

# **Comparative Transcriptional Analyses in the Nucleus Accumbens Identifies RGS2 as a Key Mediator of Depression-Related Behavior**

## **Supplemental Information**

### **Supplementary Methods and Materials**

#### **Animals and housing conditions**

All studies on California mice (*Peromyscus californicus*) were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Mice were bred at UC Davis and housed in clear polypropylene cages containing sanichip bedding, cotton nestlets and enviro-dri. Food (Harlan Teklad 2016) was provided *ad libitum*. Mice were housed 2-4 per cage and maintained on a 16L:8D light cycle with lights out at 2 PM Pacific Standard Time. No systematic biases in the distribution of estrous stage across treatment groups were detected across experiments.

#### **Social Defeat Stress (SDS)**

Mice were randomly assigned to control handling or social defeat for 3 consecutive days as previously described (1). Control mice were placed in a clean cage for 7 min. Mice assigned to social defeat were placed in the home cage of a sexually experienced aggressive same-sex mouse for 7 min or until the resident mouse attacked the focal mouse 7 times (whichever occurred first). No animal was physically injured during social defeat. Immediately after defeat or control handling, mice were returned to their home cage. Behavior tests and brain tissue collection were conducted two weeks after the last episode of social defeat.

#### **Sucrose Anhedonia**

Sucrose preference was assessed using a two-bottle choice test as previously described(2). Mice were housed for two days with two bottles of tap water to allow for habituation. Following habituation, each mouse was individually housed and water in one bottle was replaced with a 1% sucrose solution. Bottles were weighed before and after a 24 hr observation period. Placement of

tap water and sucrose solution was counterbalanced across mice. Percent sucrose preference was calculated as the amount of sucrose solution consumed (mL) over total amount of solution consumed (water and sucrose solution combined, mL).

### **Social Interaction Test**

Social interaction testing was performed as previously described(2,3). Mice were placed in an open arena for 3 min (open field). Next, an empty cage was placed in the arena and the time spent within 8 cm of the cage (interaction zone) was scored for 3 min (acclimation phase). For the last phase, an unfamiliar same-sex mouse was placed into the cage and time spent near the interaction zone was scored for 3 min (interaction phase). We define time spent in the interaction zone with a target mouse as social approach. Social vigilance was scored during the acclimation and interaction phases by recording the amount of time the focal mouse spent with its head oriented towards the target mouse while outside the interaction zone. For the sequencing experiment (Fig. 1), a perforated plexiglass cage was used for the acclimation and interaction phase. Due to an equipment malfunction, behavior data for two stressed females was not recorded. For all other experiments, a wire cage was used for the acclimation and interaction phases.

### **RNA extraction and RNA-sequencing library preparation**

Adult mice (n = 7-8 per group) were anesthetized with isoflurane directly from the home cage 1 day after the final behavioral test. Brains were removed rapidly, placed into ice-cold PBS, and sliced into 1mm-thick coronal sections in a slice matrix. Bilateral punches were made from VTA (16 gauge), NAc (14 gauge), and PFC (12 gauge) and flash-frozen in tubes on dry ice. Total RNA was isolated with TriZol reagent (Invitrogen) and purified with RNeasy Micro Kits (Qiagen). All RNA samples were determined to have A260/280 values  $\geq 1.8$  (Nanodrop); samples for RNA-seq had RIN values  $> 9$  (BioAnalyzer, Agilent). 500 ng of purified RNA was used to prepare libraries for sequencing using the Truseq mRNA library prep kit (Illumina RS-122-2001/2). VTA, NAc, and PFC samples were prepared from individual animals (NAc: 8 females/group, males 7-8 per/group; VTA: 8 females/group; PFC: 8 females/group) and sequenced with 125-nt single-end reads at Beckman Coulter Genomics (currently Genewiz). Samples were multiplexed to produce  $>30M$  reads/sample. All reads have been deposited RNA-

seq files are available through NCBI BioProject (ID: PRJNA700778). RNAseq data from human subjects used for analysis was published previously(4).

### **Rank-Rank hypergeometric overlap (RRHO)**

Full threshold-free differential expression lists were ranked by the  $-\log_{10}(p\text{-value})$  multiplied by the sign of the fold change from the DeSeq2 analysis, filtered to all genes with a mean base expression of  $>2$  Reads Per Kilobase of transcript, per million mapped reads (RPKM) to avoid low-expression artifacts. RRHO was used to evaluate the overlap of differential expression lists (an uncorrected  $p < 0.05$  and a  $\log_{2}FC > |1.15|$ ) between stress comparisons RRHO difference maps were produced for pairs of RRHO by calculating for each pixel the normal approximation of difference in log odds ratio and standard error of overlap between each matched comparison. This  $Z$  score was then converted to a  $P$ -value and corrected for multiple comparisons across pixels(5).

### ***In-situ* hybridization**

In-situ hybridization was performed as previously described(6). Briefly, brains were dissected from paraformaldehyde-perfused animals, postfixed, and cryoprotected in 30% sucrose before embedding in Tissue-Tek OCT (Sakura Finetek). Brains were coronally cryosectioned at  $60\ \mu\text{m}$  and mounted on slides. Sections were subsequently fixed, treated with proteinase K, acetylated, permeabilized, and equilibrated in hybridization solution. A riboprobe ( $0.3\text{mg/mL}$ ) directed against *Rgs2* corresponding to bases 74-550 of cDNA sequence XM\_028884620.1 was hybridized overnight at  $65^{\circ}\text{C}$ . Slides were then washed in high-stringency buffers, incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin primary antibody (1:1000; Roche) overnight at  $4^{\circ}\text{C}$ , then developed in staining solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Roche) at  $37^{\circ}\text{C}$  for 24 hrs. Slides were subsequently washed, fixed in 4% PFA, and coverslipped.

### **Western blots**

Punch samples from the NAc were homogenized in ice cold buffer (7.4 pH, 20% glycerol, 0.4 M NaCl, 20 mM HEPES, 5 mM  $\text{MgCl}_2$ , 0.5 mM EDTA in  $\text{H}_2\text{O}$ ) with protease inhibitor (1% PMSF in EtOH). Laemmli buffer (Sigma, St. Louis, MO) was added to the homogenate at a 1:1 dilution

and samples were placed on a shaker at 4°C for 1 h. Proteins were denatured at 98°C for 5 min, chilled on ice, and separated with gel electrophoresis (15% bis-acrylamide resolving gel). Protein was transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA), rinsed, and blocked with 5% skim milk in 0.1% Triton-X with tris-buffered saline (TBST). Membranes were incubated overnight in primary rabbit anti-RGS2 (Abcam, Boston, MA; ab155762) 1:1000 at 4°C. Antibody specificity was determined by use of samples of NAc from *Rgs2* knockout mice (Supplemental Fig. 1). Membranes were then incubated for 1 h in peroxidase-conjugated anti-rabbit secondary antibody (Vector, Burlingame, CA) diluted 1:1000 in TBST in 5% skim milk. Following TBST washes, developing solution was applied (Bio-Rad), and the blot was imaged on a Bio-Rad ChemiDoc. We quantified the mature form of the RGS2 protein at ~26 kDa. Blots were probed for  $\beta$ -actin as a loading control (Cell Signaling, Danvers, MA, 1:1000), and RGS2 protein bands were normalized to their respective  $\beta$ -actin controls. RGS2/ $\beta$ -actin ratios were further normalized to control mice on each blot. Levels of protein are displayed as the relative levels of RGS2 quantified from control females.

### **Quantitative Real-Time PCR**

RNA was extracted as above from NAc tissue punches from independent cohorts of SDS male and female mice (n = 5–8 per group) using an RNAeasy micro kit (Qiagen, 74004) and converted to cDNA using an iScript cDNA synthesis kit (Bio-Rad; **1708891**). Primer efficiency and specificity were verified; sequences are given in Supplemental Table 1. Real-time qPCR was performed using SybrGreen Fast master mix (Applied Biosystems) and standard cycling conditions on a ViiA 7 Real-Time PCR system and analyzed by the  $2^{-\Delta\Delta Ct}$  method with *Hprt1* as a reference gene.

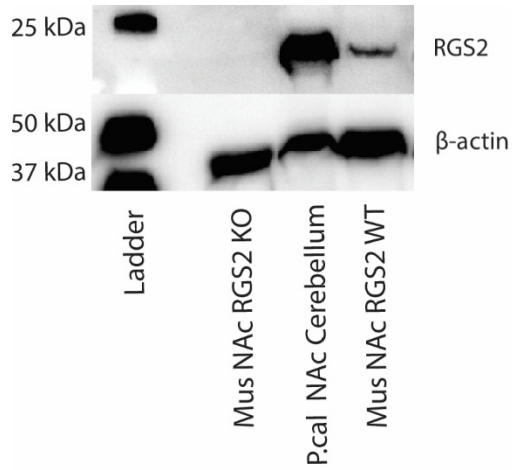
### **Overexpression of *Rgs2* within the NAc by HSV-mediated gene transfer.**

To overexpress RGS2, we used a bicistronic p1005 herpes simplex virus (HSV) expressing GFP or GFP and *Rgs2* (Origene). In this system, GFP expression is driven by a cytomegalovirus (CMV) promoter, while the gene of interest is driven by the IE4/5 promoter(7). Viral construction and surgery were carried out according to standardized methods(7,8). One week following social defeat, stressed mice received one bilateral 0.6 $\mu$ L injection of either the RGS2 overexpression vector or vector containing GFP alone into the NAc core (A/P: 0.84, M/L:  $\pm$ 1.5,

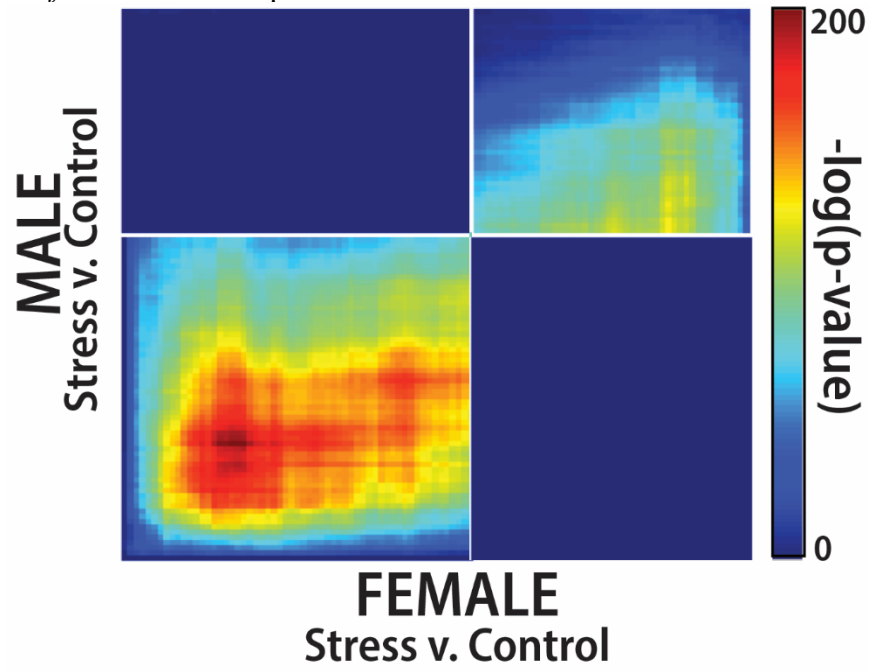
D/V: 6.0; based on a California mouse brain atlas (brainmaps.org)). One week later mice were run through a sucrose anhedonia test followed by a social interaction test. To confirm that expression was limited to NAc, 40  $\mu$ m sections of the NAc (4 slices per animal) were imaged under fluorescent microscopy to visualize GFP with red (530/615) Neurotrace (ThermoFisher) staining to confirm neural expression. RGS2 overexpression by viral gene transfer was confirmed via western blot.

## Supplemental Figures

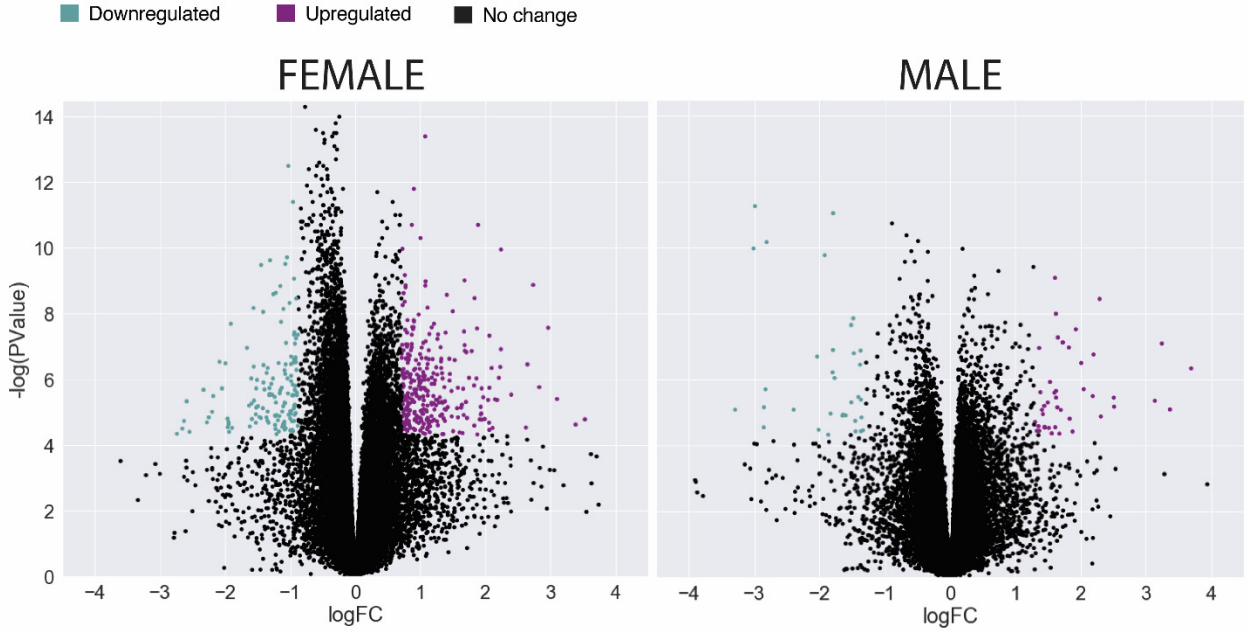
Supplemental Figure S1: Validation of RGS2 antibody. Western blot of protein samples from *Mus musculus* RGS2 knock-out (KO), *Peromyscus californicus* cerebellum, and wild-type *M. musculus*. RGS2 bands were approximately 24 kDa while corresponding  $\beta$ -actin bands were approximately 42 kDa.



Supplemental Figure S2: Rank-rank hypergeometric plot comparing male and female *P. californicus* RNAseq data from control and stressed animals.

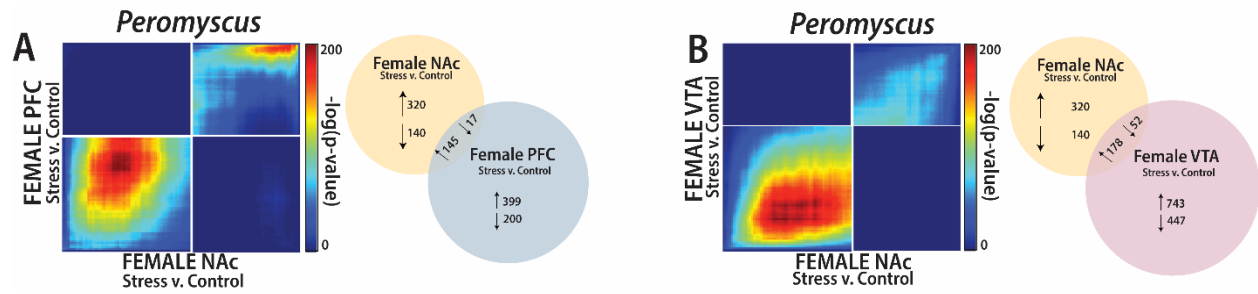


Supplemental Figure S3: Volcano plots for control versus stress comparisons for female and male *P. californicus* RNAseq data.

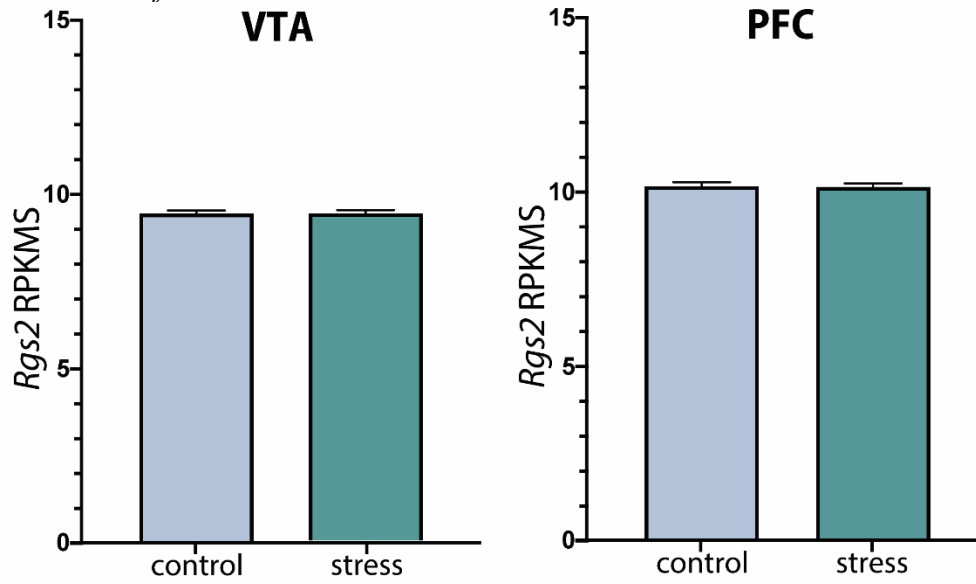




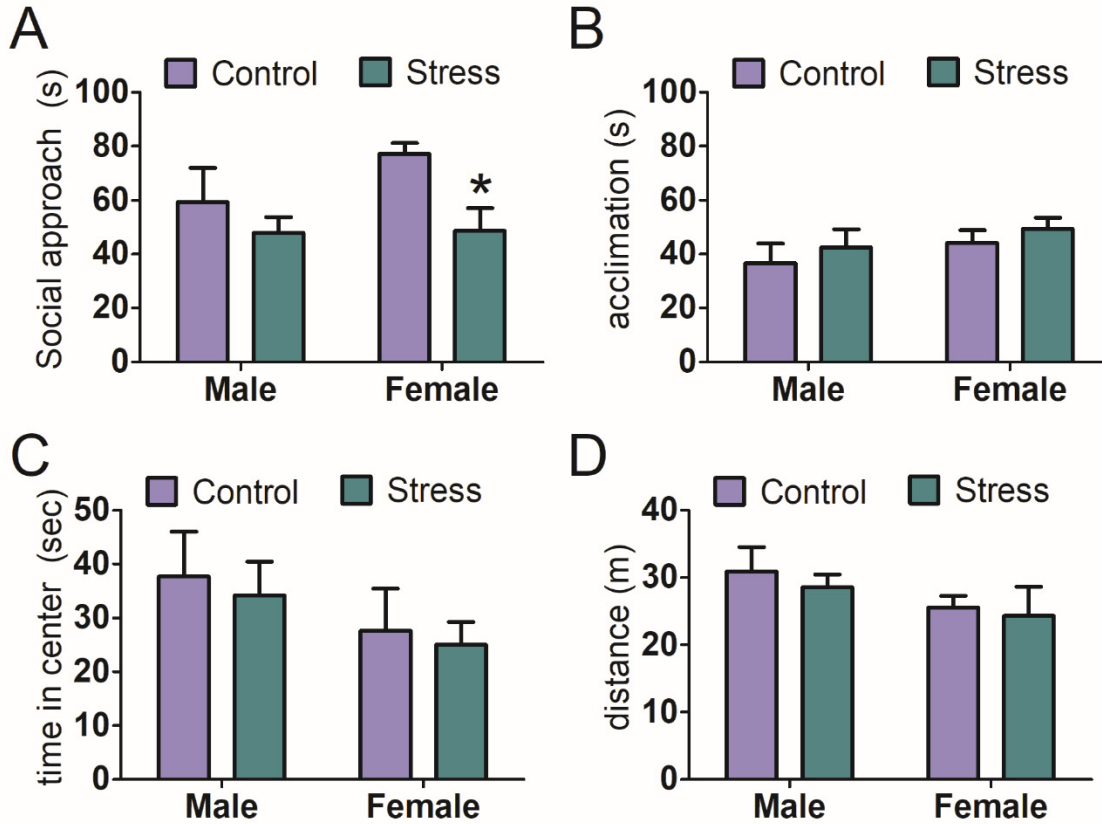
Supplemental Figure S4: Rank-rank hypergeometric plots comparing female *P. californicus* nucleus accumbens (NAc) RNAseq data from females with female prefrontal cortex (PFC) RNAseq data (A) and female ventral tegmental area (VTA) RNAseq data (B).



Supplemental Figure S5: There were no differences in the number of *Rgs2* reads between control and stressed *P. californicus* females in the VTA or PFC.



Supplemental Figure S6: Social defeat reduced social approach in female *P. californicus* but not males (A). There were no differences in approach to an empty cage during acclimation (B), time spent in the center during the open field (C), or total distance traveled in the open field (D). \*  $p < 0.05$  vs control.



Supplementary Table S1:  
RT-qPCR Primer Sequences

<i>Hprt1</i> F: CCCAGCGTCGTGATTAGTGATG	<i>Hprt1</i> R: GTGATGGCCTCCCATCTCTTT
<i>Rgs2</i> F: TTGCAAATTCCTCCACTCC	<i>Rgs2</i> R: CTGAACGCAGCAAGTCCATA

See separate Excel file for Supplementary Table S2, which presents RNA sequencing results

Supplementary Table S3:  
NAc transcripts affected by stress in both male and female *Peromyscus californicus*

Upregulated	Downregulated
ASB4	SIC25A1
CDKL	
LSP1	
DAF	
CD55	
KRAB	
TSPAN26	
TRADD	
MRPL14	
TTC30	
IFT81	

## Supplemental References

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3. Williams AV, Duque-Wilckens N, Ramos-Maciel S, Campi KL, Bhela SK, Xu CK, *et al.* (2020): Social approach and social vigilance are differentially regulated by oxytocin receptors in the nucleus accumbens. *Neuropsychopharmacol* 45: 1423–1430.
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5. Cahill KM, Huo Z, Tseng GC, Logan RW, Seney ML (2018): Improved identification of concordant and discordant gene expression signatures using an updated rank-rank hypergeometric overlap approach. *Sci Rep* 8: 9588.
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