual male rats were weaned at PND 21 and then shipped from WSU to UTA. On receipt, each V-L male was paired with a vehicle C-L male and housed together thereafter. On the day after receipt, half of the pairs were subjected to a regimen of CRS daily for 6 h for 21 d, whereas the other half of the pairs were not stressed. (*B*) Effects of germ line-dependent (V-L) and context-dependent (stress) epigenetic modifications on BW. Animals were weighed at weaning (PND 21) at WSU and again on receipt at UTA (PND 25). Illustrated is group mean BW (g) ± SEM. Purple, C-L nonstress condition; blue, V-L nonstress condition; yellow, C-L stress condition; and red, V-L stress condition. BW in each group was also compared. C-L stress condition and V-L stress condition are significantly lower than C-L nonstress condition at PND 29 ($P = 0.003$ and $P = 0.012$, respectively), PND 32 ($P < 0.001$ for both), PND 37 ($P < 0.001$ for both), PND 40 ($P < 0.001$ for both), PND 46 ($P = 0.007$ and $P = 0.008$, respectively), and PND 52 ($P = 0.016$ and $P = 0.003$, respectively). At PND 57 only, C-L stress group has a lower BW than C-L nonstress group ($P = 0.009$). V-L nonstress condition had a significantly higher BW than C-L nonstress condition at PND 46 ($P = 0.048$), PND 52 ($P = 0.013$), PND 61 ($P = 0.021$), and PND 67 ($P = 0.046$). In the nonstress groups, the V-L males had higher BW than did C-L at PND 52 ($P = 0.026$) and PND 96 ($P = 0.046$). There was no significant BW difference between the C-L stress condition and V-L stress condition males. In C-L, CRS had a significant effect on BW at PND 29 ($P = 0.002$), PND 32.5 ($P < 0.002$), PND 37 ($P < 0.001$), PND 40 ($P < 0.001$), PND 46 ($P = 0.003$), PND 52 ($P = 0.003$), PND 57 ($P = 0.003$), PND 61 ($P = 0.028$), and PND 67 ($P = 0.03$). In the nonstress groups, V-L males tend to have a higher BW than C-L males do at PND 52 and PND 96. However, this lineage-specific BW difference was not seen in stress groups. CRS resulted in a decrease in BW in both lineages. This effect diminished after animals had recovered from the CRS treatment (PND 67) in the C-L males. However,

Legend continued on following page

BW in the stress condition continued to be lower than in the nonstress condition in the V-L animals. Exposure to CRS did not seem to have differential effect between two lineages; however, it had a significant effect within a lineage, particularly in V-L males. In these males, the decrease in BW because of the CRS lasted longer than in the C-L males. Therefore, the transgenerational influence of vinclozolin does not affect BW in a synergistic fashion with exposure to CRS; rather, it changes sensitivity toward to external stress during this important life-history stage. (*Inset*) Graph shows the moving average (n = 3 consecutive weighings) of weight differences within each dyad for stress (red) and nonstress (blue) (V-L minus C-L). (C) Circulating concentrations of CORT and TESTO in V-L and C-L males that received CRS (gray bars). Comparison is with males that did not receive CRS (white bars).

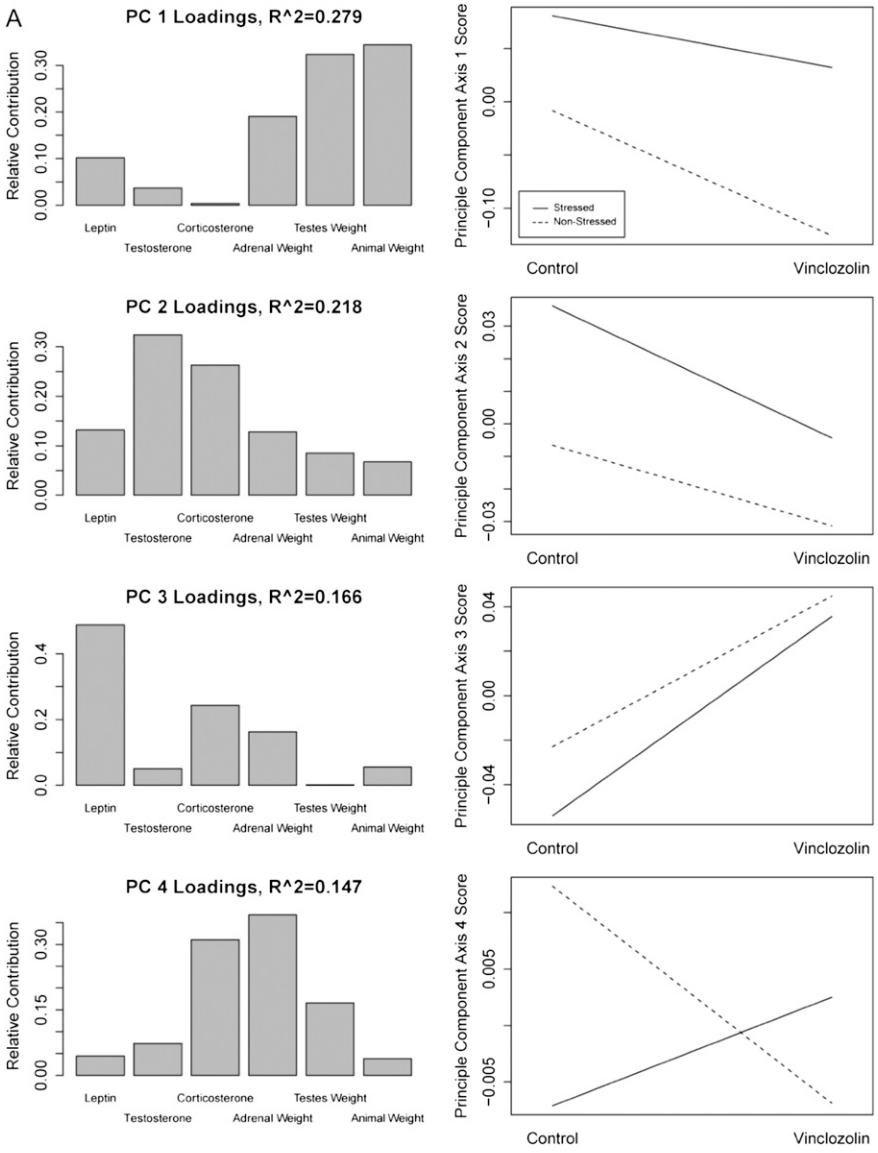


Fig. S2. (Continued)

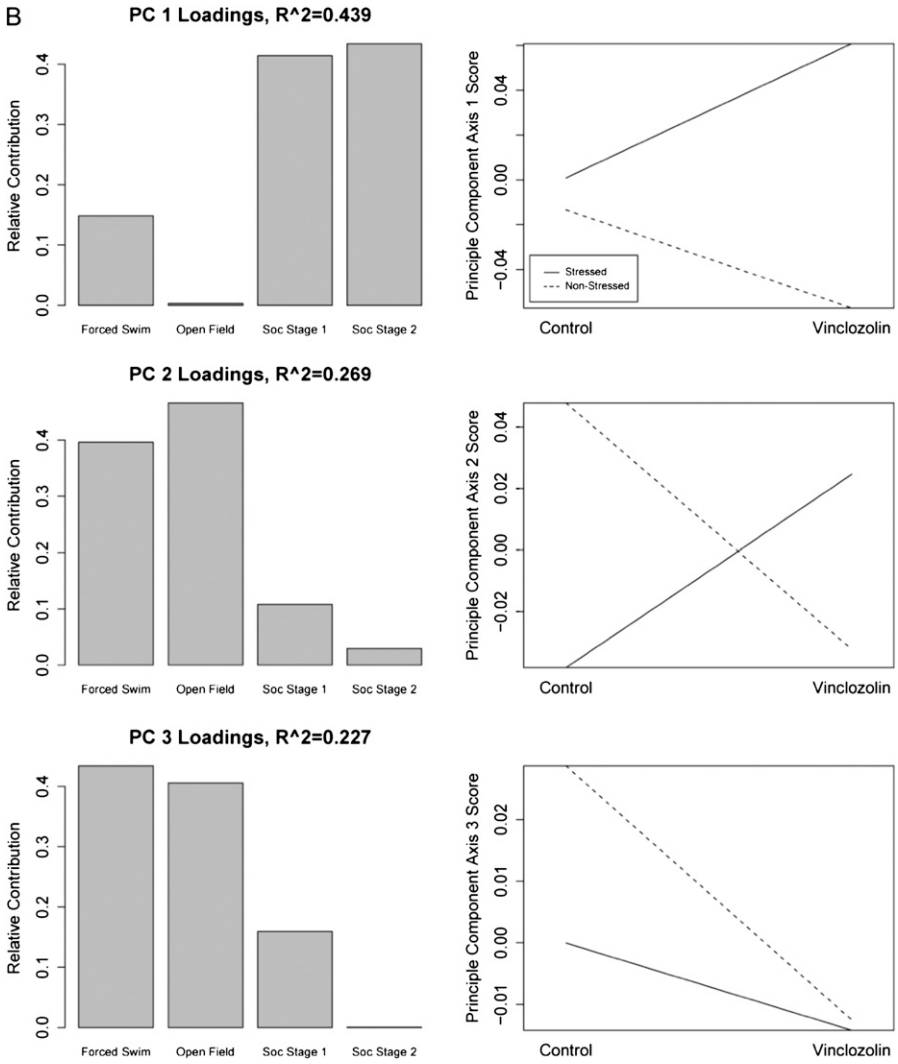


Fig. S2. (Continued)

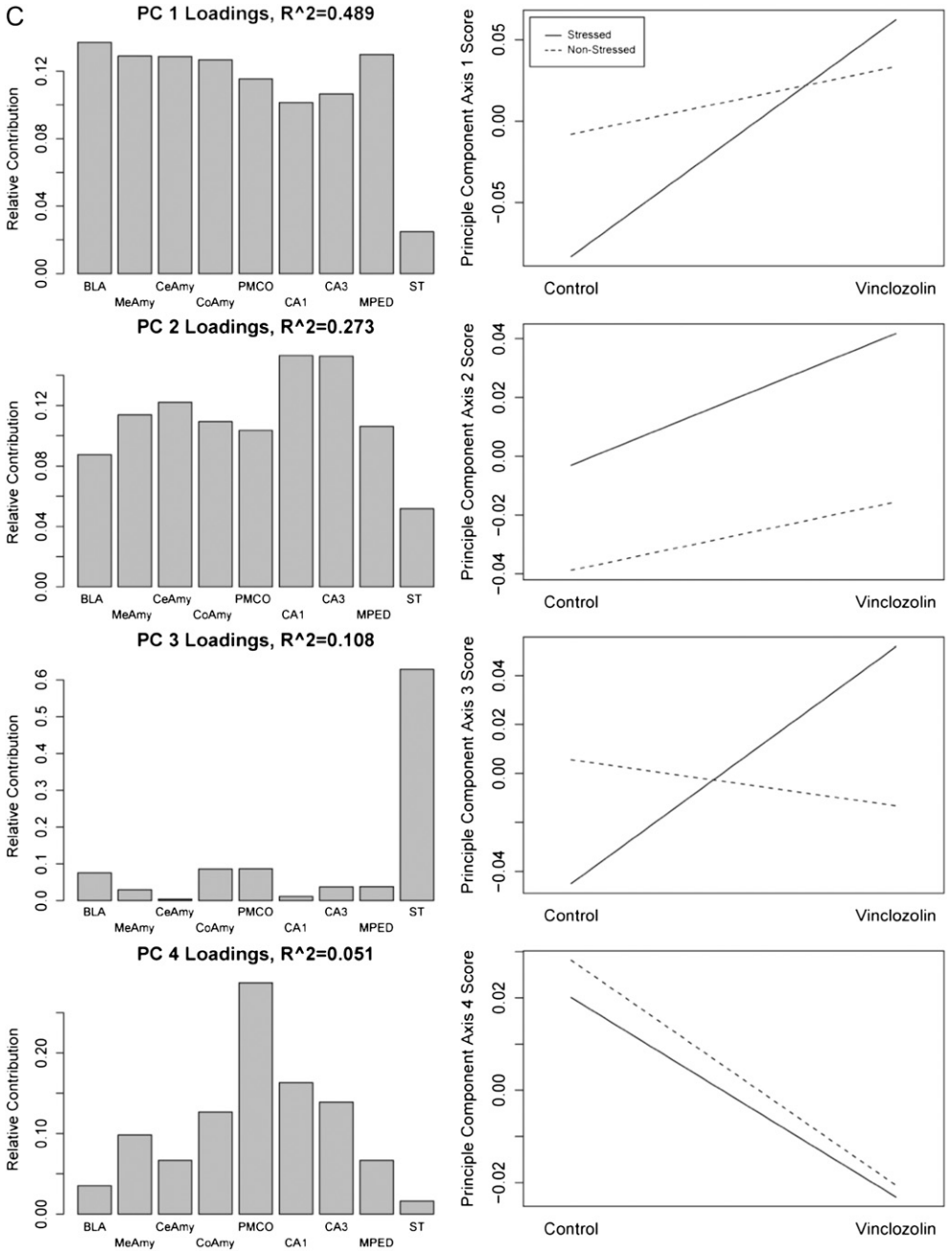


Fig. S2. Phenotype analysis. (*A*) Relative importance for each physiological trait (leptin, TESTO, CORT, adrenal weight, testes weight, and BW) across the four principal component axes. On the left are the four principal component axes (PC1–PC4). The loading score for each nuclei is transformed into the relative weight by taking the absolute value and dividing by the sum of the loadings across all nuclei. A value of 1 indicates that the axis is entirely determined by that nucleus, whereas a value of 0 indicates that nucleus contributes no information to that axis. On the right are the interaction plots for each principal component axis. NS, not significant. PC1: stress ($F = 17.8816$, $P < 0.001$, qFDR = 0.002), lineage ($F = 7.5362$, $P = 0.001$, qFDR = 0.019), interaction (NS). PC2: stress (NS), lineage (NS), interaction (NS). PC3: stress (NS), lineage ($F = 7.3091$, $P = 0.01$, qFDR = 0.021), interaction (NS). PC4: stress (NS), lineage (NS), interaction (NS). Significance for both main effects and interactions were determined by using ANOVAs and a false discovery rate correction (with qFDR < 0.05 being significant). (*B*) Relative importance for each behavioral trait (FS, OF, Soc 1, and Soc 2) across the three principal component axes. On the left are the three principal component axes (PC1–PC3). The loading score for each nuclei is transformed into the relative weight by taking the absolute value and dividing by the sum of the loadings across all nuclei. A value of 1 indicates that the axis is entirely determined by that nucleus, whereas a value of 0 indicates that nucleus contributes no information to that axis. On the right are the interaction plots for each principal component axis. NS, not significant. PC1: stress (NS), lineage (NS), interaction (NS). PC2: stress (NS), lineage (NS), interaction (NS). PC3: stress (NS), lineage (NS), interaction (NS). Significance for both main effects and interactions were determined by using ANOVAs and a false discovery rate correction (with qFDR < 0.05 being significant). (*C*) Relative importance for metabolic activity in each brain nucleus (BLA, MeAmy, CeAmy, CoAmy, PMCo, CA1, CA3, MePD, and ST) measured across the first four principal component axes. On the left are the four principal component axes (PC1–PC4). The loading score for each nuclei is transformed into the relative weight by taking the absolute value and dividing by the sum of the loadings across all nuclei. A value of 1 indicates that the axis is entirely determined by that nucleus, whereas a value of 0 indicates that nucleus contributes no information to that axis. On the right are the interaction plots for each principal component axis. An interaction plot represents the effect of treatment and stress for each of the four axes. NS, not significant. PC1; stress, (NS), lineage ($F = 6.545$, $P = 0.016$, qFDR = 0.028), interaction (NS). PC2: stress (NS), lineage (NS), interaction (NS). PC3: stress (NS), lineage (NS), interaction ($F = 4.639$, $P = 0.040$, qFDR = 0.055). PC4: stress (NS), lineage ($F = 8.60$, $P = 0.007$, qFDR = 0.021). Significance for both main effects and interactions were determined by using ANOVAs and a false discovery rate correction (with qFDR < 0.05 being significant).

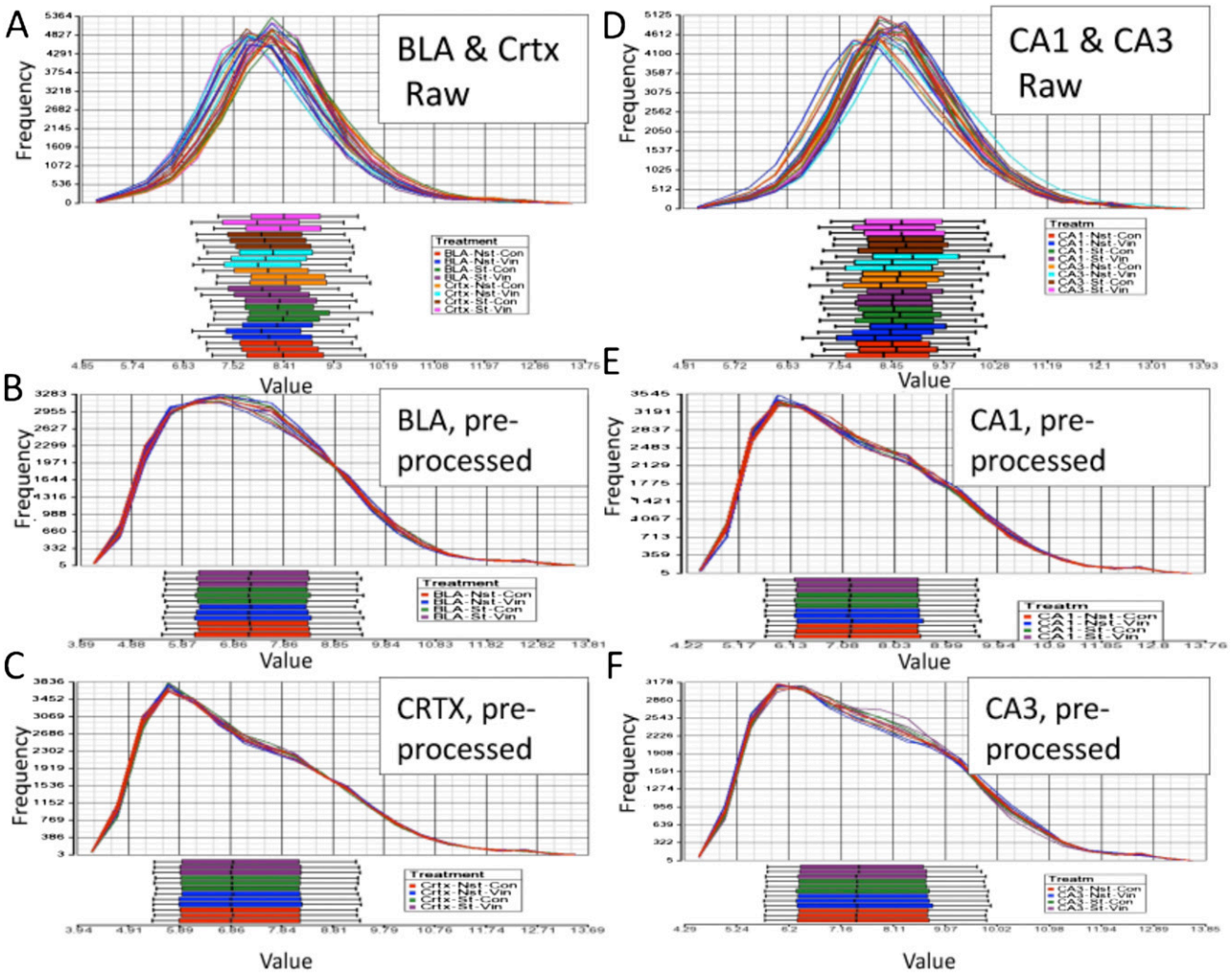


Fig. S3. Sample histograms and box plots for raw microarray signals (*A* and *D*) or for signals preprocessed with RMA GC content-adjusted algorithm (*B*, *C*, *E*, and *D*) for CRTX (*A* and *C*), BLA (*A* and *B*), CA1 (*D* and *E*), and CA3 (*D* and *F*).

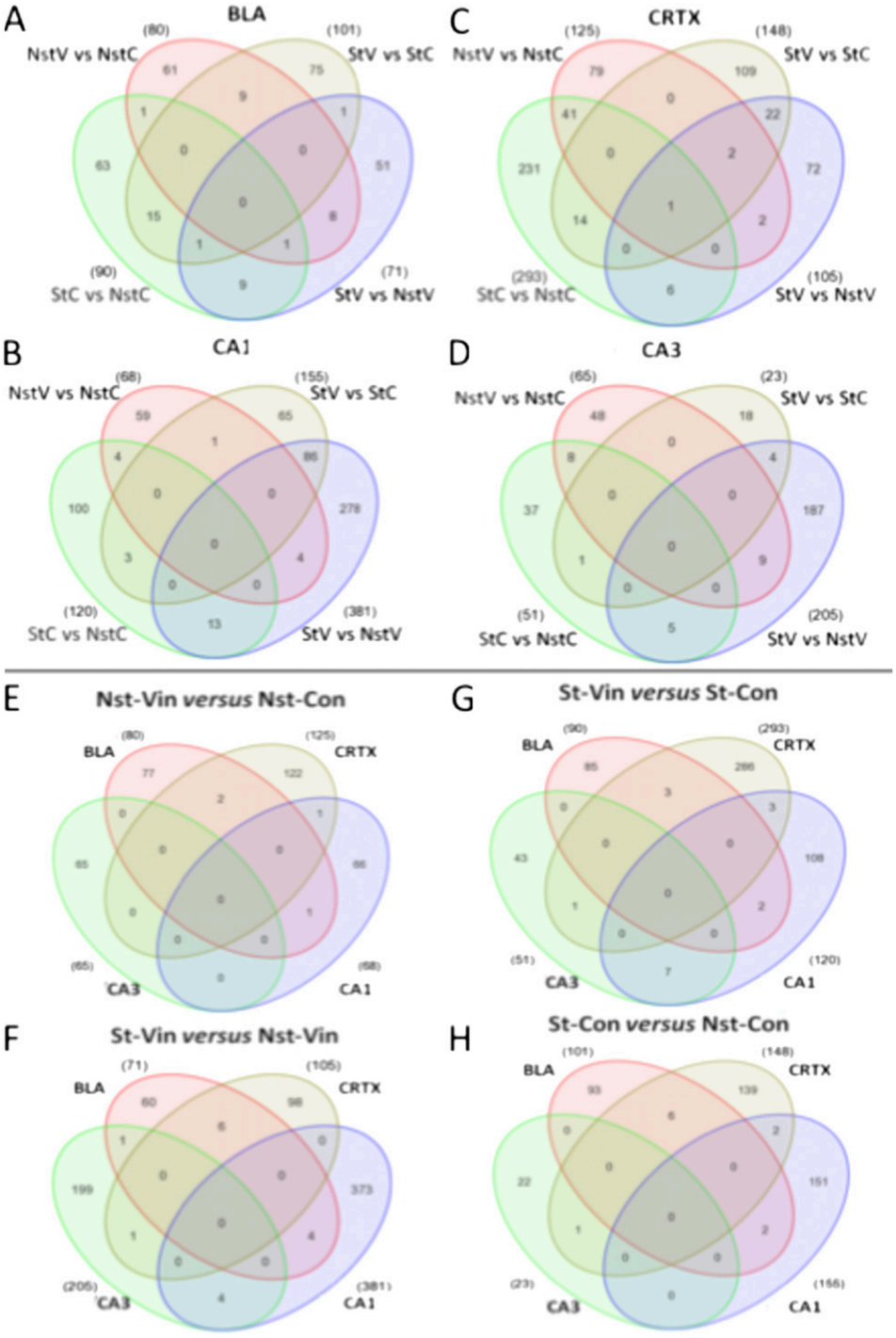


Fig. S4. Venn diagrams of four differentially expressed gene lists obtained by contrasting VNS V-L nonstress condition vs. C-L nonstress condition (NstV vs. NstC), V-L stress condition vs. C-L stress condition (StV vs. StC), C-L stress condition vs. C-L nonstress condition (StC vs. NstC), or V-L stress condition vs. V-L nonstress condition (StV vs. NstV) shown for CRTX (*C*), BLA (*A*), CA1 (*B*), and CA3 (*D*). (*E*, *F*, *G*, and *H*) Venn diagrams for the same lists showing how each of the four brain regions overlap. Numbers in brackets show number of differentially expressed probe sets.

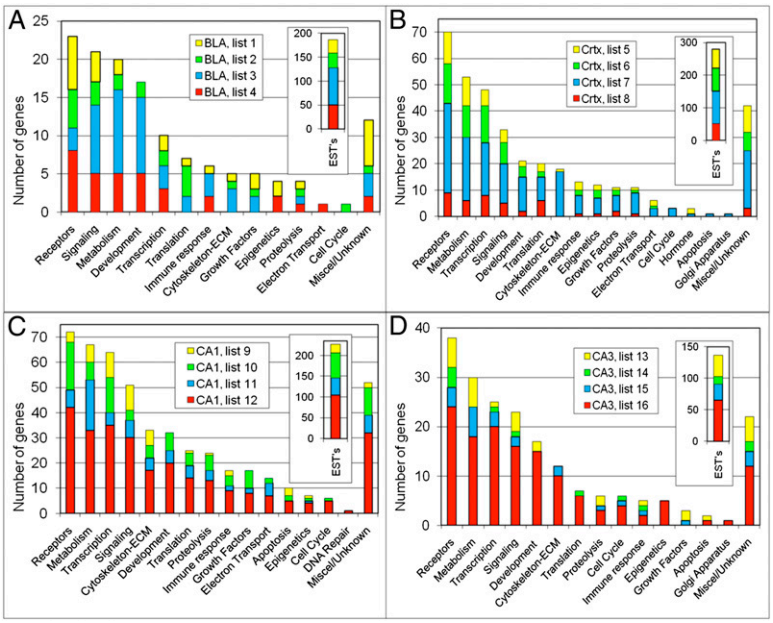


Fig. S5. Bar graph showing the number of genes differentially expressed and associated with functional categories in the brain. (*A*) BLA. (*B*) CRTX. (*C*) CA1. (*D*) CA3. Yellow, V-L nonstress vs. C-L nonstress differentially expressed genes (lists 1, 5, 9, and 13); green, V-L stress vs. C-L stress (lists 2, 6, 10, and 14); blue, C-L stress vs. C-L nonstress (lists 3, 7, 11, and 15); and red, V-L stress vs. V-L nonstress (lists 4, 8, 12, 16).

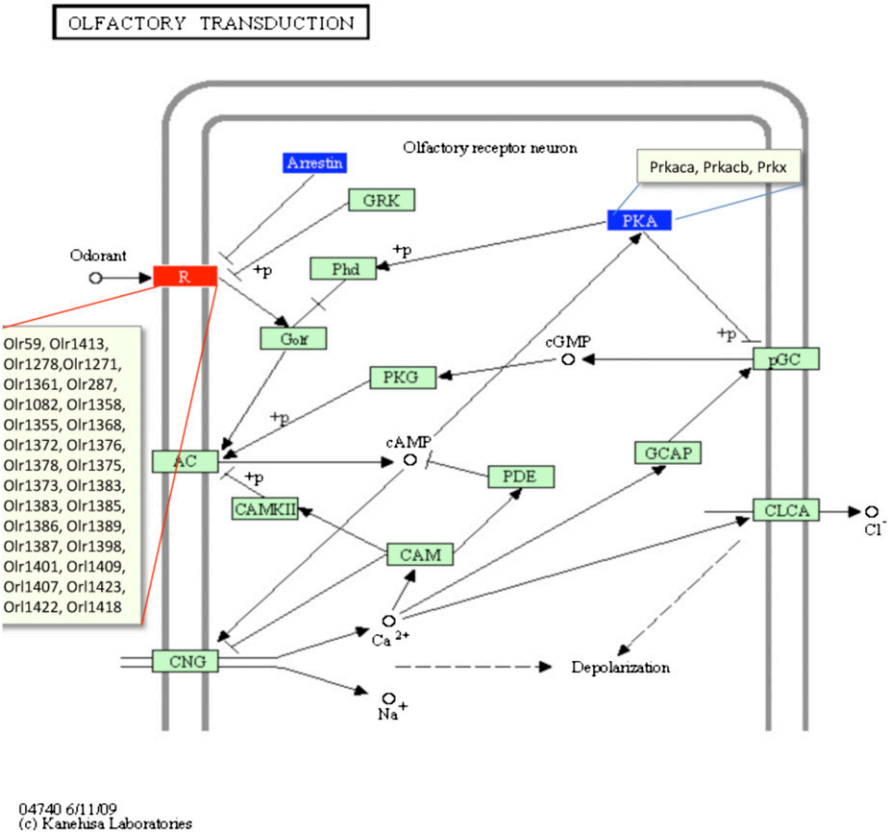


Fig. S6. Olfactory transduction pathway (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) showing genes affected by either CRS during adolescence (stress) or transgenerational epigenetic modification by vinclozolin (lineage) at least in one of four studied brain regions: CRTX, BLA, CA1, or CA3. Red box, up-regulated ; blue boxes, down-regulated; green boxes, not affected. Extended lists of genes for two pathway entities (R and PKA) are shown in beige boxes.

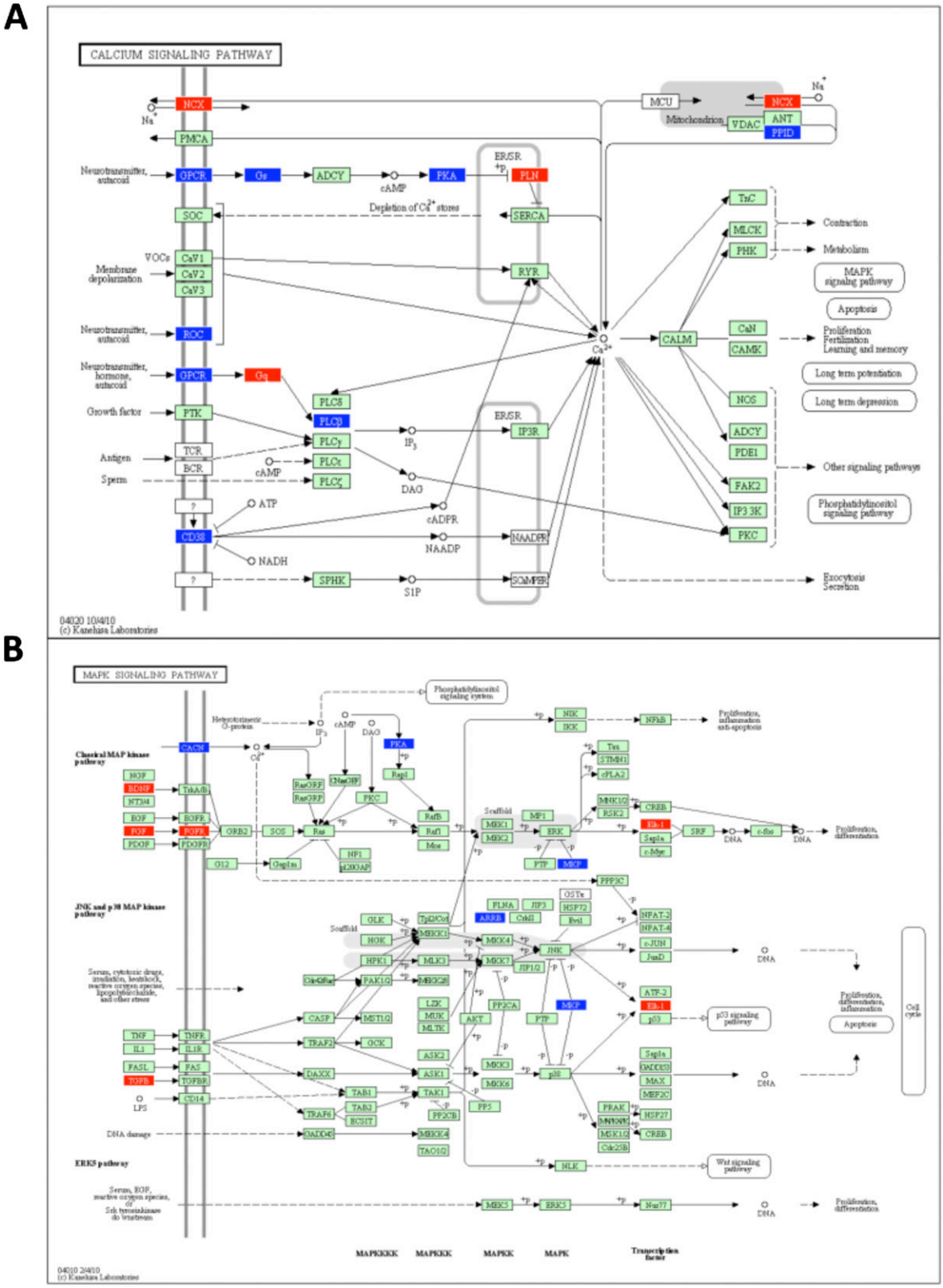


Fig. S7. Calcium signaling pathway (*A*) and MAPK signaling pathway (*B*) (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) showing genes affected by either stress or lineage at least in one of four studied brain regions: CRTX, BLA, CA1, and CA3. Red boxes are up-regulated; blue boxes are down-regulated; and green boxes are not affected.

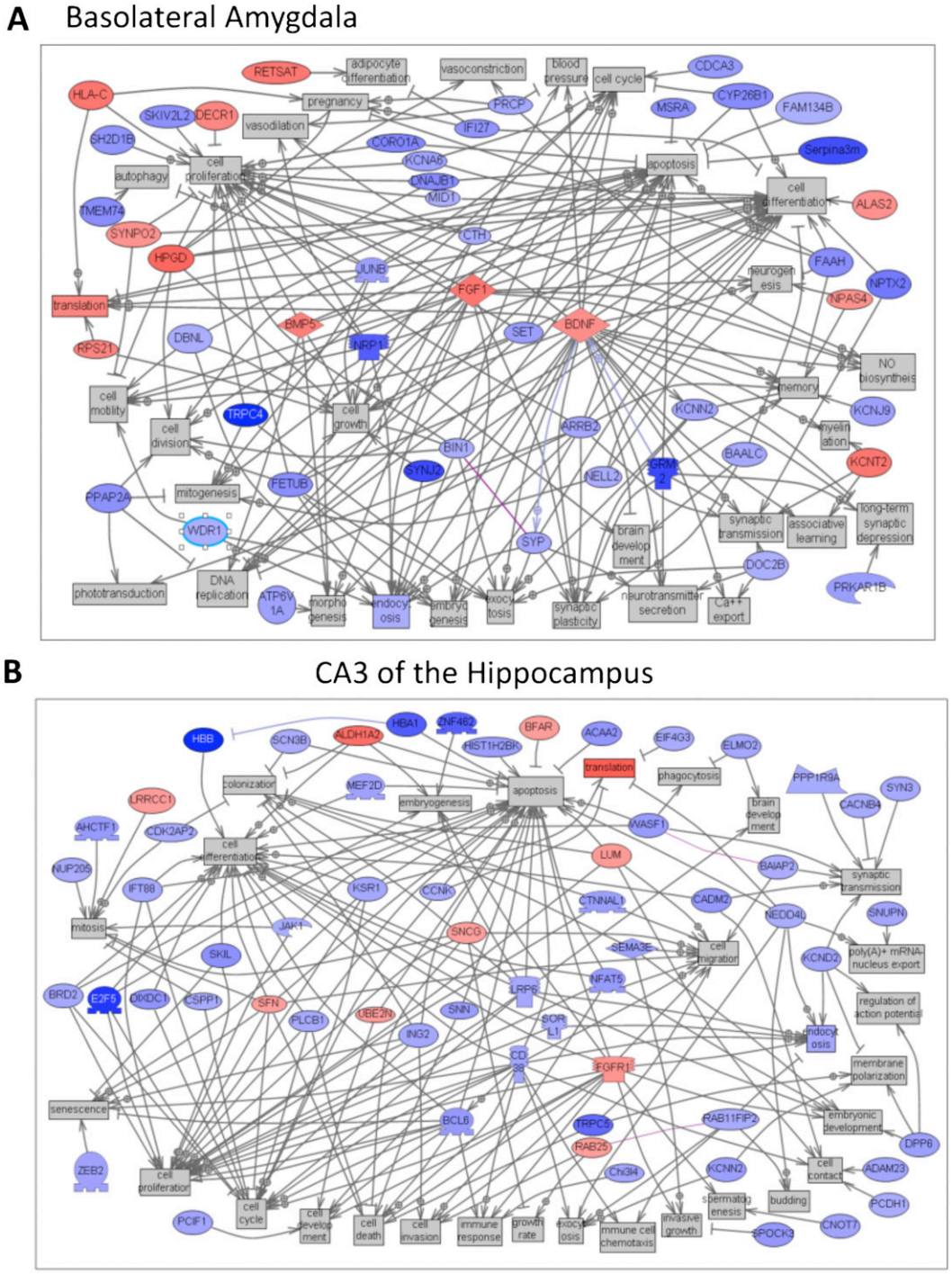


Fig. S8. Subnetworks of shortest connections including cell processes for genes affected by either stress or lineage in BLA (combined gene lists 1–4; *A*) or CA3 (combined gene lists 13–16; *B*) obtained by global literature analysis using Pathway Studio 7.0 software (Ariadne Genomics). (*A*) For BLA, 49 affected genes of 125 genes (no ESTs included) are shown as red or blue. (*B*) For CA3, 60 affected genes of 198 genes (no ESTs) are shown as red or blue. The rest of affected genes are not connected and not shown. Oval and circle, protein; diamond, ligand; ice cream cone, receptor; circle/oval on tripod platform, transcription factor; crescent, protein kinase and kinase; irregular polygon, phosphatase. Red, up-regulated genes; blue, down-regulated genes; arrows with plus sign, positive regulation/activation; arrows with minus sign, negative regulation/inhibition; gray arrows, regulation; lilac, expression; purple, binding; green, promoter binding.

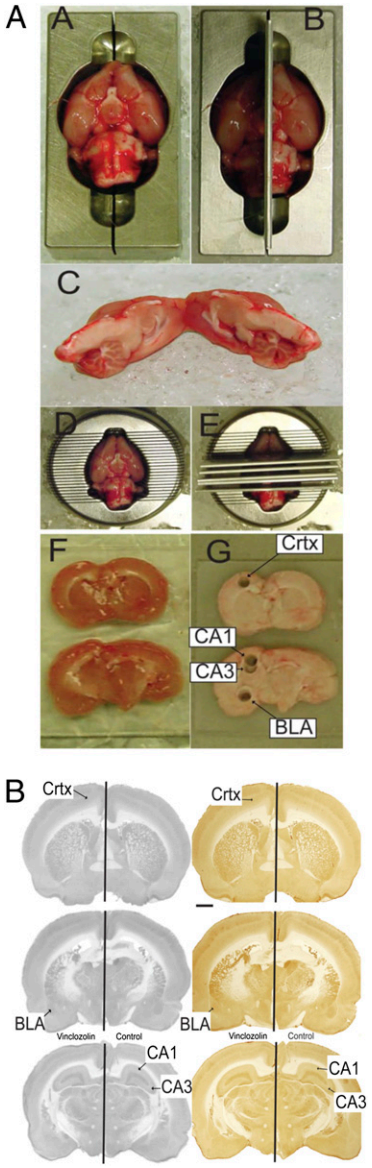
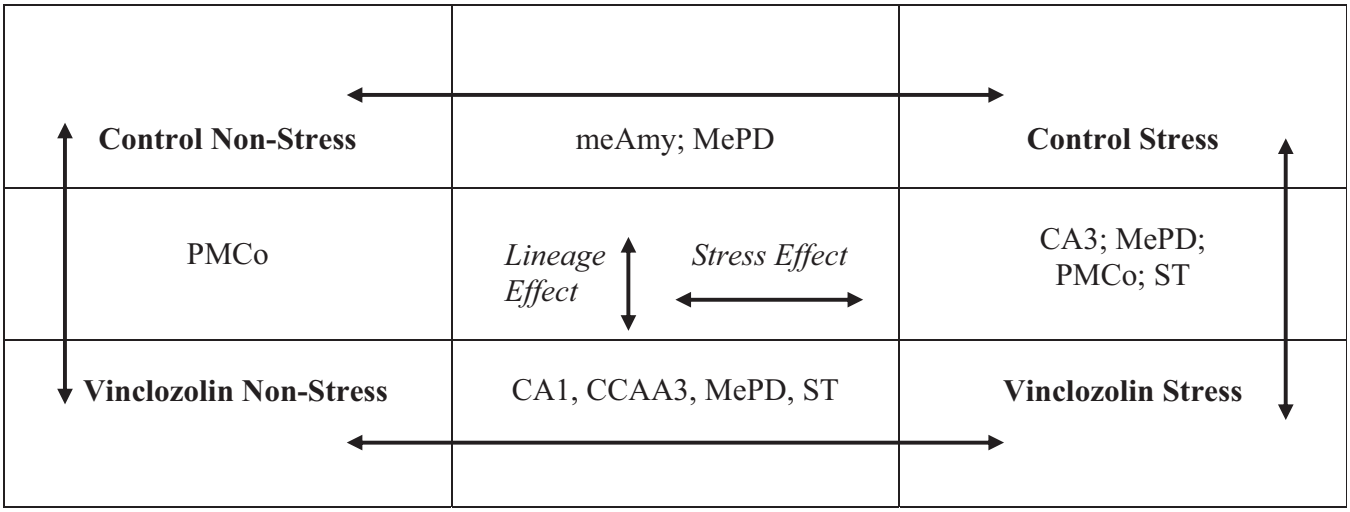


Fig. S9. Images of different stages of brain processing. (*A*) Rats from one dyad were processed simultaneously. Before freezing, the brain was cut sagittally along the midline. The symmetrical brain halves were then recombined with one side from the vinclozolin individual and the other from the control individual; the sides used were alternated to control for possible asymmetries in activity. One such "brain" was used for metabolic studies and the other for RNA studies. (*AA*) Brain in sagittal block. (*AB*) Razor cutting brain along midline in sagittal plane. (*AC*) Two halves of brain shown. (*AD*) Recombining of brain halves; one half is from the V-L individual and the other from the C-L individual in the dyad. (*AE*) Brain being blocked into 2-mm slices. (*AF*) Target slices of recombined brain. (*AG*) Frozen slices of recombined brain showing 1-mm punches on one half (one individual) of sampled brain areas. (*B*) Cytochrome oxidase histochemistry of 40-μm sections of a recombined brain (left half is from V-L individual and right half is from C-L individual). Different levels indicate different brain regions analyzed: BLA, CA1, and CA3.

Table S1. Metabolic activity (abundance of cytochrome oxidase) in brain nuclei in V-L and C-L male rats that received CRS during adolescence

	Mean activity value				Percentage of maximum				Percentage change			
Nucleus	CNS	VNS	CS	VS	CNS	VNS	CS	VS	CNS vs. VNS	CS vs. VS	CNS vs. CS	VNS vs. VS
BLA	207.70	198.80	213.36	200.69	97.3	93.2	100.0	94.1	−4.2	−5.9	2.7	0.9
BnSTl	208.82	204.36	203.06	207.47	100.0	97.9	97.2	99.4	−2.1	2.1	−2.8	1.5
BnSTm	205.58	197.59	204.33	201.59	100.0	96.1	99.4	98.1	−3.9	−1.3	−0.6	1.9
CA1	157.95	157.41	152.34	141.65	100.0	99.7	96.4	89.7	−0.3	−6.8	−3.6	**−10.0**
CA3	159.54	156.31	160.11	142.16	99.6	97.6	100.0	88.8	−2.0	**−11.2**	0.4	**−8.8**
CeAmy	175.72	178.02	174.78	168.64	98.7	100.0	98.2	94.7	1.3	−3.5	−0.5	−5.3
CoAmy	159.91	152.69	161.40	157.46	99.1	94.6	100.0	97.6	−4.5	−2.4	0.9	3.0
CRTX	223.69	236.90	235.57	229.76	94.4	100.0	99.4	97.0	5.6	−2.5	5.0	−3.0
MeAmy	152.50	162.73	166.48	165.13	91.6	97.7	100.0	99.2	6.1	−0.8	**8.4**	1.4
MePD	205.56	203.31	226.58	185.28	90.7	89.7	100.0	81.8	−1.0	**−18.2**	**9.3**	**−8.0**
MPOA	201.14	197.50	198.19	189.12	100.0	98.2	98.5	94.0	−1.8	−4.5	−1.5	−4.2
PMCo	221.05	182.06	210.09	180.53	100.0	82.4	95.0	81.7	**−17.64**	**−13.4**	−5.0	−0.7
ST	245.28	247.57	241.44	274.92	89.2	90.0	87.8	100.0	0.8	**12.2**	−1.4	**10.0**
VMH	202.36	191.03	207.71	202.27	97.4	92.0	100.0	97.4	−5.5	−2.6	2.6	5.4



Boldface values in the percentage change columns indicate comparisons that were equal to or greater than 8% change. Schematic indicates those bolded nuclei in terms of comparison (lineage comparisons vertical vs. stress comparisons horizontal), indicated in the center cell of the 3 × 3 grid.

Table S2. Abbreviations for brain nuclei imaged for cytochrome histochemistry abundance (per ref. 1)

Abbreviation	Definition
BLA	Basolateral amygdaloid nucleus
BnSTl	Bed nucleus of the stria terminalis, anterolateral division
BnSTm	Bed nucleus of the stria terminalis, anteromedial division
CA1	CA1 area of the hippocampus
CA3	CA3 area of the hippocampus
CeAmy	Central amygdaloid nucleus
CoAmy	Anterior cortical amygdaloid nucleus
CRTX	Primary and secondary motor cortex
MeAmy	Medial amygdaloid nucleus
MePD	Medial amygdaloid nucleus, posterior dorsal part
MPOA	Medial preoptic area
PMCo	Posteromedial cortical amygdaloid nucleus
PVN	Periventricular hypothalamic nucleus
ST	Stria terminalis
VMH	Ventromedial hypothalamic nucleus

1. Paxinos G, Watson C (2007) *The Rat Brain in Stereotaxic Coordinates* (Academic, New York).

Table S3. Altered gene sets for V-L and C-L male rats subjected to CRS during adolescence

List no.	Effect	Comparison	No. of altered genes
BLA			
1	Lineage	Nonstress V-L vs. nonstress C-L	79
2	Lineage	Stress V-L vs. stress C-L	101
3	Stress	Stress C-L vs. nonstress C-L	81
4	Stress	Stress V-L vs. nonstress V-L	62
CRTX			
5	Lineage	Nonstress V-L vs. nonstress C-L	118
6	Lineage	Stress V-L vs. stress C-L	142
7	Stress	Stress C-L vs. nonstress C-L	290
8	Stress	Stress V-L vs. nonstress V-L	96
CA1			
9	Lineage	Nonstress V-L vs. nonstress C-L	68
10	Lineage	Stress V-L vs. stress C-L	155
11	Stress	Stress C-L vs. nonstress C-L	118
12	Stress	Stress V-L vs. nonstress V-L	379
CA3			
13	Lineage	Nonstress V-L vs. nonstress C-L	64
14	Lineage	Stress V-L vs. stress C-L	23
15	Stress	Stress C-L vs. nonstress C-L	50
16	Stress	Stress V-L vs. nonstress V-L	202

Table S4. Number of altered genes in specific brain regions according to signaling pathways and cellular processes

		No. of genes altered in tissue list no.															
		BLA				CRTX				CA1				CA3			
Pathway name	No. of altered genes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Olfactory transduction	78	3	4	1	4	6	8	17	2	3	9	3	17		4	2	9
Calcium signaling pathway	18	1				1	4	3		1	1		6		1		1
Neuroactive ligand–receptor interaction	16	1			1	2	3	2					7				
MAPK signaling pathway	13		1	2	1		1		1		2	1	4	1			2
Huntington disease	13		1	1		1	1	2			1	4	3				1
Pathways in cancer	12			1		1		2	1	1	1	1	3	1			2
Endocytosis	11		1	1	2			1			2		4	1			2
Alzheimer's disease	11					1		2				4	3				1
Cell adhesion molecules (CAMs)	10	1			1			3			3	1	3				
Cytokine–cytokine receptor interaction	10							4			5		5				
Axon guidance	9			3		1					1		2	1			3
Metabolism of xenobiotics by P450	9					1		1			1	2	4	1			
Drug metabolism, cytochrome P450	9					1		1			1	2	4	1			
Phagosome	9			2	1			1			4		4				
Oxidative phosphorylation	9			1		1		2				4	1				
Parkinson disease	9					1		2			1	4	2				
Retinol metabolism	8	1			1					1	1		2	1			2
Regulation of actin cytoskeleton	8			1				1	1				2	1			2
PPAR signaling pathway	8						1	2			1		4				1

Dataset S1. Genes expressed differentially in four brain regions in lineage and stress comparisons

Dataset S1 (PDF)