



Published in final edited form as:

Nature. 2011 February 24; 470(7335): 492–497. doi:10.1038/nature09856.

## Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor

Kerry J. Ressler<sup>1,2,4</sup>, Kristina B. Mercer<sup>1</sup>, Bekh Bradley<sup>2,3</sup>, Tanja Jovanovic<sup>2</sup>, Amy Mahan<sup>4</sup>, Kimberly Kerley<sup>1</sup>, Seth D. Norrholm<sup>2,3</sup>, Varun Kilaru<sup>2</sup>, Alicia K. Smith<sup>2</sup>, Amanda J. Myers<sup>5</sup>, Manuel Ramirez<sup>5</sup>, Anzhelika Engel<sup>5</sup>, Sayamwong E. Hammack<sup>6</sup>, Donna Toufexis<sup>4,6</sup>, Karen M. Braas<sup>7</sup>, Elisabeth B. Binder<sup>2,8</sup>, and Victor May<sup>7</sup>

<sup>1</sup> Howard Hughes Medical Institute, Chevy Chase, MD

<sup>2</sup> Department of Psychiatry and Behavioral Sciences Emory University School of Medicine, Atlanta, GA

<sup>3</sup> Atlanta VA Medical Center, Atlanta, GA

<sup>4</sup> Yerkes National Primate Research Center, Atlanta, GA

<sup>5</sup> University of Miami, Miller School of Medicine, Miami, FL

<sup>6</sup> Department of Psychology, University of Vermont, Burlington, VT

<sup>7</sup> Departments of Anatomy and Neurobiology and Pharmacology, University of Vermont College of Medicine, Burlington, VT

<sup>8</sup> Max Planck Institute of Psychiatry, Munich, Germany

### Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is known to broadly regulate the cellular stress response. In contrast, it is unclear if the PACAP/PAC1 receptor pathway has a role in human psychological stress responses, such as posttraumatic stress disorder (PTSD). In heavily traumatized subjects, we find a sex-specific association of PACAP blood levels with fear physiology, PTSD diagnosis and symptoms in females (N=64, replication N=74,  $p < 0.005$ ). Using

---

Users may view, print, copy, download and text and data- mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: [http://www.nature.com/authors/editorial\\_policies/license.html#terms](http://www.nature.com/authors/editorial_policies/license.html#terms)

Corresponding Author: Kerry J. Ressler, MD, PhD, Investigator, Howard Hughes Medical Institute, Associate Professor, Department of Psychiatry and Behavioral Sciences, Yerkes Research Center, Emory University, 954 Gatewood Dr Atlanta, GA 30329, USA, off: 404-727-7739, fax: 404-727-8070, [kressle@emory.edu](mailto:kressle@emory.edu).

Supplementary Online Material: *Detailed Materials and Methods* (13 pages), with 4 Tables and 3 Figures

Reprints and permissions information is available at [www.nature.com/reprints](http://www.nature.com/reprints).

The authors declare no competing financial interests.

**Author Contributions:** K.J.R. designed the experiments and wrote the paper. K.J.R., B.B., and V.M. organized collaborations, obtained funding, supervised data collection and analyses, and revised the paper. K.B.M., K.K., and E.B.B. performed the genetics experiments and analyses on the primary, replication, and GAIN cohorts, and revised the paper. T.J. and S.D.N. performed and supervised the human physiology studies and revised the paper. V.K. and A.K.S. performed and supervised the methylation studies and revised the paper. A.M., S.E.H., D.T., K.J.R., and V.M. performed and supervised the rodent fear conditioning and estrogen replacement studies and rtPCR analyses and revised the paper. A.J.M., M.R., and A.E. performed and supervised the human mRNA expression analyses and the Alzheimer's disease gene association studies. K.M.B. and V.M. performed and supervised the PACAP38 radioimmunoassays and related data analyses.

a tag-SNP genetic approach (44 single nucleotide polymorphisms, SNPs) spanning the PACAP (*ADCYAP1*) and PAC1 (*ADCYAP1R1*) genes, we find a sex-specific association with PTSD. rs2267735, a SNP in a putative estrogen response element within *ADCYAP1R1*, predicts PTSD diagnosis and symptoms in females only (combined initial and replication samples: N=1237;  $p < 2 \times 10^{-5}$ ). This SNP also associates with fear discrimination and with *ADCYAP1R1* mRNA expression. Methylation of *ADCYAP1R1* is also associated with PTSD ( $p < 0.001$ ). Complementing these human data, *ADCYAP1R1* mRNA is induced with fear conditioning or estrogen replacement in rodent models. These data suggest that perturbations in the PACAP/PAC1 pathway are involved in abnormal stress responses underlying PTSD. These sex-specific effects may occur via estrogen regulation of *ADCYAP1R1*. PACAP levels and *ADCYAP1R1* SNPs may serve as useful biomarkers to further our mechanistic understanding of PTSD.

---

PACAP was first isolated from ovine hypothalamic extracts based on its ability to stimulate cAMP production in anterior pituitary cells<sup>1</sup>. It is a highly conserved member of the VIP/secretin/glucagon peptide family with pleiotropic functions in development, cell signaling, metabolism, homeostasis and cell protection<sup>2–6</sup>. Among these myriad functions are studies demonstrating 1) high expression of PACAP peptide and its selective PAC1 receptor in hypothalamic and limbic structures, 2) PACAP regulation of corticotropin releasing hormone (CRH) and autonomic function, 3) actions of PACAP in stress-related behavior, 4) reduced anxiety-like phenotypes in PACAP and PAC1 receptor null mice, and 5) blunted corticosterone response in knockout animals after emotional stressors. Thus, PACAP/PAC1 receptor signaling is integrally involved in stress mechanisms<sup>7,8</sup>. We hypothesized that PACAPergic systems may be important mediators of abnormal stress responses following psychological trauma contributing to PTSD, an extreme maladaptive and debilitating psychiatric disorder affecting up to 40% of individuals over lifetime exposure to traumatic events<sup>9,10</sup>.

Little is known about the biological processes regulating PTSD and other stress-related responses. To examine whether the PACAP/PAC1 pathway is associated with PTSD in a high risk, heavily traumatized population, we analyzed blood levels of PACAP, and genetic variation and methylation of the PACAP (*ADCYAP1*) and PAC1 receptor (*ADCYAP1R1*) genes in a cohort of over 1200 highly traumatized subjects with and without PTSD (See Suppl. Tables 1–2 for demographic information).

## PACAP levels associated with PTSD in females

Using radioimmunoassay, we first examined PACAP peptide levels in peripheral blood samples from a previously described, highly traumatized, at risk population<sup>11–13</sup> that had been matched on age, sex, and trauma histories (n = 64, see Suppl. Tables 1–3 for demographics). We found that PTSD symptoms (PTSD Symptom Scale<sup>14</sup>) were significantly correlated with PACAP38 (PACAP peptide containing 38 residues) blood levels in females ( $p < 0.005$ ,  $r = 0.497$ , Fig 1A), but not in males ( $p > 0.5$ ). Also in females, PTSD diagnosis was associated with PACAP38 levels ( $p = 0.001$ ), with higher PACAP38 found in the PTSD cohort. Furthermore, PACAP levels (median split, low vs. high) were differentially associated with PTSD symptoms in females (Fig 1B). PACAP38 levels also predicted differential response on all three symptom clusters necessary to fulfill diagnostic

criteria for PTSD (intrusive re-experiencing (e.g., trauma flashbacks), avoidance (e.g., avoidance of trauma reminders) and hyperarousal (e.g., increased startle response)) in females but not males (Fig 1C). These analyses were repeated in a second, all female cohort (N=74) with similar findings (Fig 1D; high vs. low PACAP38 levels, controlling for age, substance abuse and total trauma exposure, one-tailed t-tests: total symptoms,  $p = 0.05$ , hyperarousal symptoms,  $p = 0.001$ ; and % with clinically significant symptoms,  $\chi^2=4.9$ ,  $p<0.05$ ). These observations were especially notable since females may be at twice the risk for PTSD as compared to males<sup>9,11</sup>, implicating roles for sex hormones, especially estrogen, in the disorder<sup>15–17</sup>. When we controlled for common stress-related phenomena (depression and history of substance abuse), the effect of PACAP level on PTSD remained ( $p<0.05$ ). In contrast, there was no effect of PACAP level on depression symptoms or substance abuse when controlling for PTSD.

In addition to the psychological symptoms that define the syndrome, subjects with PTSD have been found to have abnormally high conditioned fear responses. This high level of fear may result from a combination of an inability to habituate to aversive stimuli, a decreased ability to extinguish (learn to inhibit) fear memories, and possibly an over-consolidation of the original fear memory<sup>18–22</sup>. Hence, we determined the physiological (electromyographic) levels of conditioned fear for 27 participants (16 male, 11 female) with PACAP blood levels. Fear potentiation was determined by measuring the acoustic startle reflex in the presence of startle cues alone, or startle cues combined with stimuli paired (conditioned stimulus, CS+) or unpaired (CS-) with an aversive airblast (Fig 1E). Female (but not male) subjects with high PACAP38 levels demonstrated markedly increased startle reflex responses to both CS+ and CS- cues. This was particularly pronounced during the late acquisition phase when normal subjects had habituated to the fearful stimuli (Fig 1F). In aggregate, these data suggest that PACAP38 peptide is strongly associated with the psychological and physiological symptoms of PTSD in women with a history of trauma.

### **ADCYAP1R1 associated with PTSD in females**

To assess whether there may be a genetic association of PTSD with polymorphisms in either the PACAP (*ADCYAP1*) or PAC1 receptor (*ADCYAP1R1*) locus, we performed a tag-SNP analysis ( $r^2 = 0.8$ ; MAF = 0.1) across both genes with a total of 44 SNPs (14 *ADCYAP1* and 30 *ADCYAP1R1* SNPs). Using logistic regression, we examined whether each SNP was associated with PTSD diagnosis in this cohort of highly traumatized urban civilian subjects ( $n = 798$ )<sup>11,12,23</sup>, in total, or stratified by gender (females:  $n = 503$ ; males:  $n = 295$ ). Only the *ADCYAP1R1* receptor SNP rs2267735 ( $p = 0.0002$  in females; *NS* in males) remained significant after experiment-wide multiple correction for sex and 44 independent tests (Figure 2A–B and Suppl. Fig. 1). No SNPs in the peptide *ADCYAP1* gene met experiment-wide criteria for association (Suppl. Fig. 2). Given these striking gender differences and recent data demonstrating that *ADCYAP1R1* gene expression may be dynamically modulated by estrogen<sup>24</sup>, the distribution of estrogen response elements (EREs) within *ADCYAP1R1* gene was examined (Suppl. Table 4). We found that rs2267735 was within a predicted ERE (Fig 2C, Genomatix; matrix similarity = 0.877, core similarity = 1.0). Because rs2267735 is positioned within the central variable region of the consensus

sequence, *in silico* analyses do not currently allow us to predict how the ‘C’ vs ‘G’ allele may differentially alter ERE function and further *in vitro* analyses are warranted.

We next determined if the association between rs2267735 and PTSD diagnosis could be replicated in an additional 439 subjects. These subjects were from the same overall study, but were interviewed and had DNA collected after the original discovery population. Thus they served as a replication source from the same population but distinct in time and with different interviewing staff. The table in Figure 3A shows the logistic regression results for males and females separately in the initial population described in the tag-SNP analysis, the replication sample from the same population, and the combined sample of 1237 individuals. The main effect of the SNP on PTSD diagnosis could be replicated in women ( $p < 0.05$ ) and combining both samples increased the significance of the association ( $N = 763$ ,  $p < 0.00002$ ). As in the discovery sample, no effects were observed in males (male combined sample  $N = 474$ ,  $p = 0.7$ ).

To further examine *ADCYAP1R1* rs2267735 SNP associations with continuous PTSD symptom levels in females, we analyzed both an additive and a dominant model with total PTSD symptoms and symptom subscales using the combined samples (Fig 3B – E). The ‘CC’ allele was most robustly associated with total PTSD symptoms and among subscales, hyperarousal symptoms were the most strongly associated with rs2267735. Notably, even after controlling for childhood trauma history and adult trauma, age and race, (which slightly reduces total N due to missing data) the rs2267735 ‘CC’ allele was associated with higher levels of PTSD hyperarousal symptoms compared to ‘G’ carriers in women ( $p = 0.0008$ , Fig 3E), but not men ( $p = 0.51$ ).

We repeated the above analyses with Beck Depression Inventory (BDI) symptoms and history of lifetime substance abuse, and found no associations with these measures and rs2267735 (Suppl. Fig. 3), suggesting that this association may be relatively specific to PTSD. To address whether rs2267735 might be associated with other severe psychiatric illnesses, we performed analyses using bipolar disorder, schizophrenia, and Alzheimer’s disease samples. From the data of the Genetic Association Information Network (GAIN) publicly accessible database (<http://www.ncbi.nlm.nih.gov/projects/gap>), we analyzed the association of rs2267735 (included on the Affymetrix 6.0 SNP array) with bipolar disorder as well as schizophrenia. We did not observe a significant association of this SNP with these two disorders in subjects with African American (954 cases, 1195 controls) or European (1378 cases, 1351 controls) ancestry. Specifically, we found that all pre-computed p-values for associations of rs2267735 with schizophrenia or bipolar disorder were higher than the multiple-testing correction p-value of 0.01, indicating no major contribution of this variant.

Additionally, we examined the association of rs2267735 and Alzheimer’s in a previously characterized Alzheimer’s disease sample<sup>25</sup>. In this cohort of 342 subjects, we found no association with rs2267735 and Alzheimer’s disease diagnosis using either the additive genetic model ( $p = 0.19$ ) or the dominant/recessive model ( $p = 0.89$ ). These data suggest that we find robust associations with rs2267735 in women, but not men, with PTSD. In contrast, we find no association with depression symptoms, substance abuse, Alzheimer’s disease, bipolar disorder, and schizophrenia across different samples. Note that for all of these

negative results, due to the limited sample sizes, we cannot rule out the possibility that rs2267735 may be associated with PTSD in men or with other disorders with a smaller effect size than we see with PTSD in women.

To parallel our results with plasma PACAP38 levels, we next examined whether physiological measures of fear are differentially associated with the *ADCYAP1R1* rs2267735 SNP. In PTSD, but not depression<sup>18</sup>, fear response to an inhibitory CS-, or 'safety signal', is exaggerated. The discrimination between CS+ and CS- improves across the training procedure in controls, but not in those with PTSD. We examined whether rs2267735 was associated with impaired fear discrimination late in conditioned acquisition, during the same period noted in Figure 1E. Notably, females with the 'CC' genotype were significantly less able to discriminate CS+ from CS- signals (Figure 3F, sex by genotype interaction,  $p < 0.05$ , and 'CC' vs 'G' carriers in females,  $p < 0.05$ ).

We next examined whether a difference in dark-enhanced startle, a measure of increased anxiety in humans that is similar to light-enhanced startle in rodents<sup>26–28</sup>, was differentially associated with rs2267735. Again, we found that females, but not males, with the 'CC' genotype showed significantly more startle in the dark compared to the light (Fig 3G, males,  $N=35$ ,  $p=0.71$ ; females,  $N=53$ ,  $p=0.02$ ). Together, these data suggest that the *ADCYAP1R1* rs2267735 SNP may be relatively specific in its association with PTSD psychological and physiological phenotypes. Further, the robust association of rs2267735 with hyperarousal symptoms suggests that the role of PACAP/PAC1 may be specifically involved in the normalization of the stress response, a process which is particularly dysregulated in PTSD.

## ADCYAP1R1 methylation and mRNA expression

Environmental, genetic and epigenetic mechanisms likely moderate the long-term effects of trauma exposure. Using the Illumina HumanMethylation<sup>27</sup> BeadChip, we interrogated methylation in DNA extracted from peripheral blood at the first site within the *ADCYAP1R1* CpG island (Suppl. Fig. 2). Methylation at this site was significantly associated with total PTSD symptoms (Figure 4A,  $N=94$ ,  $r = 0.354$ ,  $p < 0.0005$ ) in a sex-independent manner. Further, CpG methylation level (median split) was associated with PTSD diagnosis (Figure 4B,  $\chi^2=8.1$ ,  $p < 0.005$ ), but not depression ( $p > 0.05$ , Suppl. Fig. 3E). There was no significant association between methylation of *ADCYAP1* and PTSD symptoms. These data suggest that *ADCYAP1R1* is regulated, in part, through epigenetic mechanisms that contribute to differential function of the PAC1 receptor in PTSD.

To examine the potential relationship of genotype and brain mRNA expression as described previously<sup>29</sup>, we utilized a brain mRNA expression data set<sup>30</sup> to test whether *ADCYAP1R1* rs2267735 is associated with differential gene expression. We first examined whether cortical *ADCYAP1R1* and *ADCYAP1* mRNA levels were correlated. As shown in Fig 4C, these mRNA levels were significantly inversely correlated ( $r = -0.219$ ,  $p < 0.001$ , including males and females), suggesting that brain levels of PACAP peptide and PAC1 mRNA are tightly regulated.

We next utilized a prior analyzed genome-wide association and brain mRNA expression data<sup>30</sup> to examine whether *ADCYAP1R1* rs2267735 imputed genotypes were associated

with differential *ADCYAP1R1* expression in brain. We found a sex x genotype effect (Fig 4D,  $F(3,99) = 4.3, p < 0.05$ ) with females with the 'CC' genotype expressing significantly less *ADCYAP1R1* mRNA than males ( $F(1,33) = 5.5, p < 0.05$ ) or than females who are 'G' carriers (one-tailed,  $F(1, 45) = 2.87, p < 0.05$ ). Thus, mRNA encoding the PACAP peptide and PAC1 receptor appeared to be tightly regulated within the human cortex, and *ADCYAP1R1* mRNA levels were associated with the *ADCYAP1R1* rs2267735 SNP.

## Fear induces *ADCYAP1R1* in mouse amygdala

Despite prior studies examining PACAP/PAC1 receptor function in CNS/PNS development, endocrine homeostasis, metabolism, cellular protection/regeneration, and chronic stress responses<sup>2,3,6,31–34</sup>, a role for PACAP signaling in fear conditioning has not been evaluated. Given our data implicating PACAP in PTSD, we wondered if *ADCYAP1R1* mRNA was differentially regulated in mice using Pavlovian fear conditioning<sup>35–40</sup>, a means of studying acute fear and trauma responses that has been proposed to model PTSD<sup>19,22</sup>. We performed classical fear conditioning experiments using male mice, in which a previously neutral tone CS (6kHz) was paired with 10 footshocks (1mA, 0.5sec; Figure 5A). This conditioning paradigm consistently provides robust fear learning in mice leading to changes in gene expression within the amygdala, a region critical for fear learning and expression. Quantitative PCR analyses shows that amygdala *ADCYAP1R1* mRNA increased ~1.5-fold during the consolidation of fear (Figure 5B,  $p < 0.05$ ), with a similar trend within the mPFC. When peak freezing was compared with brain mRNA levels, we find a significant correlation between fear learning and *ADCYAP1R1* mRNA (Fig 5C,  $r^2 = 0.49, p < 0.05$ ).

## Estrogen induces *ADCYAP1R1* in rat BNST

To further establish the relationship between PACAP/PAC1 receptors and estrogen in a validated model of sex hormone regulation, we examined estrogen-induced changes in *ADCYAP1* and *ADCYAP1R1* transcripts in the bed nucleus of stria terminalis (BNST) in female rats. The BNST is a component of the extended amygdala that is subject to significant gonadal hormonal control<sup>7,27,28</sup>. In rodents, it is critical for emotional behavior, mediating stress responses and the light-enhanced startle response. We examined gene expression in the BNST in ovariectomized (OVX) female rats following 21-day implantation of continuous release estrogen pellets. Compared to control implants, estradiol increased *ADCYAP1* transcripts in the dorsal and ventral BNST 2.1- and 3.4-fold, respectively ( $p < 0.01$ , Figure 5D). Additionally, estradiol increased *ADCYAP1R1* mRNA 1.5-fold in the dorsal BNST samples ( $p < 0.05$ , Figure 5E), and future studies should also examine estradiol sensitivity of these genes in amygdala. While these rodent studies are complex and have differing experimental designs, these data clearly illustrate dynamic PACAP/PAC1 receptor regulation within central areas mediating fear, stress and estrogen responsiveness.

## Discussion

Since its identification more than 20 years ago, PACAP has been increasingly implicated in diverse cellular stress response pathways and neurotrophic function. However, the

organizational role of the PACAP system in orchestrating behavioral stress responses has yet to be clarified. Our data suggest that PACAP/PAC1 receptor expression and signaling may be integrally involved in regulating the psychological and physiological responses to traumatic stress. Further, we report association of an ERE-embedded *ADCYAP1R1* SNP with PTSD, and we demonstrate fear- and estrogen-dependent regulation of PACAP systems within stress-responsive regions of the brain. These data may begin to explain sex-specific differences in PTSD diagnosis, symptoms, and fear physiology. Future work targeting the PACAP/PAC1 receptor system may lead to novel and robust biomarkers as well as to further our understanding of the neural mechanisms underlying pathological responses to stress with potential therapeutic targets towards the prevalent and debilitating syndrome of PTSD.

### Methods Summary (Please see detailed Online Supplementary Methods)

This highly traumatized, civilian, cross-sectional cohort has been previously described in candidate gene-association studies of PTSD and depression<sup>11–13</sup>. Research interviews, salivary DNA and blood samples were collected from patients receiving services in the primary care clinics at Grady Memorial Hospital (Atlanta, GA). All study procedures have been reviewed and approved by the Emory Institutional Review Board and the Grady Hospital Research Oversight Committee. PTSD measures in this manuscript are based on the PTSD symptom scale 14, which has been validated within this population using the Clinician Administered PTSD Scale. PACAP38 radioimmunoassay (1:30,000, Peninsula Laboratories, Belmont, CA) was performed at University of Vermont, using double antibody immunoprecipitation as previously described<sup>41</sup>. For genotyping, pairwise tagging ( $R^2 > 0.8$ , MAF  $> 0.1$ ) was used to choose tag-SNPs for both *ADCYAP1* and *ADCYAP1R1*. The coordinates were chr18:885000-906000 and chr7:31048667-31117836 for *ADCYAP1* and *ADCYAP1R1*, respectively (NCBI B36) which includes approximately 10kb upstream and 5kb downstream of the coding regions for both genes. Genotypes for the tag-SNPs were generated using Sequenom iPLEX with follow-up analyses using Taqman. For methylation analyses, DNA was whole-genome amplified, fragmented, and hybridized to the HumanMethylation27 BeadChip (Illumina, San Diego, CA). Individual samples were stratified to separate BeadChips according to PTSD status to limit bias. The BeadChips were scanned using a BeadStation 500GX, and the methylation level (beta value) was calculated using the Methylation Module of the BeadStudio software. The eyeblink component of the acoustic startle response was measured by EMG recordings of the right orbicularis oculi muscle with two 5-mm Ag/AgCl electrodes filled with electrolyte gel, as previously described<sup>18–19</sup>. The mouse fear conditioning and rat estrogen replacement studies are described in detail in Supplementary Methods.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

This work was primarily supported by National Institutes Health Grants MH071537, DA019624 (KJR) and HD27468 (VM). Support was also received from Emory and Grady Memorial Hospital General Clinical Research

Center, NIH National Centers for Research Resources (M01RR00039 and P20RR16435), the American Foundation for Suicide Prevention (BB) and the Burroughs Wellcome Fund (KJR). RNA samples were run using the Oncogenomics Core Facility at University of Miami. Dr Myers is supported by The National Institute on Aging (AG034504). We thank Drs. Joseph F. Cubells, Yilang Tang, and Karen Conneely for excellent discussions. We thank the Grady Trauma Project, including Drs. Charles F. Gillespie, Ann Schwartz, Aliza Wingo, David A. Gutman, and Tamara Weiss for medical support; Allen Graham, Angelo Brown, Justine Phifer, Daniel Crain, Asante Kamkwala, James Poole, Dorthie Cross, Negar Fani, and Dr. Ami Smith for clinical research support; and Kristin Schutz, Emily Reiser and Caitlin Fitzgerald for excellent molecular/genetics technical support. Methylation chip assays were performed by the Emory University Biomarkers Service Center.

## References

1. Miyata A, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun*. 1989; 164:567–74. [PubMed: 2803320]
2. Ghzili H, et al. Role of PACAP in the physiology and pathology of the sympathoadrenal system. *Front Neuroendocrinol*. 2008; 29:128–41. [PubMed: 18048093]
3. Hashimoto H, Shintani N, Baba A. New insights into the central PACAPergic system from the phenotypes in PACAP- and PACAP receptor-knockout mice. *Ann N Y Acad Sci*. 2006; 1070:75–89. [PubMed: 16888150]
4. Spengler D, et al. Differential signal transduction by five splice variants of the PACAP receptor. *Nature*. 1993; 365:170–5. [PubMed: 8396727]
5. Watanabe J, et al. Localization, characterization and function of pituitary adenylate cyclase-activating polypeptide during brain development. *Peptides*. 2007; 28:1713–9. [PubMed: 17719696]
6. Zhong Y. Mediation of PACAP-like neuropeptide transmission by coactivation of Ras/Raf and cAMP signal transduction pathways in *Drosophila*. *Nature*. 1995; 375:588–92. [PubMed: 7791875]
7. Hammack SE, et al. Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): roles for PACAP in anxiety-like behavior. *Psychoneuroendocrinology*. 2009; 34:833–43. [PubMed: 19181454]
8. Vaudry D, et al. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev*. 2009; 61:283–357. [PubMed: 19805477]
9. Breslau N. The epidemiology of posttraumatic stress disorder: what is the extent of the problem? *J Clin Psychiatry*. 2001; 62 (Suppl 17):16–22. [PubMed: 11495091]
10. Hoge CW, Auchterlonie JL, Milliken CS. Mental health problems, use of mental health services, and attrition from military service after returning from deployment to Iraq or Afghanistan. *Jama*. 2006; 295:1023–32. [PubMed: 16507803]
11. Binder EB, et al. Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. *JAMA*. 2008; 299:1291–305. [PubMed: 18349090]
12. Bradley RG, et al. Influence of child abuse on adult depression: moderation by the corticotropin-releasing hormone receptor gene. *Arch Gen Psychiatry*. 2008; 65:190–200. [PubMed: 18250257]
13. Gillespie CF, Phifer J, Bradley B, Ressler KJ. Risk and resilience: genetic and environmental influences on development of the stress response. *Depress Anxiety*. 2009; 26:984–92. [PubMed: 19750552]
14. Foa EB, Tolin DF. Comparison of the PTSD Symptom Scale-Interview Version and the Clinician-Administered PTSD scale. *J Trauma Stress*. 2000; 13:181–91. [PubMed: 10838669]
15. Bangasser DA, et al. Sex differences in corticotropin-releasing factor receptor signaling and trafficking: potential role in female vulnerability to stress-related psychopathology. *Mol Psychiatry*. 2010; 15:877, 896–904. [PubMed: 20548297]
16. McEwen BS. Steroid hormones: effect on brain development and function. *Horm Res*. 1992; 37 (Suppl 3):1–10. [PubMed: 1330863]
17. Shansky RM, et al. Estrogen promotes stress sensitivity in a prefrontal cortex-amygdala pathway. *Cereb Cortex*. 2010; 20:2560–7. [PubMed: 20139149]



18. Jovanovic T, et al. Impaired fear inhibition is a biomarker of PTSD but not depression. *Depress Anxiety*. 2010; 27:244–51. [PubMed: 20143428]
19. Jovanovic T, Ressler KJ. How the neurocircuitry and genetics of fear inhibition may inform our understanding of PTSD. *Am J Psychiatry*. 2010; 167:648–62. [PubMed: 20231322]
20. Rauch SL, Shin LM, Phelps EA. Neurocircuitry models of posttraumatic stress disorder and extinction: human neuroimaging research—past, present, and future. *Biol Psychiatry*. 2006; 60:376–82. [PubMed: 16919525]
21. Shin LM, Liberzon I. The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology*. 2010; 35:169–91. [PubMed: 19625997]
22. Yehuda R, LeDoux J. Response variation following trauma: a translational neuroscience approach to understanding PTSD. *Neuron*. 2007; 56:19–32. [PubMed: 17920012]
23. Gillespie CF, et al. Trauma exposure and stress-related disorders in inner city primary care patients. *Gen Hosp Psychiatry*. 2009; 31:505–14. [PubMed: 19892208]
24. Aenlle KK, Kumar A, Cui L, Jackson TC, Foster TC. Estrogen effects on cognition and hippocampal transcription in middle-aged mice. *Neurobiol Aging*. 2009; 30:932–45. [PubMed: 17950954]
25. Corneveaux JJ, et al. Association of CR1, CLU and PICALM with Alzheimer's disease in a cohort of clinically characterized and neuropathologically verified individuals. *Hum Mol Genet*. 2010; 19:3295–301. [PubMed: 20534741]
26. Grillon C, Morgan CA 3rd, Davis M, Southwick SM. Effect of darkness on acoustic startle in Vietnam veterans with PTSD. *Am J Psychiatry*. 1998; 155:812–7. [PubMed: 9619155]
27. Walker DL, Davis M. Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. *J Neurosci*. 1997; 17:9375–83. [PubMed: 9364083]
28. Walker DL, Davis M. Light-enhanced startle: further pharmacological and behavioral characterization. *Psychopharmacology (Berl)*. 2002; 159:304–10. [PubMed: 11862363]
29. McMahon FJ, et al. Meta-analysis of genome-wide association data identifies a risk locus for major mood disorders on 3p21.1. *Nat Genet*. 2010; 42:128–31. [PubMed: 20081856]
30. Myers AJ, et al. A survey of genetic human cortical gene expression. *Nat Genet*. 2007; 39:1494–9. [PubMed: 17982457]
31. Botia B, et al. Neurotrophic effects of PACAP in the cerebellar cortex. *Peptides*. 2007; 28:1746–52. [PubMed: 17544170]
32. Brenneman DE. Neuroprotection: a comparative view of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Peptides*. 2007; 28:1720–6. [PubMed: 17513014]
33. Dejda A, et al. Inhibitory effect of PACAP on caspase activity in neuronal apoptosis: a better understanding towards therapeutic applications in neurodegenerative diseases. *J Mol Neurosci*. 2008; 36:26–37. [PubMed: 18506634]
34. Hamelink C, et al. Pituitary adenylate cyclase-activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. *Proc Natl Acad Sci U S A*. 2002; 99:461–6. [PubMed: 11756684]
35. Davis M. The role of the amygdala in fear and anxiety. *Annu Rev Neurosci*. 1992; 15:353–75. [PubMed: 1575447]
36. Ehrlich I, et al. Amygdala inhibitory circuits and the control of fear memory. *Neuron*. 2009; 62:757–71. [PubMed: 19555645]
37. Fanselow MS, LeDoux JE. Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala. *Neuron*. 1999; 23:229–32. [PubMed: 10399930]
38. LeDoux JE. Emotional memory: in search of systems and synapses. *Ann N Y Acad Sci*. 1993; 702:149–57. [PubMed: 8109874]
39. Maren S, Quirk GJ. Neuronal signalling of fear memory. *Nat Rev Neurosci*. 2004; 5:844–52. [PubMed: 15496862]
40. Pape HC, Pare D. Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiol Rev*. 2010; 90:419–63. [PubMed: 20393190]

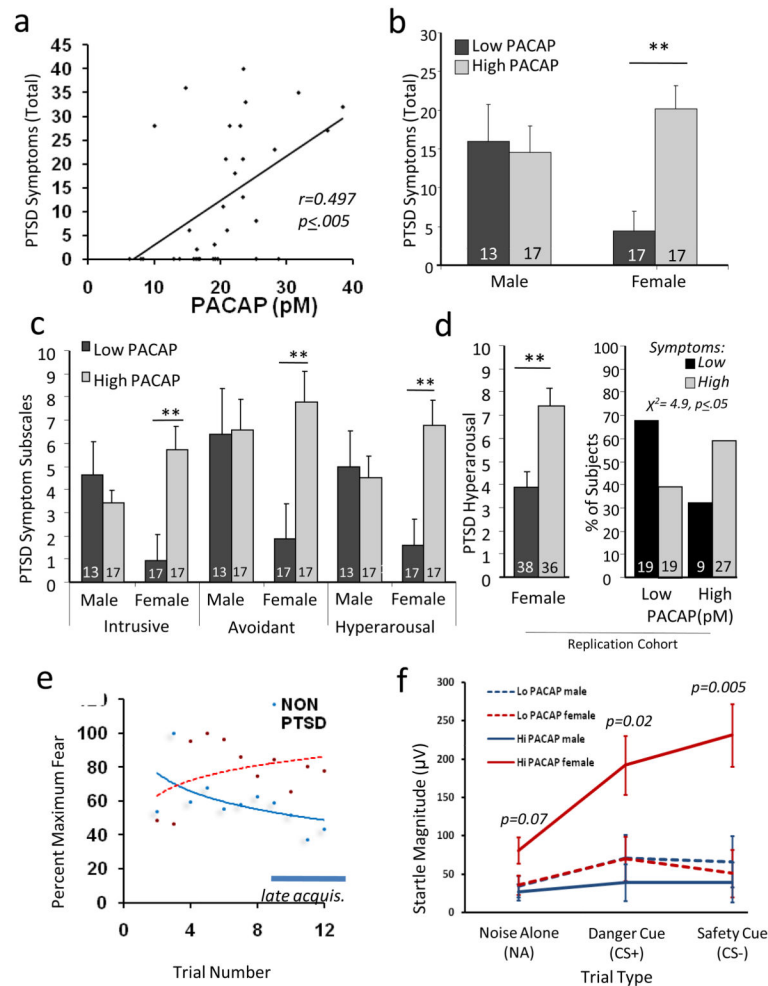
41. Girard BA, et al. Noncompensation in peptide/receptor gene expression and distinct behavioral phenotypes in VIP- and PACAP-deficient mice. *J Neurochem.* 2006; 99(2):499–513. [PubMed: 17029602]

Author Manuscript

Author Manuscript

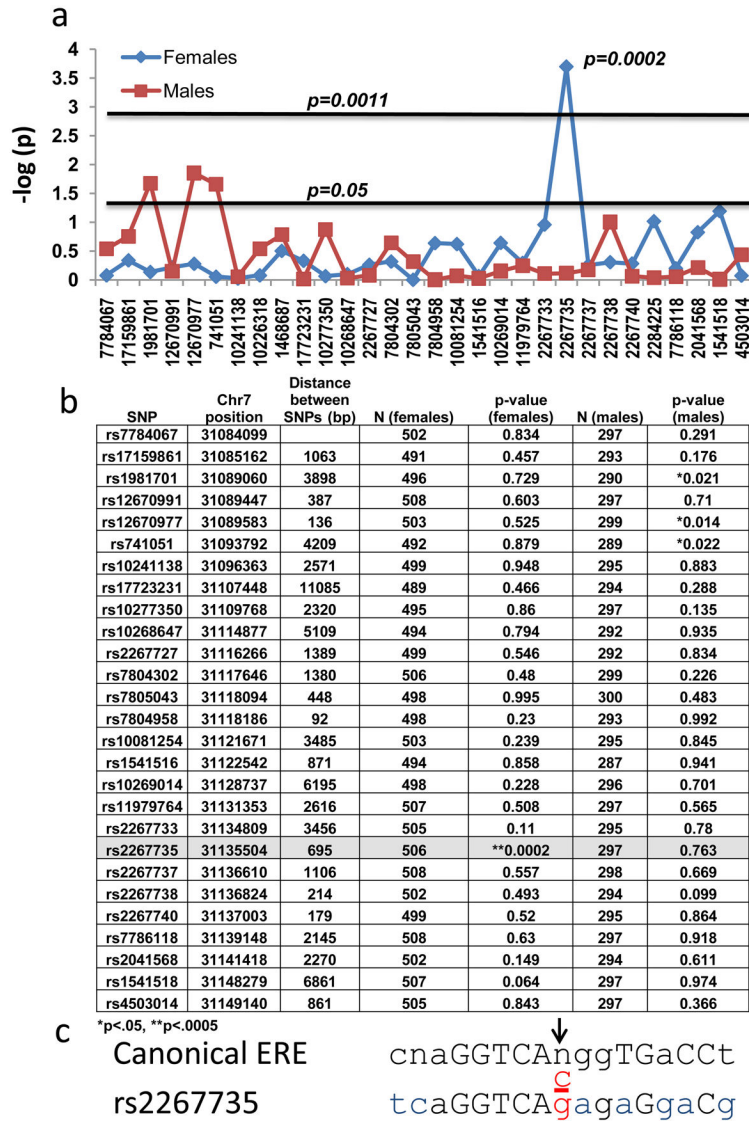
Author Manuscript

Author Manuscript



### Figure 1. PACAP blood levels predict PTSD symptoms in females

A) PTSD Symptoms (scale range 0–51), relative to Plasma PACAP38 blood levels (pM); (N=34 females;  $r = 0.497$ ,  $p = 0.005$ ). B) Total PTSD symptoms graphed relative to sex and levels of plasma PACAP38 (N=64, low: < 20pM, high: > 20pM); females with high PACAP blood levels have increased symptoms (\*\*,  $p < 0.0005$ ). C) PACAP levels (low vs. high) are also differentially associated with PTSD Intrusive, Avoidance, and Hyperarousal symptoms in females (N = 64, \*\*  $p < 0.005$ ). D) PACAP levels (low vs. high) were examined in a replication sample of highly traumatized women, with differential association in hyperarousal symptoms (left, N=74, \*\*  $p=0.002$ ) and in percent of subjects with significant symptoms (right,  $\chi^2=4.9$ ,  $p < 0.05$ ). E) Acoustic startle reflex (EMG) relative to the fear conditioning trial in subjects without (non-PTSD) vs with PTSD. Habituation is seen in non-PTSD subjects during late acquisition (bar). F) Startle magnitude during the late acquisition period vs. trial type (noise alone, CS+, and CS–), showing that females with high PACAP levels show enhanced startle responses to both fear cues (CS+,  $p = 0.02$ ) and safety cues (CS–,  $p = 0.005$ ) (N=27; 16 male, 11 female). Bars represent mean  $\pm$  SEM., N's for each group at bottom of bar graphs.



**Figure 2. Genetic Association of PAC1 Receptor (*ADCYAP1R1*) with PTSD**

A) 30 single nucleotide polymorphisms (SNPs) spanning the *ADCYAP1R1* gene (x-axis), with the  $-\log(p\text{-value})$  of logistic regression for each SNP association with PTSD (diagnosis based on DSM-IV criteria from PTSD Symptom Scale). Subjects were analyzed with logistic regression in females only (N=503) or males only (N=295). Horizontal lines represent the nominal  $p = 0.05$  or the corrected p-value,  $p = 0.0011$  (44 SNPs, correcting for 30 *ADCYAP1R1* SNPs and 14 *ADCYAP1* SNPs (Supp. Fig. 1)). rs2267735 is the only SNP remaining significant after multiple corrections ( $p = 0.0002$ ). B) Table of p-values resulting from the association of each genotyped, *ADCYAP1R1* SNP with PTSD diagnosis (by gender). The location on Chromosome 7 (GRCh37) for each SNP including the distance (bp) between the SNPs is given. The average distance between SNPs is 2.2 kb. SNP rs2267735 is located in an intron of *ADCYAP1R1*, and is not in LD with other SNPs (for African Americans in our population, data not shown). C) rs2267735 (C/G), in red, is located within a canonical estrogen response element (ERE) binding site (Capitol letters –

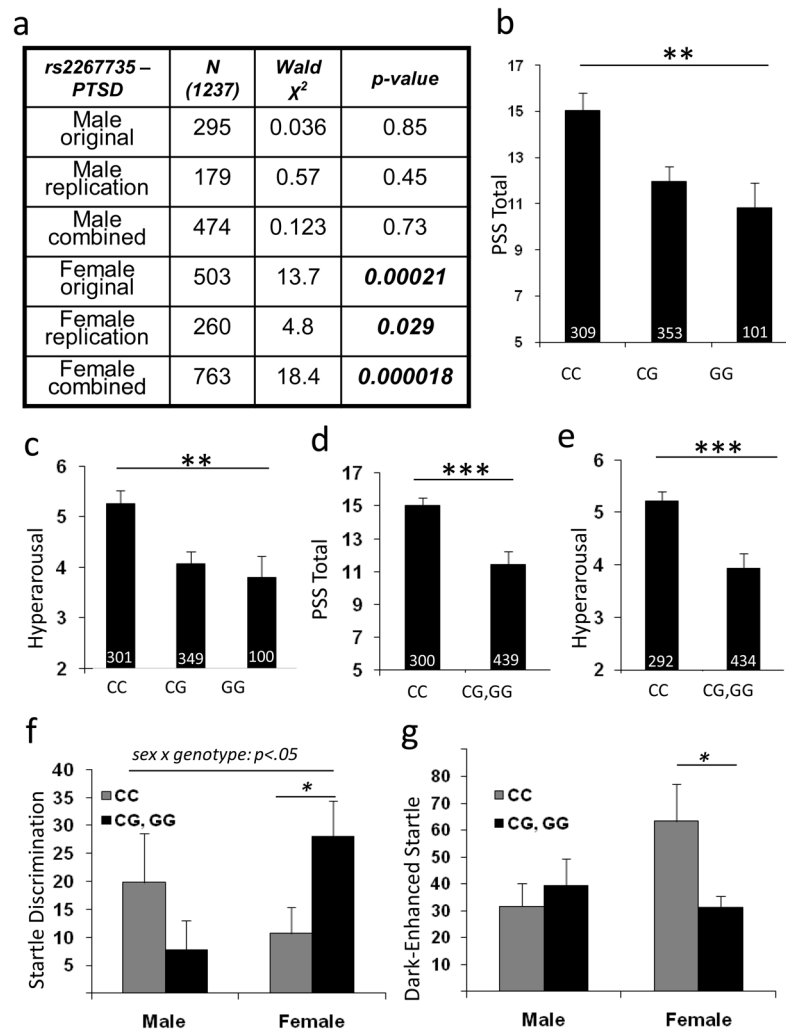
conserved canonical ERE nucleotides; blue letters – mismatches with the *ADCYAP1R1* gene and canonical ERE; Reverse strand shown).

Author Manuscript

Author Manuscript

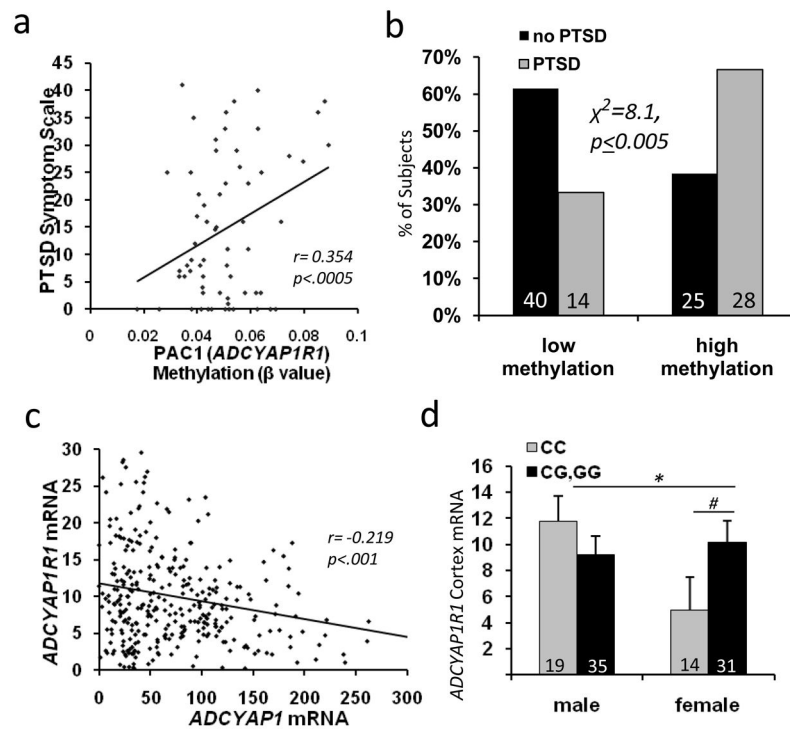
Author Manuscript

Author Manuscript



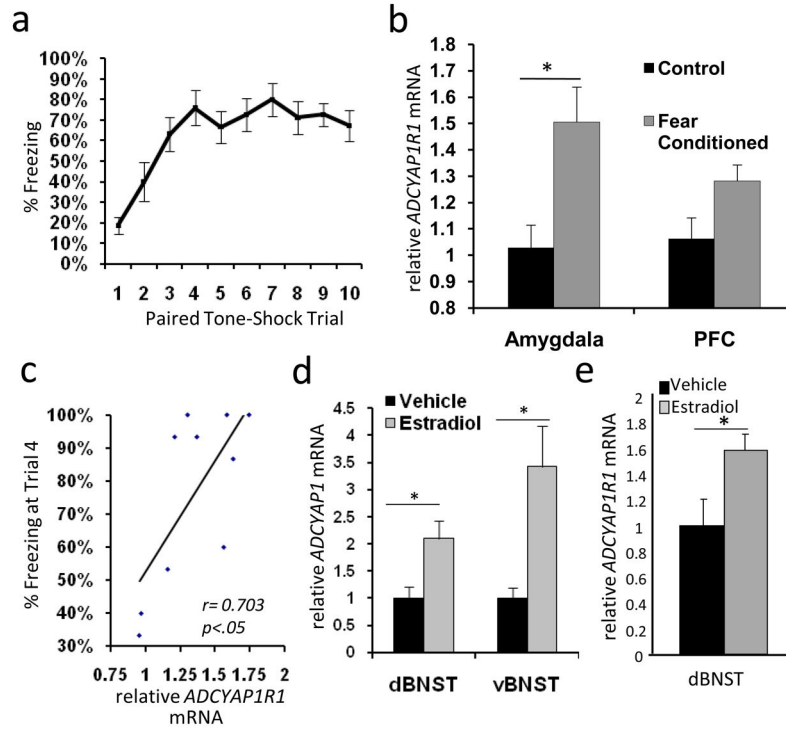
**Figure 3. Association of *ADCYAP1R1* rs2267735 with PTSD symptoms and physiological fear responses**

A) Table demonstrating the N, Wald  $\chi^2$ , and p-value, in males and females, in the original, replication, and combined samples for logistic regression of rs2267735 with PTSD diagnosis. B) Total PTSD symptoms (PSS total) are differentially associated with rs2267735 genotype in females ( $p = 0.001$ ). C) Hyperarousal is the most robustly associated symptom with rs2267735 genotype ( $p = 0.0009$ ). D) In a dominant/recessive model, even after controlling for childhood trauma, adult trauma, and age, genotype predicts total PTSD symptoms ( $p = 0.001$ ) and (E) hyperarousal symptoms ( $p = 0.0001$ ). F) Fear Discrimination (CS+ Startle minus CS– Startle) is impaired in females with rs2267735 ‘CC’ genotype. G) Dark enhanced startle (Startle<sup>Dark</sup> – Startle<sup>Light</sup>) is significantly increased in females with rs2267735 ‘CC’ genotype. (N’s are shown at base of each bar, bars represent mean  $\pm$  SEM. N’s are slightly different across analyses due to differences in number of subjects across measures. \*  $p < 0.05$ ; \*\*  $p < 0.001$ ; \*\*\*  $p < 0.0002$ )



#### Figure 4. *ADCYAP1R1* Methylation and mRNA expression

A) Methylation within the first CpG island of *ADCYAP1R1* (beta value, Illumina #cg27076139) is positively correlated with total PTSD symptoms (both sexes;  $N=107$ ;  $r = 0.354$ ,  $p < 0.0005$ ). B) Subjects with PTSD have higher levels of *ADCYAP1R1* methylation (median split,  $N = 107$ ; chi-squared analyses,  $p < 0.005$ ). C) *ADCYAP1R1* mRNA levels are inversely correlated with *ADCYAP1* mRNA levels in cortex (from prior dataset13)( $r = -0.219$ ;  $p < 0.001$ ). D) *ADCYAP1R1* mRNA levels are differentially expressed in females compared to males based on imputed *ADCYAP1R1* rs2267735 genotype (from prior dataset13)(\* $p < 0.05$  male vs. female CC carriers, #  $p < 0.05$ , one-tailed, CC vs G-carriers). Bars represent mean  $\pm$  SEM, N's for each group at bottom of each graph.



**Figure 5. Regulation of *ADCYAP1R1* and *ADCYAP1* mRNA in rodent models**

A) Percentage of time freezing, in mice, to the conditioned tone (CS+) following tone-shock pairings during the conditioned fear trials. B) rtPCR analyses of mRNA levels within mouse amygdala and mPFC 2 hrs after fear conditioning or in control handling conditions, showing a significant increase in amygdala *ADCYAP1R1* mRNA (N = 15, 1.47 fold,  $p < 0.05$ ) and a non-significant trend in mPFC (1.19 fold change). C) Correlation between average amygdala and PFC *ACYAP1R1* mRNA and % freezing at trial 4, demonstrating an association between *ADCYAP1R1* mRNA with rate of fear learning ( $r^2=0.49$ ,  $p < 0.05$ ). D) *ADCYAP1* mRNA in rat BNST in female rats (N = 12/group) following ovariectomy and estradiol implant vs. vehicle replacements. *ADCYAP1* mRNA is increased in both dorsal (2.1-fold) and ventral (3.4-fold) BNST after estradiol implantation. E) *ADCYAP1R1* transcripts are also increased in dorsal BNST (1.6-fold, N=4 per group). Bars represent mean  $\pm$  SEM, (\*  $p < 0.05$ ).