# SUPPLEMENT

# Crystallin Mu in Medial Amygdala Mediates the Impact of Social Experience on Cocaine Seeking in Males but not Females

Walker *et al.*

**Supplemental Figures:**

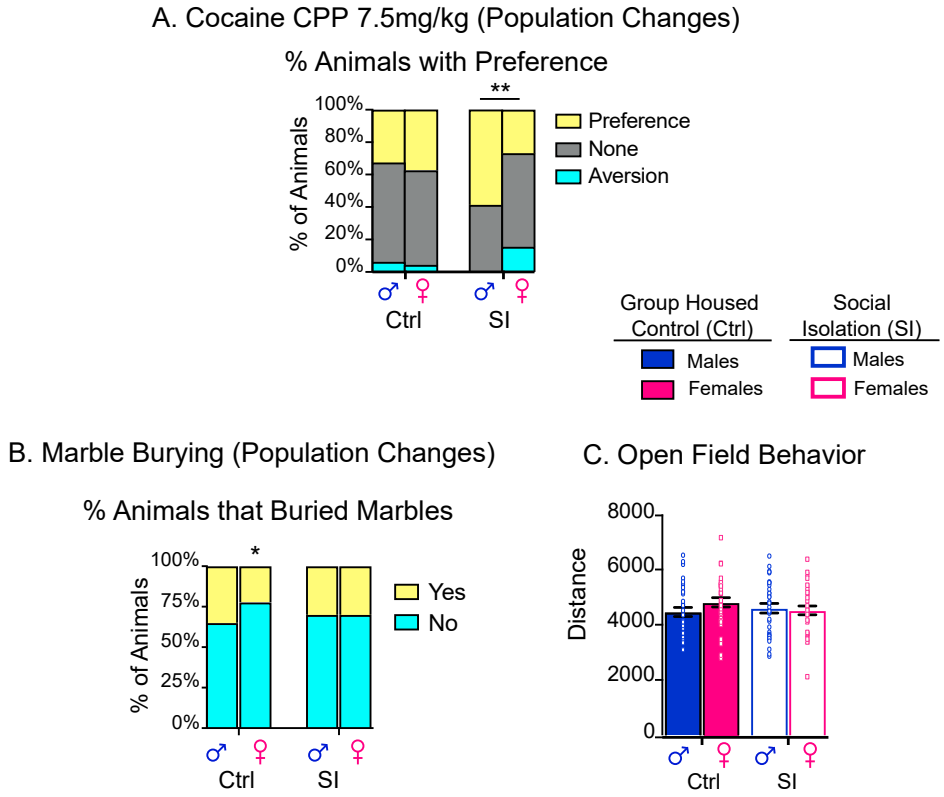


**Figure S1: Adolescent SI Impacts Cocaine CPP and Marble Burying But Has No Effect on Open Field Behavior.**
(A) Adolescent SI results in a gain of sex differences in the proportion of animals that formed a preference (yellow) or aversion (blue) for cocaine in a CPP paradigm (Ctrl males vs. females $\chi^2$=0.29; p=0.87; Isolated males vs. females: $\chi^2$=9.41; p<0.01). (B) Adolescent SI results in the loss of a sex difference in the proportion of animals that buried marbles in marble burying (distribution of animals that buried marbles: Chi squared – group-housed males [Ctrl male] ($\chi^2$=1.8; p=0.2), group-housed females [Ctrl female] ($\chi^2$ = 5.56; p=0.02), Isolated males [SI males] ($\chi^2$=3.2; p=0.07), isolated females [SI females] ($\chi^2$=3.2; p=0.07)). (C) Adolescent SI has no effect on distance traveled in an open field when compared to group-housed control mice (Ctrl). Significant effects indicated as: * = p<0.05; ** = p<0.001.

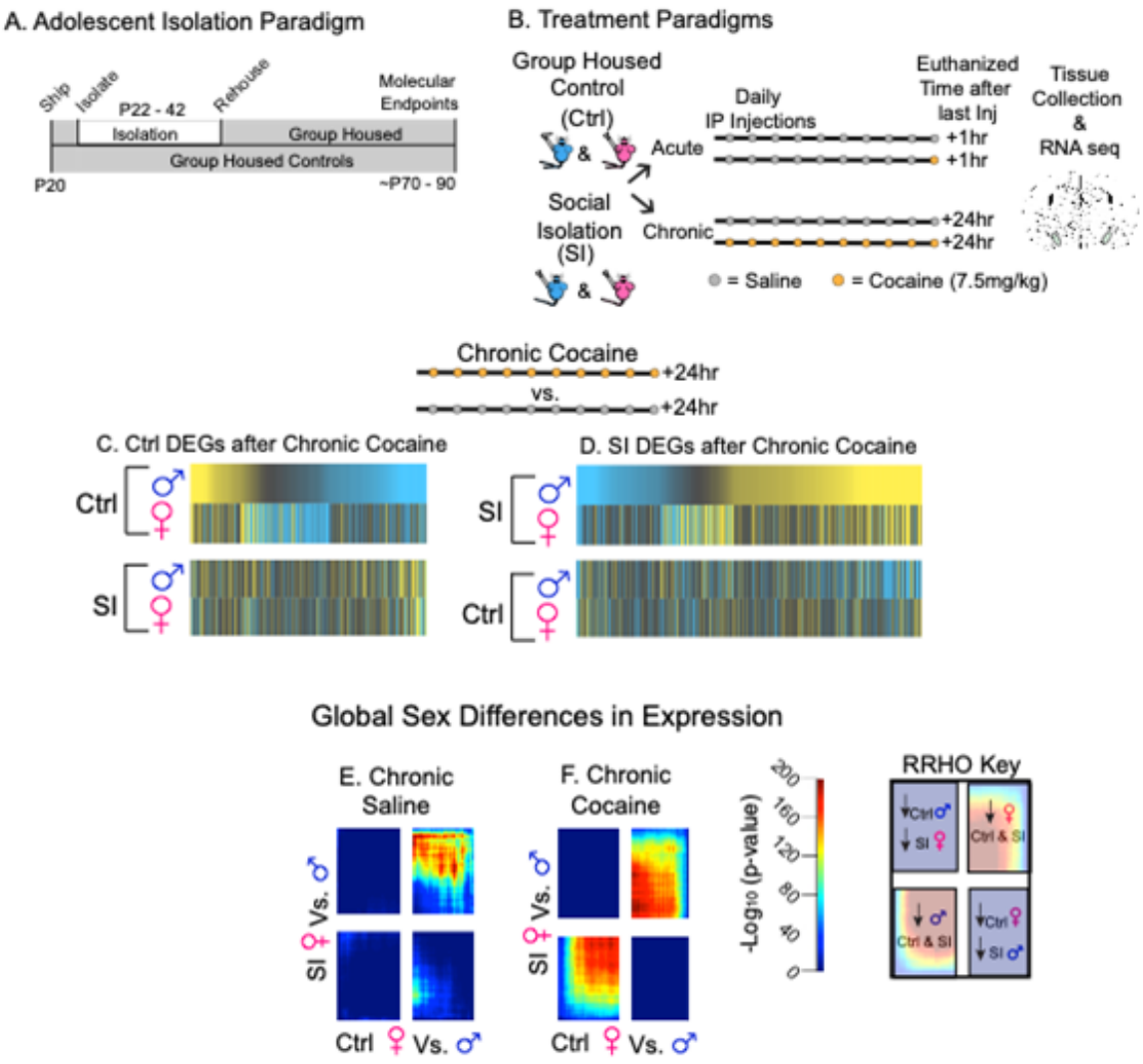


**Figure S2: Adolescent SI Disrupts Cocaine-Induced Gene Expression.**
(A-B) Schematic of isolation (A) and treatment (B) paradigms. (C-D) Union heatmaps of DEGs in group-housed control (C; Ctrl) or isolated mice (D) after chronic cocaine. As with acute cocaine, chronic cocaine induces more robust and opposite transcriptional effects in SI males when compared to their control counterparts (C-D). (E-F) RRHO analysis of transcriptome-wide changes in sex-specific patterns of expression after chronic saline (E) or cocaine (F). Sex differences in expression are disrupted by SI after chronic saline injections (E) but not by repeated cocaine injections (F).

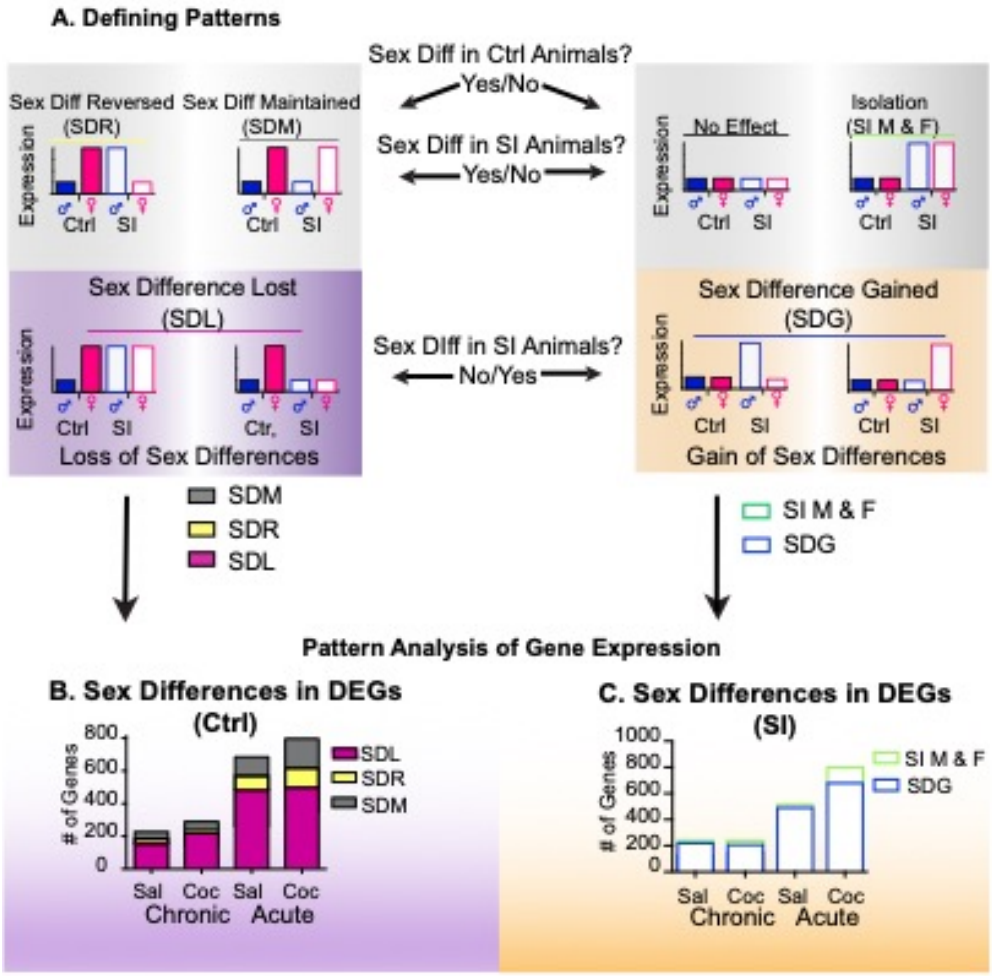


**Figure S3: Pattern Analysis Reveals that Sex Differences in Gene Expression in meA are Lost and Gained after SI.**

(A) Decision tree for categorizing patterns of genes: Genes were first categorized based differences across males and females in group-housed controls (Ctrl) and then categorized based on how that difference changed after SI. Genes that were different between Ctrl males and females (purple background) were categorized as follows: Sex Diff Lost (SDL; pink) if a baseline sex difference is lost after SI; Sex Difference Reversed (SDR; yellow) if a baseline sex difference is reversed by SI; and Sex Difference Maintained (SDM; gray) if a baseline sex difference is maintained after SI. Genes that were not different in Ctrl males vs. females (orange background) that were only affected in SI animals were categorized as: Sex Difference Gained (SDG; blue) if a sex difference in expression was induced in SI animals; and SI males (M) & females (F) (green) if effects were observed in SI animals. (B) Total number of genes that are different between the sexes in Ctrl animals divided by their categories. Genes categorized as SDL are represented across all treatment paradigms and make up >50% of the genes that display sex differences in expression in Ctrls, suggesting that SI results in a loss of expected sex differences in expression across all treatments. (C) Total number of genes that not different between Ctrl males and females but are affected by SI. Genes categorized as SDG are represented across all treatment paradigms and make up >50% of the genes that are not sex-specific in Ctrl suggesting that SI results in a gain of sex-specific expression of genes not expected to be different in males vs. females across all treatments.

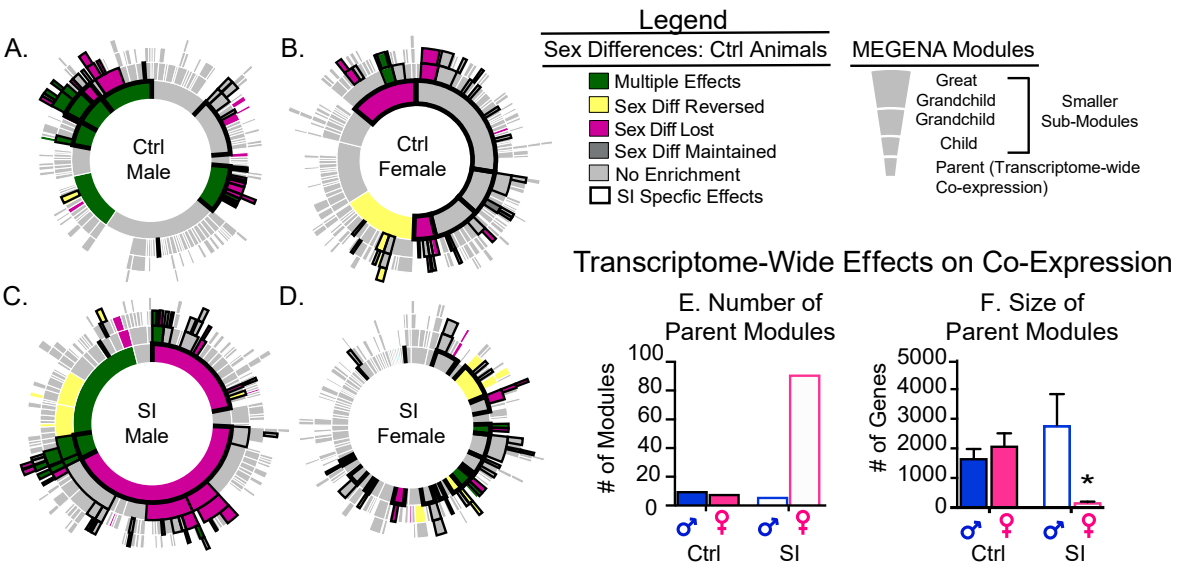


**Figure S4: Sunburst Plots Depicting Gene Modules in meA.**
(A-D) Sunburst plots derived from MEGENA of control (Ctrl) male (A) and female (B) mice compared to SI males (C) and females (D). The inner, middle and outer rings depict "parent" modules and their embedded "children" and "grandchildren" modules, respectively. Colors indicate enrichment of categories of sex-specific genes in Ctrl animals determined from the pattern analysis in each module. Bold outline indicates enrichment of genes affected only in SI animals (SI genes) determined from the pattern analysis. (E-F) Bar graphs of the number of parent modules (E) and average number of genes per parent module (F) in each group. SI females have more modules composed of fewer genes than any other group ($p<0.05$).

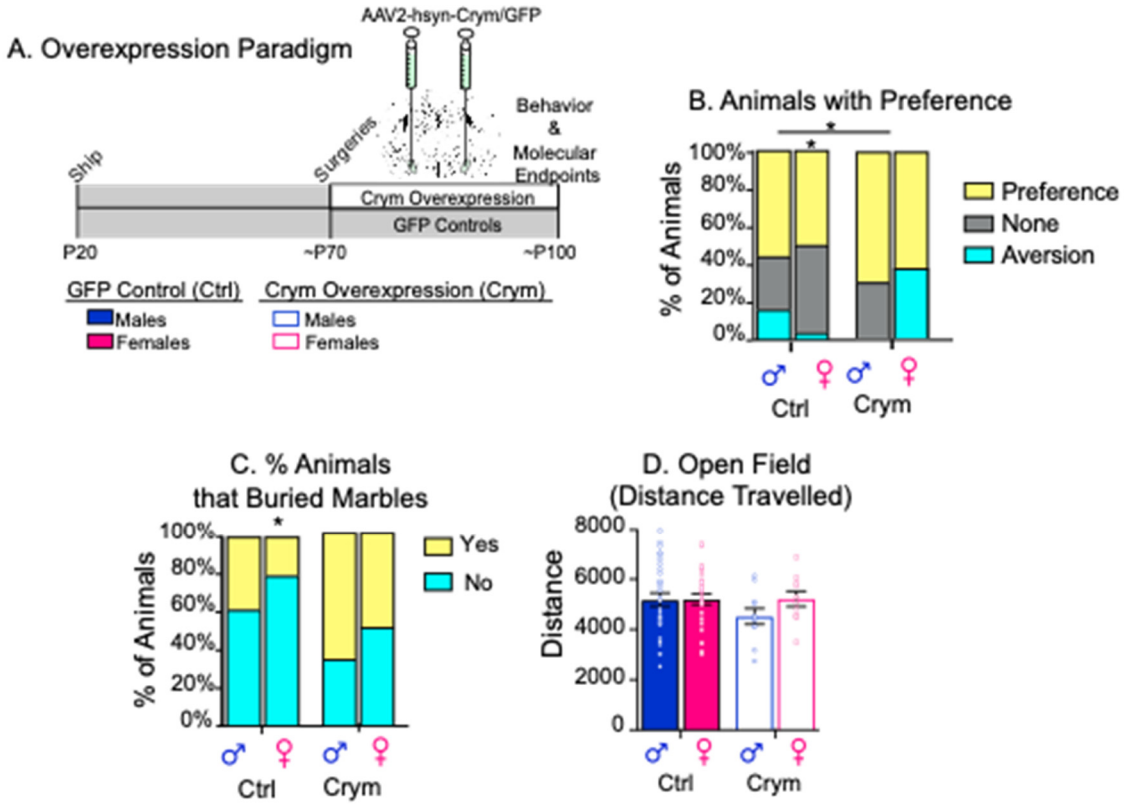


**Figure S5: *Crym* Overexpression in the meA Regulates Cocaine CPP and Marble Burying But Has No Effect on Open Field.**

(A) Schematic of experimental design. (B-D) *Crym* overexpression in meAMY regulates cocaine CPP, marble burying and open field. Similar to SI, *Crym* overexpression increases the number of males that formed a preference (yellow) for cocaine and increases the number of females that formed an aversion (blue) to cocaine (B). *Crym* overexpression in meAMY increases the proportion of animals that buried marbles in males and females similar to the effects of *SI* (C). *Crym* overexpression has no effect on distance travelled in an open field test (D). Post-hoc significant effects indicated as: * = $p<0.05$; ** = $p<0.001$.

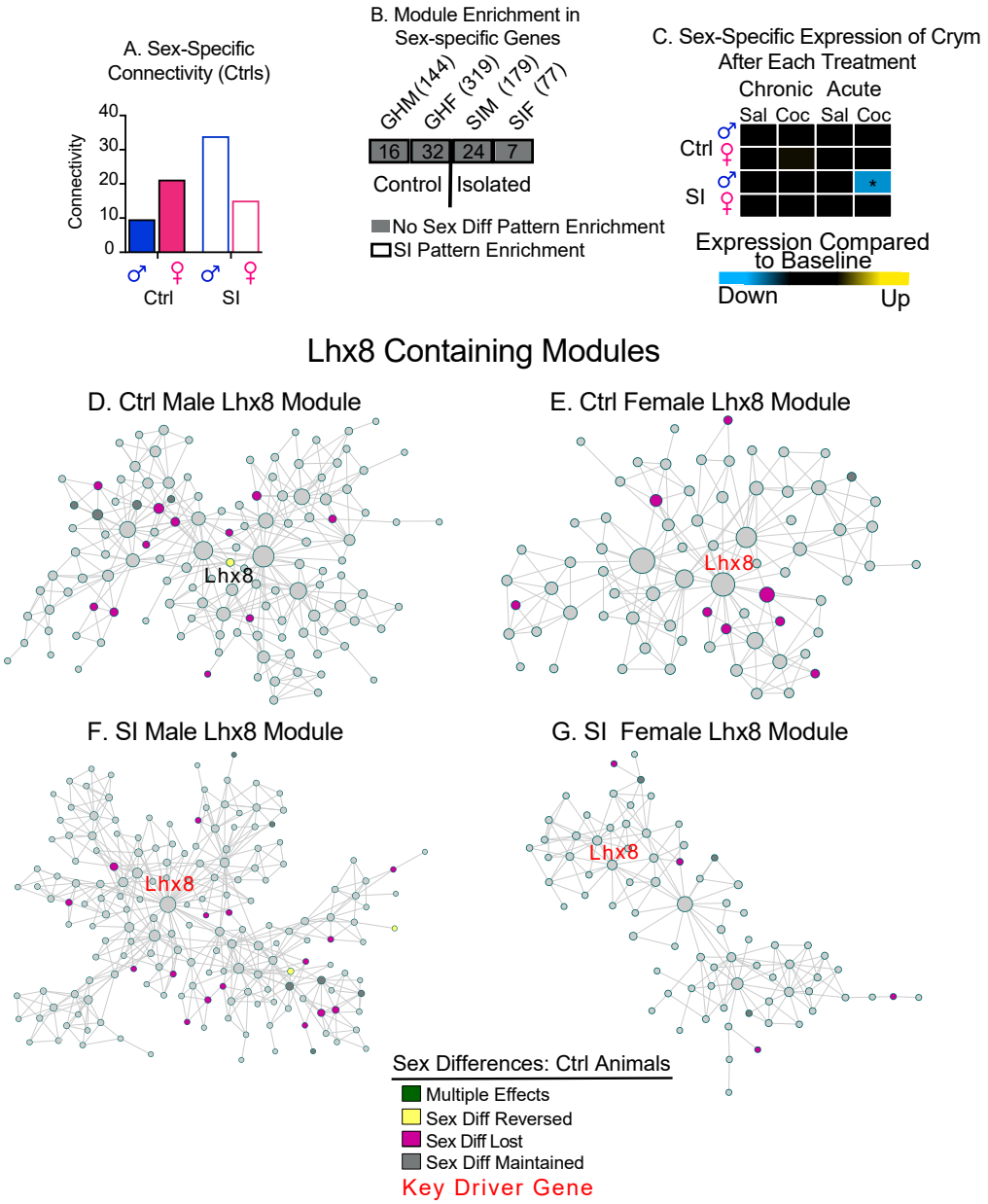


**Figure S6: *Lhx8* Does Not Meet All Criteria Used to Identify Sex-Specific Key Drivers.** (A) *Lhx8* (which encodes LIM homeobox 8) displays increased connectivity (males only, A) after SI (open bars). (B) *Lhx8*-containing modules are not enriched in sex-specific genes but are enriched in genes altered in SI males. (C) Finally, *Lhx8* does display sex-specific expression patterns after acute cocaine in SI animals when compared to the same baseline (Ctrl females after chronic saline). *Expression is significantly different ($p<0.05$) from baseline and meets the 30% threshold for change in expression. Blue = significantly down from baseline; yellow = significantly up from baseline; black = no change in expression from baseline. (D-G) Arachne plots of *Lhx8* containing modules in all four groups. Ctrl males (D), Ctrl females (E), SI males (F) and SI females (G). *Lhx8* is a key driver in modules for all conditions except Ctrl males as indicated by red text. Genes that are significantly different between Ctrl males and females which fall into different categories of expression are color-coded according to the patterns that they display.

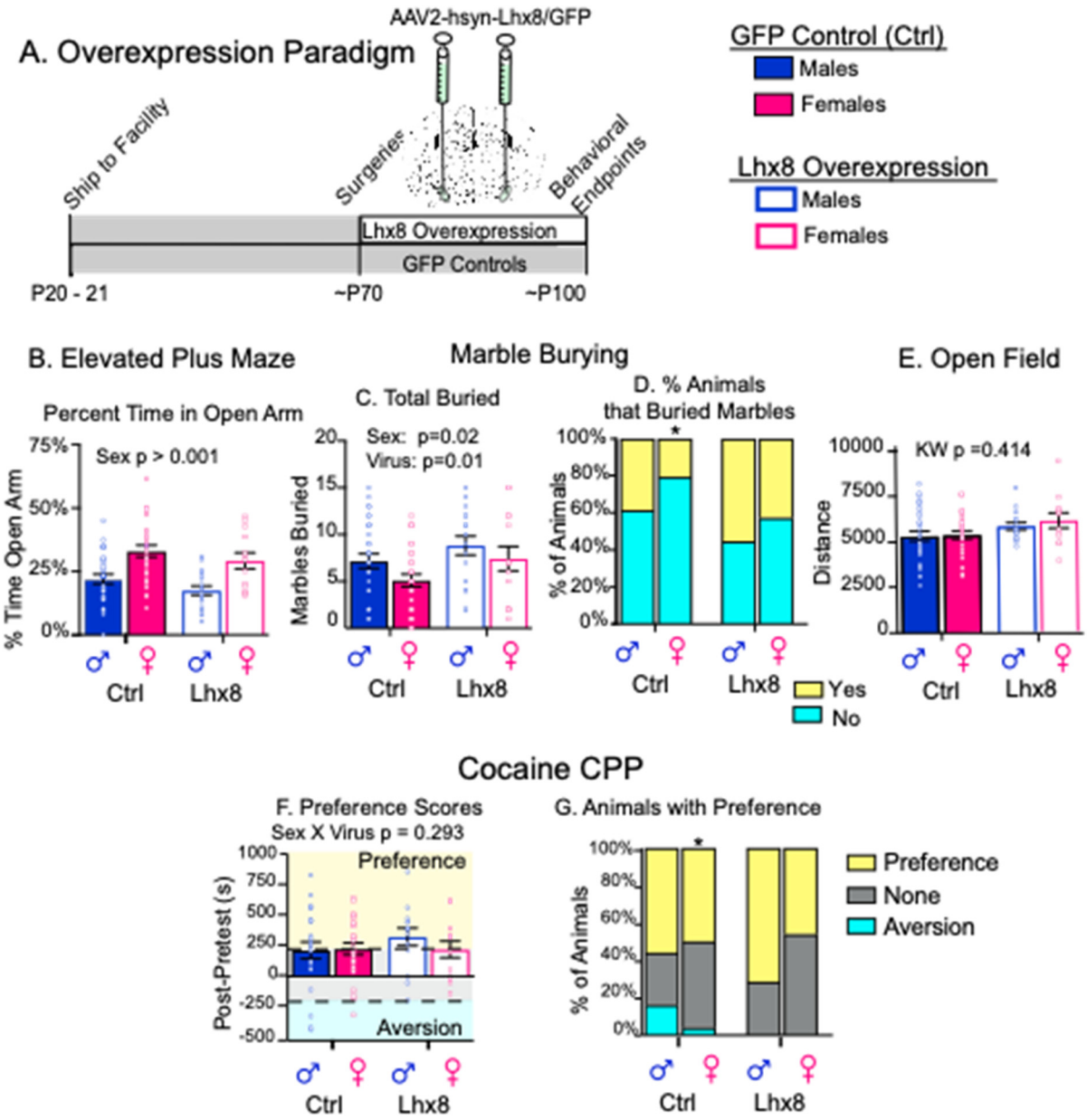


**Figure S7: *Lhx8* Overexpression in Adult meA Has No Effect on Behavior.**
(A) Schematic of experimental design for *Lhx8* overexpression in meA. (B–G) *Lhx8* overexpression in this brain region has no effect on sex-specific behaviors in elevated plus maze (Sex X virus $F_{(3,83)}$=0.15; p=0.7; Sex: $F_{(3,33)}$=18.43; p<0.01), (B-C) marble burying (Sex X virus $F_{(3,83)}$ = 0.11; p=0.74; Sex: $F_{(3,83)}$ =5.83; p=0.02; D &F) or open field (KW H=2.86; p=0.41; D). However, *Lhx8* overexpression increases the number of marbles buried in both males and females (B) and increases the proportion of animals that buried marbles in males and females (C). *Lhx8* overexpression also has no effect on cocaine CPP ($F_{(3,73)}$=1.12; p=0.29; Sex: $F_{(3,73)}$=.24; p=0.63; F & G) (compared to same GFP controls presented in Figure 5 and Suppl Figure 5). Lhx8 overexpression in meA does not affect the number of females that formed an aversion (blue) to cocaine (G). Main effects or interactions are indicated on graphs Post-hoc significant effects indicated as: * = p<0.05; ** = p<0.001, error bars indicate SEM. KW, Kruskal Wallace

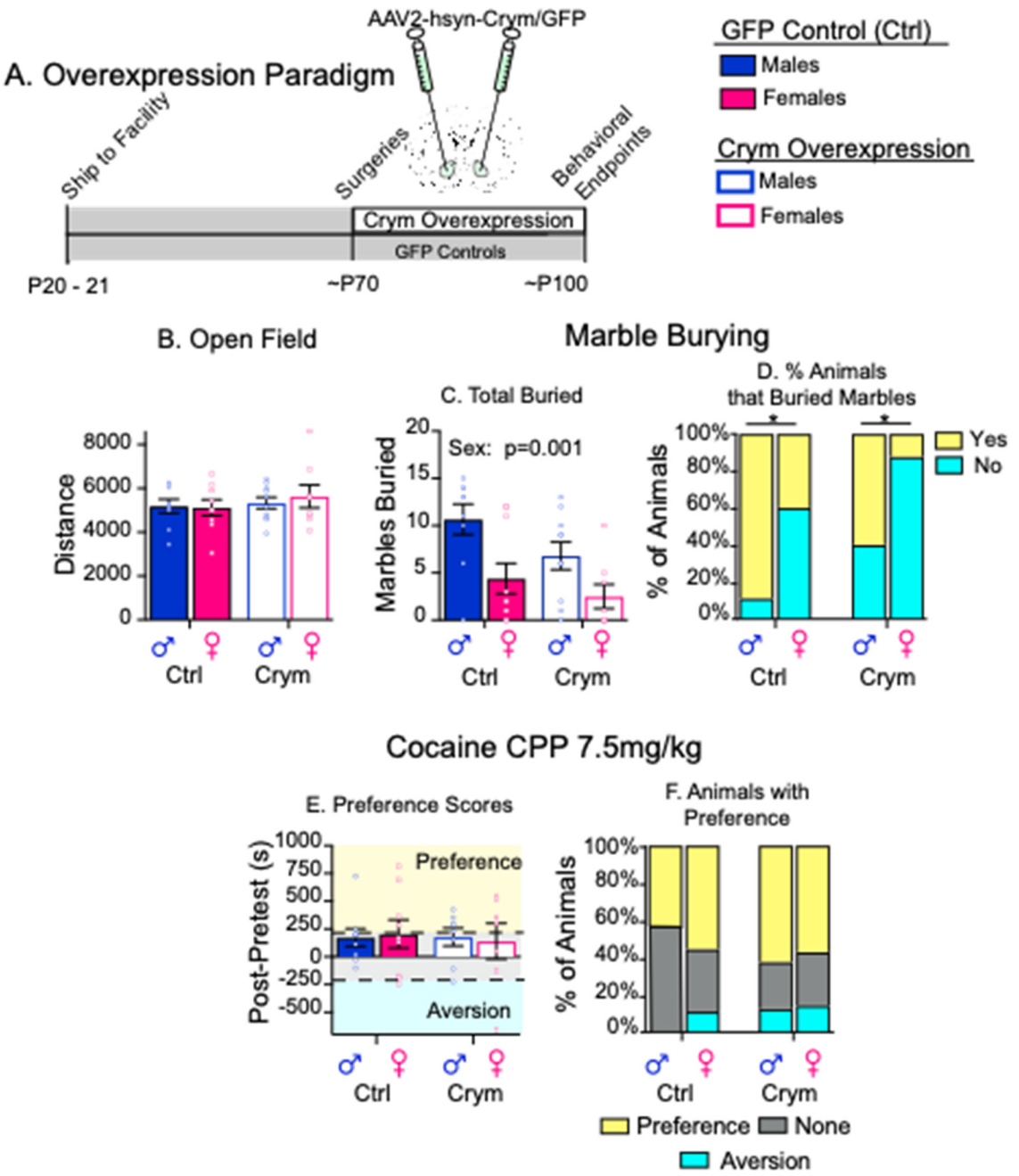


**Figure S8: *Crym* Overexpression in Adult Nucleus Accumbens Has No Effect on Behavior.**

Schematic of experimental design for *Crym* overexpression in nucleus accumbens. (B-F) *Crym* overexpression in this region has no effect on open field (Sex X virus $F_{(3,32)}$ = 0.25; p=0.62; Sex: $F_{(3,33)}$ =0.1; p=0.75; B), marble burying (Sex X virus $F_{(3,33)}$ = 0.42; p=.52; Sex: $F_{(3,33)}$ =12.0; p<0.01; C-D) or cocaine CPP (Sex X virus $F_{(3,33)}$ = 0.061; p=0.81; Sex: $F_{(3,33)}$ =0.38 p=0.54 E-F) when compared to GFP controls. Main effects or interactions are indicated on graphs; Post-hoc significant effects indicated as: * = p<0.05; ** = p<0.001, error bars indicate SEM.

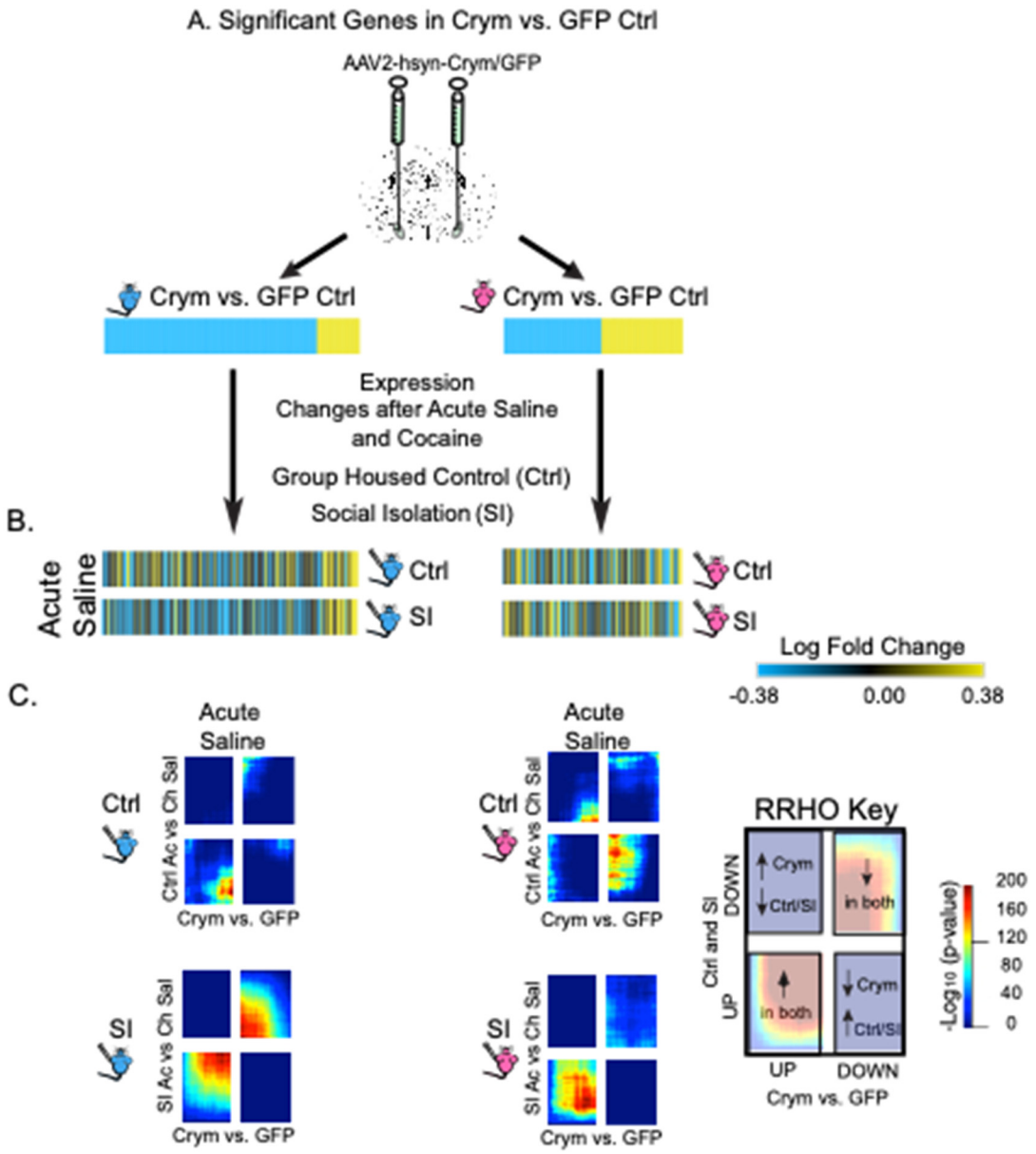


**Figure S9: Genes Regulated by *Crym* Overexpression are Responsive to Acute Saline and Cocaine in a Sex-Specific Manner**.
(A). Genes altered by *Crym* overexpression in meA of males (left) and females (right). (B-C) Expression profiles of genes altered by *Crym* overexpression in group-housed control (Ctrl) and SI males and females after acute saline (B). The expression pattern of Crym-regulated genes (A) are reflected in the expression of genes in SI males but not Ctrl males (B) suggesting that *Crym* overexpression reflects stimulus induced expression patterns in SI males. A similar effect was observed in global transcriptional patterns (C). This effects was not observed in females (C).

**Supplemental Tables:** *(see separate Excel files)*

Supplemental Table 1: Differentially Expressed Genes in meA of Group-Housed and SI Animals

Supplemental Table 2: Pattern Analysis of Gene Expression in the meA.

Supplemental Table 3: Differentially Expressed Genes in meA After *Crym* Overexpression

**DETAILED METHODS:**

**Animals**

All experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee at Mount Sinai. Age-matched male and female C57BL/6J mice were shipped from Jackson Laboratories (Bar Harbor, ME) and delivered to the facility on postnatal day (P) 21. Animals' sex was determined by examination of the external genitalia. For those animals used in the transcriptomic analysis, the presence of x- and y-linked genes (e.g., Xist) were used to confirm a match between chromosomal sex and assigned sex. After 24 hr of acclimation, animals were isolated on P22 by placing one animal in a transparent polycarbonate cage. Animals had olfactory, visual and auditory interactions with others but were not allowed tactile interactions. After three weeks of isolation (P42), animals were rehoused with their original same-sex cage mates and group housed until ~P90 when behavioral and molecular testing was conducted. For behavioral endpoints, 6 different cohorts of animals were used (3 cohorts for anxiety-related behaviors and 3 cohorts for cocaine CPP, n = 10-15/group/cohort). For sequencing endpoints, all animals were collected in one cohort. For each cohort, group-housed controls were used as comparisons. A separate cohort of animals was generated to confirm the effects of SI on Crym expression using qPCR. Animals were maintained on a 12 hr light-dark cycle (lights on at 7:00) at 22-25°C with *ad libitum* access to food and water.

*Vaginal Cytology:* For all endpoints, vaginal cytology was monitored prior to and during behavioral testing and was included in the analysis to confirm that estrus cycle had no effect on

the above behavioral endpoints. There were no effects of estrus cycle phase on anxiety-related behaviors, but others have observed cycle-specific effects on cocaine CPP (1). Therefore, during CPP testing, females underwent training on proestrus and estrus and were tested on the 3rd day of their cycle. Because of the cycle-specific effects on cocaine CPP, only females in diestrus on the test day were included in the transcriptional analysis.

**Behavioral Testing**

*Elevated Plus Maze:* Sex differences in elevated plus maze are well established (2-4) and have been used previously as a measure of hormonal reorganization of sexually specific behaviors (5). Generally, females spend more time in the open arm of an elevated plus maze (2-4). On the day of testing, animals were moved into a separate testing room and given at least 1 hr to habituate before testing commenced. Males and females were studied on 2 different acrylic elevated plus mazes (one for each sex) standing ~92 cm tall with 4 cross shaped arms (12 x 50 cm), 2 arms enclosed with opaque walls (40 cm tall) and 2 open arms under dim red light. Animals were placed at the center of the maze facing one of the open arms and behavior was recorded for 6 min and tracked using Ethovision 13.0. Amount of cumulative time spent in the open arms, closed arms and center were recorded. In a few cases during testing, the mouse was not detected by the tracking software because of a shadow from the closed arm. Therefore, total tracking time was used to calculate percent time in the open and closed arms. If tracking was lost for greater than 20% of total time, the data were not used in the analysis. In the experiment examining *Crym* overexpression in the nucleus accumbens, tracking was lost (>20%) in over half of the animals. Therefore, this test was excluded from analysis. All testing was conducted between 10:00 and 17:00 hr and groups were balanced throughout the testing.

*Open Field:* Sex differences in open field are sensitive to the time of day, species and strain (4, 6) but as with elevated plus maze this behavioral test has been used as a measure of disruption

of sex-specific programming of behavior by hormones (7, 8). On the day of open field testing, animals were moved into a separate testing room and given at least 1 hr to habituate before testing commenced. Animals were placed in the corner of an arena (44 x 44 cm) and allowed to explore the open arena for 10 min. Behavior was recorded and tracked using Ethovision 13.0 and time in the center and periphery as well as distance travelled and velocity were recorded. Once again, males and females were studied in separate arenas and groups were balanced throughout the testing day. All testing was conducted between 10:00 and 17:00 hr under dim red light.

*Marble Burying:* Sex differences in marble burying are well established (7, 9) and sensitive to reorganization by gonadal hormones during puberty (7). Males bury more marbles in this task than females and testosterone exposure during puberty increases burying in females (7). On the day of testing, animals were moved into a separate testing room and given at least 1 hr to habituate before testing commenced. Animals were placed in a plexiglass cage (19.56 x 30.91 x 13.34 cm) containing 9 cm of corn cob bedding and 20 sterilized glass marbles (13 mm diameter) placed in a 4 x 5 grid and evenly distributed across the entire cage. Animals were placed in the center of the arena and a lid was placed gently on the cage. After 15 min, animals were removed from the testing arena and the number of marbles partially and completely buried were counted and recorded. Marbles were counted by two researchers blind to treatment group. Each animal was run in its own cage with clean bedding and marbles. All testing was conducted between 10:00 and 17:00 hr under dim red light and groups were balanced throughout the testing day.

*Cocaine Conditioned Place Preference:* Sex differences in cocaine-related behaviors, including CPP, are less consistent and appear to be dependent on dose, experimental procedure and

species. However, it is notable that, when sex differences are observed, females form a greater preference for cocaine compared to males (1, 10, 11). On the days of testing, animals were moved into a separate testing room and given at least 1 hr to habituate before testing commenced. Three chamber CPP apparati were used with 2 large outer chambers and one small middle chamber. All chambers differed in tactile and visual cues. One large chamber had striped walls and small wire mesh floors, while the other had gray walls and large wire mesh floors. The middle smaller chamber had white walls, metal rods as the floor and two overhead lights (vs. one in each of the larger chambers). During pretesting, animals were placed in the middle chamber and were allowed to explore all three chambers for 20 min. Infrared beam breaks were monitored using Med Associates (San Diego, CA) software to track locomotor activity and time spent in each chamber. After pretesting, pre-CPP scores were calculated as time spent in the gray chamber minus time spent in the stripe chamber. Any animals spending greater than 300 sec on one side during the pre-test were excluded from the study, which amounted to <10% of all animals.

An unbiased approach was utilized to determine conditioning. For each group, an equal number of animals were conditioned with cocaine on their preferred or non-preferred side. On each of two conditioning days animals were injected with saline between 10:00-13:00 hr and placed in one chamber for 30 min. In the afternoon (14:00-18:00), animals were injected with cocaine (7.5 mg/kg) and placed in the opposite chamber for 30 min. The following day, animals were placed in the apparatus and allowed equal access to all three chambers. An animal was considered to have a preference for cocaine if they spent more than 50% (>625 sec) of their time in the cocaine-paired chamber or considered to have an aversion to cocaine if they spent more than 50% of their time during testing on the saline-paired side. Half of the males in each cohort were run in one week followed by females over the next ~10 days. The second half of the males were run after the females were completed to account for any time that passed during testing. Groups were balanced between testing conditions and time of testing was included in the analysis

to confirm that time was not a factor in the development of CPP. For females, vaginal cytology was monitored 7-10 days before behavioral testing commenced to confirm that females were cycling regularly. The pretest was conducted on one day for all of the females. However, because evidence suggests that cocaine CPP is dependent on estrous cycle stage (1), females were conditioned when they were in proestrus and estrus and testing occurred ~24 hr after the last conditioning day. This means that females were in metestrus or diestrus I when behavioral testing occurred.

**Statistical Analysis of Behavioral Data**

All behaviors were analyzed using a 2-way ANOVA or a Kruskall-Wallis non-parametric test depending on the significance of a Levene's test for homogeneity of variance. If an interaction was identified, a Tukey post-hoc analysis was conducted to determine specific differences between groups. If an effect was identified via Kruskall-Wallis, follow-up Mann-Whitney tests were run to determine specific differences between groups. Chi-square tests were used to determine differences in the distribution of behavioral phenotypes within a group (marble burying and CPP). Pearson's Chi-square tests were used to account for the lack of representation of all categories across groups. All analyses were conducted using SPSS Statistical Software, V25 (IBM, Armonk, NY).

**Cocaine Injections and Tissue Collection for Immunohistochemistry and RNA-seq**

On P80, animals were divided into 8 groups of males and 8 groups of females: group-housed controls + chronic cocaine/saline; adolescent SI + chronic cocaine/saline; group-housed controls + acute cocaine/saline; and adolescent SI + acute cocaine/saline. In total, 200 animals were utilized. For chronic treatment, animals were given one injection (IP) of cocaine or saline per day for 10 days (between 10:00 and 14:00 hr) and were euthanized 24 hr after their last injection (n=6-8 animals/group; total = 116 samples). For acute treatment, animals were given

saline injections (IP) for 9 days in an effort to habituate the animals to handling and injection stress. On the 10$^{th}$ day, animals were injected with saline or cocaine (7.5 mg/kg) and euthanized ~1 hr after the injection. We chose these dosing paradigms to model sex-specific behavioral effects of adolescent SI. Thus, we observed sex-specific behavioral impact of SI on cocaine CPP (reflective of acute exposure to cocaine), whereas sex-specific effects of SI on cocaine self-administration (reflective of chronic exposure to cocaine) are less pronounced (12).

For protein analysis, mice were rapidly anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and transcardially perfused with a fixative solution containing 4% PFA paraformaldehyde (PFA) (w/v) in 1X phosphate buffered saline, pH 7.5 at 4°C delivered at 20 ml/min for ~7 min with a peristaltic pump. Brains were post-fixed for 24 hours in 4% PFA at 4°C and stored in PBS + Azide (0.1%) until processing. For RNA analysis, all animals were euthanized via cervical dislocation, brains were removed and sectioned on ice in a brain block (1 mm thick) and bilateral micropunches (15 gauge) of meA were snap-frozen on dry ice and stored at -80°C until use. Vaginal cytology was monitored throughout the injections and only those females in metestrus/diestrus were used for library preparation.

**FOS Immunohistochemistry**

Brains were prepared for sectioning transferring in 30% sucrose for 24-48 hrs followed by snap freezing in isopentane on dry ice. Once frozen, free floating sections (40 μm) were sectioned in the frontal plane on a cryostat (Leica Biosystems, Buffalo Grove IL). Sections were stored at 4°C in PBS + Azide (0.01%) until staining occurred. Free-floating sections were processed for immunohistochemistry as follows. On day 1, sections were rinsed three times for 10 min in PBS before permeabilization 15 min in PBS containing 0.2% Triton X-100 (PBST; Fisher). After three PBS washes, a blocking step was performed and sections were incubated one hour in PBS containing 3% normal donkey serum (v/v). Sections were rinsed three times in

PBS and incubated over night with the primary antibody against FOS (Abcam, ab190289) in PBS containing 3% normal donkey serum (v/v). Sections were then washed three times in PBS and incubated with secondary antibodies (Alexa Fluor 594 donkey anti-rabbit; 1:1000; Jackson ImmunoResearch, 1:1000 dilution) for 2 h at room temperature. After three rinses in PBS, sections were mounted in Vectasheild mounting medium with DAPI (Vector labs, Burlingame, CA).

Immunofluorescence was visualized using a LSM 710 laser-scanning confocal microscope (Carl Zeiss) at 10X objective using identical illumination parameters for all prepared sections. Z-stack images (2 μm planes) were obtained and stacked images were subsequently overlaid with the corresponding atlas sections (13). For quantification, an identical contrast threshold range was applied to sections and the average intensity and density were calculated using Image J software (version 1.53, National Institutes of Health, USA).

**RNA Isolation, Library Preparation, and Sequencing**

RNA was isolated as described previously (14) using RNAeasy Mini Kit (Qiagen, Fredrick, MD) with a modified protocol from the manufacturer allowing for the separation and purification of small RNAs from total RNA. Briefly, after cell lysis and extraction with QIAzol (Qiagen, Fredrick, MD), small RNAs were collected in the flow-through and purified using the RNeasy MinElute spin columns and total RNA was purified using RNeasy Mini spin columns. Samples were treated with DNAse to rid them of genomic DNA and run on nanodrop and an Agilent Bioanalyzer 2100 to confirm RNA purity, integrity and concentration. All samples exhibited RINs >8.

Libraries were prepared using the TruSeq Stranded mRNA HT Sample Prep Kit protocol (Illumina, San Diego, CA). Briefly, poly A selection and fragmentation of 300 ng of RNA was performed, and the resulting RNA was converted to cDNA with random hexamers. Adapters were ligated and samples were size-selected with AMPur XP beads (Beckman Coulter, Brea,

CA). Barcode bases (6 bp) were introduced at one end of the adaptors during PCR amplification steps. Library size and concentration were assessed using Tape Station (Life Technologies, Grand Island, NY) before sequencing. Libraries were pooled for multiplexing (pool of 21 samples) and sequenced on a HighSeq2500 System using V4 chemistry with 50 base pair single-end reads at UCLA Neuroscience Genomics core. Each pool was sequenced 3 times with the goal of obtaining ~30 million reads per sample. QC revealed an average of 29 million reads per sample (min = 19 million; Max = 51 million) with an average mapping rate of 90.2%. The number of independent tissue samples included in the final analysis was between 6-8 per group.

**Differential Expression Analysis**

Gene expression raw read counts were normalized as counts per million (CPM) using trimmed mean of M-values normalization (TMM) method (15) to adjust for the differences in library size among samples. Genes expressed at ≤1 CPM in ≥5 samples were excluded from further analysis. DEGs in comparisons of male vs. female, adolescent SI vs. group-housed controls, cocaine vs. saline and acute vs. chronic were identified using the Bioconductor package *limma* (16) with the following thresholds: nominal p-value <0.05 and fold-change of 30% (Suppl Table 1).

**Pattern Analysis**

Pattern analysis was conducted as described previously (14). Briefly, each expression change from the same baseline was converted into 0 or 1/-1 (0 = no effect; -1 = significantly downregulated; 1 = significantly upregulated) for each condition. Output from the analysis resulted in a list of combinations of 0s and -1/1s observed in the dataset. The patterns of expression were defined by two investigators unfamiliar with the experiment to avoid bias. Each gene was then assigned to a defined category. Patterns included genes that were differentially

expressed when compared to group-housed females injected with chronic saline ($p<0.05$; fold change >30%). While many patterns were observed in the dataset (Suppl Table 2), we focused on two: those genes that were differentially regulated between control males vs. females under each exposure paradigm (sex differences; Suppl Figure 3) and those that were only affected in SI animals (SI-only Effects). Importantly, a gene can only be defined as one category under each treatment condition. Thus, we identified genes that are uniquely regulated by each stimulus.

**Multiscale Embedded Gene Co-Expression Network Analysis**

MEGENA (17) was used to construct gene co-expression networks for group-housed and SI male and female samples. First, MEGENA constructed a planar filtered network by utilizing parallel computation, early termination and prior quality control. Next, MEGENA performed a multiscale clustering analysis to obtain co-expression modules by introducing compactness of modular structures characterized by a resolution parameter. Lastly, MEGENA conducted a multiscale hub analysis to identify highly connected hubs (or driver genes) of each module at each scale.

DEGs were then laid onto the modules to perform enrichment analysis: namely, ranking modules associated with different treatment conditions according to the adjusted enrichment p-value. In addition, gene ontology (GO)-function enrichment analysis (18) was applied to the modules to identify enriched biological processes with p-values adjusted by BH correction. Sunburst plots showing the enrichment of sex differences in changes (SD), SI changes or GO-biological processes in individual modules of the networks were visualized using the R package *sunburstR* (19). Top-ranked modules in each network were also visualized in circos plots with the significance of DEG enrichment using the R package *Netweaver* (20). Module subnetworks were visualized by Cytoscape_v3.3 (21).

**Enrichment Analysis**

Fisher's exact tests were conducted using the Super Exact Test package in R as described previously to determine module preservation as well as enrichment of patterns within modules (14, 22)

**Rank Rank Hypergeometric Overlap (RRHO) Analysis**

We applied an RRHO test to compare gene regulation between the comparisons representing sex differences in gene expression after acute and chronic cocaine (Figure 1), sex differences in expression after Crym overexpression (Figure 3), *Crym* specific effects in males and females (Figure 3), and Crym overexpression effects compared to cocaine induced transcription in group housed controls and SI animals (Figure 4). RRHO identifies overlap between expression profiles in a threshold free manner to assess the degree and significance of overlap(23). Here we used a modified script that visualizes both positive and negative correlations and illustrates each quadrant separately based on the number of genes in each comparison as previously described (24). Full differential expression lists were ranked by the -log(p-value) multiplied by the sign of the fold change/slope of association. A one sided version of the test was used to look for over enrichment. RRHO difference maps were produced for each comparison by calculating for each pixel the normal approximation of difference in log odds ratio and standard error of overlap between the comparison representing the Pattern and the Factor. This z-score was then converted to a p-value and corrected for multiple comparisons across pixels (25)

**Viral Vectors**

*Crym* and *Lhx8* cDNA was synthesized and cloned into an pAAV plasmid (Gene Script). The plasmid was package into an AAV2 vector by the Duke viral vector core (Durham, NC).

This vector expresses both the gene of interest and eGFP under the *hSyn* promoter and were separated by a t2a sequence. AAV2-hSyn expressing eGFP only was used as the control. These AAV vectors express their transgenes solely within neurons (26).

For *in vivo* behavioral validation of key driver genes, male and female C57BL/6J mice were injected with AAV2 vectors and, ~3 wks after surgery when transgene expression is maximal, mice were subjected to all behavioral paradigms discussed above. Briefly, animals were shipped from Jackson Laboratories at ~P21 and allowed one week to acclimate to the new facility. On ~P70 we overexpressed genes of interest via stereotaxic injections of AAV2s expressing Crym or Lhx8 plus GFP, or GFP alone, in meA under ketamine (100 mg/kg IP)-xylazine (10 mg/kg IP) anesthesia. Animals were placed in a small-animal stereotaxic instrument (Kopf Instruments, Los Angeles, CA). Vectors (0.5 µl of viral titer = 1x10^12 particles/ml) were bilaterally injected using 33-gauge syringe needles (Hamilton) at a rate of 0.1 µl/min into meA (Bregma coordinates: anterior/posterior, -1.1; Medial/lateral, 2.5 mm; dorsal ventral, -5.3 mm ; 0° angle) or nucleus accumbens (Bregma coordinates: anterior/posterior, 1.6; Medial/lateral, 1.5 mm; dorsal/ventral, -4.4 mm; 10° angle). Injections were performed in three cohorts with viruses represented across each cohort. Only those animals that had correct anatomical targeting, and *Crym* expression that was ≥1.5 standard deviations greater than GFP controls, were included in the analysis.

Behavioral testing was conducted as described above, however, to limit the number of animals all 4 behaviors were run in each animal in the following order: week 1: elevated plus maze, open field and marble burying; week 2-3: cocaine CPP. As stated above, males were analyzed first in cocaine CPP and females were analyzed the following week. All females were trained on proestrus and estrus and CPP testing was conducted when females were in metestrus/diestrus 1. A pilot experiment was performed prior to surgeries in a separate cohort of animals to confirm that CPP behaviors were not affected by previous behavioral testing or handling. Behavioral endpoints were analyzed as described above. Cohort was included as a

factor in the analysis. If a cohort effect was observed for an endpoint, the direction and effect were confirmed to be the same for each cohort. If the patterns for the behaviors were the same across cohorts but small differences were observed in behaviors, cohort was included as a random factor in the ANOVA.

For the cohort of animals with *Crym* overexpression in the nucleus accumbens, CPP testing was conducted in a different room using different conditioning chambers. A small but significant difference in behavior was noted in these testing chambers. Therefore, a slightly different threshold was applied to the preference scores for this cohort only: preference = ≥50% time (600 sec) in the cocaine-paired chamber (rather than >50% time in the cocaine-paired chamber).

**RNA Isolation and qPCR Validation**

Animals were euthanized by cervical dislocation ~10 days after the last day of behavioral testing for transgene validation or on ~P72 for confirmation of SI effects on expression. Brains were removed and sectioned in a brain matrix (1 mm sections) and viral vector expression was confirmed by epifluorescence microscope. Only those animals that displayed GFP expression in meA were processed for RNA isolation. RNA was isolated as described above and converted to cDNA using High Capacity Reverse Transcriptase Kits (Catalog #4368814; ThermoFisher, Foster City, CA) according to manufacturer's protocol. qPCR was performed for 2 genes of interest (Crym and Lhx8) and 2 internal controls using Taqman® gene expression assays (Thermo Fisher, Foster City CA) and Taqman® Fast Universal Master Mix (ThermoFisher, Foster City, CA) on an ABI Quant Studio Flex 7 according to the manufacturer's protocol and using the following parameters: 1 cycle (2 min @ 50°C followed by 2 min @ 95°C); 45 cycles (1 sec @ 95°C followed by 20 sec @ 60°C). Expression was analyzed for all three viral vectors in each animal using the comparative ΔCt method (27). Each sample was normalized to its own

internal controls (geometric mean of the Ct values for *Hprt1* and *Actb)* and calibrated to the average ΔCt for the GFP groups. Only those animals that had expression values >1.5 SD from the mean expression of *Crym* or *Lhx8* in the GFP groups were used in the behavioral analysis and the follow-up RNA-seq. For validation of sequencing data, qPCR was performed for Crym and two internal controls (*Hprt1* and *Actb*) in group house control or SI males and females (n=6-8/group). Expression was calculated relative to group housed females to replicate the analysis presented using the sequencing data.

**Immunohistochemistry for Viral Targeting**

A subset of Crym and GFP animals were euthanized to validate viral placement. Animals were perfused and tissue was processed following the same protocol described above. Immunohistochemistry was conducted as described above with the following primary and secondary antibodies: chicken anti-GFP antibodies (Aves Lab, GFP10-20 1:1000 ) and donkey anti-chicken Alexa Fluor 488 (Jackson ImmunoResearch,1:1000). Imaging was conducted as described.

**RNA-Seq Analysis After *Crym* Overexpression**

Aliquots of RNA with confirmed *Crym* overexpression (or GFP controls) were sent to GeneWiz (New Jersey) for library preparation and RNA-seq (total N = 68). Paired-end sequencing reads were aligned to mouse genome GRCm38.95 using STAR aligner (28). FeatureCounts (29) was then used to assign reads to genes for quantifying gene expression levels based on GENCODE (30) release M20. Read counts were normalized as CPM using TMM method to adjust for differences in library size among samples. Principal component analysis and unsupervised clustering analysis were applied to the normalized expression levels to assess whether there were any outliers. Genes expressed at ≥1 CPM in ≥5 samples were obtained for DEG analysis. Limma was applied to the normalized expression levels to identify

DEGs between *Crym* overexpression and GFP in males and in females, as well as DEGs between male GFP vs. female GFP, and male *Crym* overexpression vs. female *Crym* overexpression.

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