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

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PACAP was first isolated from ovine hypothalamic extracts based on its ability to stimulate cyclic AMP production in anterior pituitary cells. It is a highly conserved member of the VIP/secretin/glucagon family, with pleiotropic functions in development, cell signalling, metabolism, homeostasis and cell protection. Among these myriad functions, studies have demonstrated (1) high expression of PACAP peptide and its selective PAC1 receptor in hypothalamic and limbic structures, (2) PACAP regulation of corticotropin releasing hormone and autonomic function, (3) actions of PACAP in stress-related behaviour, (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 signalling is integrally involved in stress mechanisms. We hypothesized that PACAPergic systems may be important mediators of abnormal stress responses following psychological trauma contributing to PTSD, which is an extreme maladaptive and debilitating psychiatric disorder affecting up to 40% of individuals over lifetime. 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 analysed blood levels of PACAP, and genetic variation and methylation of the PACAP (ADCYAPI) and PAC1 receptor (ADCYAPI) genes, in a cohort of more than 1,200 highly traumatized subjects with and without PTSD (see Supplementary Tables 1 and 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 that had been matched on age, sex, and trauma histories (n = 64, see Supplementary Tables 1–3 for demographics). We found that PTSD symptoms (PTSD symptom scale) 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 versus high) were differentially associated with PTSD symptoms in females (Fig. 1b). PACAP38 levels also predicted differential response on all three symptom clusters necessary to fulfil diagnostic criteria for PTSD (intrusive re-experiencing (for example, trauma flashbacks), avoidance (for example, avoidance of trauma reminders) and hyperarousal (for example, 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 versus 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 percentage with clinically significant symptoms, χ² = 4.9, P < 0.05). These observations were especially notable, as females may be at twice the risk for PTSD as compared to males, implicating roles for sex hormones, especially oestrogen, in the disorder. When we controlled for demographic information, we found that PACAP38 was associated with PTSD in females (Fig. 1e). These findings suggest that perturbations in the PACAP–PAC1 receptor pathway may be important mediators of abnormal stress responses underlying PTSD. These sex-specific effects may occur via oestrogen regulation of ADCYAPI. PACAP levels and ADCYAPI SNPs may serve as useful biomarkers to further our mechanistic understanding of PTSD.

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common stress-related phenomena (depression and history of sub-
stance abuse), the effect of PACAP level on PTSD remained
(P < 0.05). In contrast, there was no effect of PACAP level on depres-
sion symptoms or substance abuse when controlling for PTSD.

In addition to the psychological symptoms that define the syn-
drome, 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.18–22 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 airstream. Female (but not male)
subjects with high PACAP38 levels demonstrated markedly increased
startle reflex responses to both CS+ (P = 0.02) and CS− (P = 0.005)
cues. This was particularly pronounced during the late acquisition
phase when normal subjects had habituated to the fearful stimuli
(Fig. 1e,f). In aggregate, these data suggest that PACAP38 peptide
is strongly associated with the psychological and physiological symp-
toms of PTSD in women with a history of trauma.

**ADCYAPI1 associated with PTSD in females**

To assess whether there may be a genetic association of PTSD with
polymorphisms in either the PACAP (ADCYAPI) or PAC1 receptor
(ADCYAPI1) locus, we performed a tag-SNP analysis (r² = 0.8;
minor allele frequency (MAF) = 0.1) across both genes with a total
of 44 SNPs (14 ADCYAPI and 30 ADCYAPI1 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)11,12,23, in total, or stratified by gender (females: n = 503;
males: n = 295). Only the ADCYAPI1 receptor SNP rs2267735
(P = 0.0002 in females; NS in males) remained significant after
experiment-wide multiple correction for sex and 44 independent tests
(Fig. 2a and b, Supplementary Fig. 1). No SNPs in the peptide
ADCYAPI1 gene met experiment-wide criteria for association
(Supplementary Fig. 2). Given these striking gender differences and recent
data demonstrating that ADCYAPI1 gene expression may be
dynamically modulated by oestrogen24, the distribution of oestrogen
response elements (EREs) within the ADCYAPI1 gene was examined
(Supplementary 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’ versus ‘G’ allele may differ-
tentially 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
Fig. 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 1,237 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 ADCYAPI1 rs2267735 SNP associations with
continuous PTSD symptom levels in females, we analysed both an
additive and a dominant model with total PTSD symptoms and symp-
tom subscales using the combined samples (Fig. 3b-e). The ‘CC’ geno-
type was most robustly associated with total PTSD symptoms, and
among subscales, hyperarousal symptoms were the most strongly asso-
ciated with rs2267735. Notably, even after controlling for childhood
trauma history and adult trauma, age and race (which slightly reduces
total N owing to missing data), the rs2267735 ‘CC’ genotype 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 life-time substance abuse, and found
no associations with these measures and rs2267735 (Supplementary
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.genome.gov/19518664), we analysed 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, 1,195 controls) or European (1,378 cases, 1,351 controls) ancestry. Specifically, we found no association with rs226735 SNP with PTSD diagnosis (by sex). Additionally, we examined the association of rs2267735 and Alzheimer’s in a previously characterized Alzheimer’s disease sample.

In this cohort of 342 subjects, we found no association with rs226735 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, or schizophrenia across different samples. Note that for all of these negative results, owing 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 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). e, Fear discrimination, measured with potentiated startle (CS+: startle minus CS−—startle) is impaired in females with rs2267735 ‘CC’ genotype. g, Dark-enhanced startle (startle dark − startle light) is significantly increased in females with rs2267735 ‘CC’ genotype. N values are shown at base of each bar, bars represent mean ± s.e.m. N values are slightly different across analyses owing to differences in number of subjects across measures. *P < 0.05; **P < 0.001; ***P < 0.0001.

The location on chromosome 7 for each SNP including the distance (bp) between the SNPs is given. The average distance between SNPs is 2.5 kb. SNP rs2267735 is located in an intron of ADCYAP1R1, and is not in linkage disequilibrium with other SNPs (for African Americans in our population, data not shown). c, rs2267735 (C/G), in red, is located within a canonical oestrogen response element (ERE) binding site (capital letters, conserved canonical ERE nucleotides; blue letters, mismatches with the ADCYAP1 gene and canonical ERE; reverse strand shown).
ADCPYAP1R1 methylation and mRNA expression

Environmental, genetic and epigenetic mechanisms are likely to moderate the long-term effects of trauma exposure. Using the Illumina HumanMethylation27 BeadChip, we interrogated methylation in DNA extracted from peripheral blood at the first site within the ADCPYAP1R1 CpG island (Supplementary Fig. 2). Methylation at this site was significantly associated with total PTSD symptoms (Fig. 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 (Fig. 4b, \( \chi^2 = 8.1, P < 0.005 \)), but not depression (\( P > 0.05 \), Supplementary Fig. 3e). There was no significant association between methylation of ADCPYAP1 and PTSD symptoms. These data suggest that ADCPYAP1R1 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\(^26\), we used a brain mRNA expression data set\(^30\) to test whether ADCPYAP1R1 rs2267735 is associated with differential gene expression. We first examined whether cortical ADCPYAP1R1 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 used a previously analysed data set with combined genome-wide association and brain mRNA expression data\(^30\) to examine whether ADCPYAP1R1 rs2267735 SNP was associated with differential expression in controls, but not in those with PTSD, and trauma responses that has been proposed to model PTSD\(^{19,22}\). We wondered if ADCPYAP1R1 mRNA was differentially regulated in mice using Pavlovian fear conditioning, a means of studying acute fear and trauma responses that has been proposed to model PTSD\(^{19,22}\). We performed classical fear conditioning experiments using male mice, in which a previously neutral tone CS (6 kHz) was paired with 10 foot-shocks (1 mA, 0.5 s; Fig. 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 show that amygdala Adcyap1r1 mRNA increased \(~1\)-fold during the consolidation of fear (Fig. 5b, \( P < 0.05 \)), with a similar trend within the medial prefrontal cortex (mPFC). When peak freezing was compared with brain mRNA levels, we find a significant correlation between fear learning and Adcyap1r1 mRNA (Fig. 5c, \( r^2 = 0.49, P < 0.05 \)).

Oestrogen induces Adcyap1r1 in rat BNST

To further establish the relationship between PACAP–PAC1 receptors and oestrogen in a validated model of sex hormone regulation, we examined oestrogen-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\(^{27,28}\). In rodents, it is critical for emotional behaviour, mediating stress responses and the light-enhanced startle response. We examined gene expression in the BNST in ovariectomized female rats following 21-day implantation of continuous release oestrogen pellets. Compared to control implants, oestradiol increased Adcyap1 transcripts in the dorsal and ventral BNST 2.1- and 3.4-fold, respectively (\( P \leq 0.01 \), Fig. 5d). Additionally, oestradiol increased Adcyap1r1 mRNA 1.5-fold in the dorsal BNST samples (\( P < 0.05 \), Fig. 5e), and future studies should also examine oestradiol sensitivity of these genes in amygdala. While these rodent studies are complex and have differing experimental designs, our data clearly illustrate dynamic PACAP–PAC1 receptor regulation within central areas mediating fear, stress and oestrogen responsiveness.
MAF > 0.1) was used to choose tag-SNPs for both ADCYAP1 and ADCYAP1R1. The coordinates were chr. 18 885000–906000 and chr. 7 31048667–31117836 for ADCYAP1 and ADCYAP1R1, respectively (NCBI B36), which includes approximately 10 kilobases (kb) upstream and 5 kb 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, bisulphite-converted DNA was whole-genome amplified, fragmented, and hybridized to the HumanMethylation27 BeadChip (Illumina). 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 (β value) was calculated using the Methylation Module of the BeadStudio software. The eyeblink component of the acoustic startle response was measured by electromyographic recordings of the right orbicularis oculi muscle with two 5-mm Ag/AgCl electrodes filled with electrolyte gel, as described18–20. The mouse fear conditioning and rat oestrogen replacement studies are described in detail in Supplementary Methods.

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**METHODS SUMMARY**

This highly traumatized, civilian, cross-sectional cohort has been previously described in candidate gene-association studies of PTSD and depression1–11. Research interviews, salivary DNA and blood samples were collected from patients receiving services in the primary care clinics at Grady Memorial Hospital (Atlanta, Georgia, USA). 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 scale6, which has been validated within this population using the Clinician Administered PTSD Scale. PACAP38 radioimmunoassay (1:30,000, Peninsula Laboratories) was performed at University of Vermont, using double antibody immunoprecipitation as described11. For genotyping, pairwise tagging (r2 > 0.8,
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Supplementary Information is linked to the online version of this article at www.nature.com/nature.

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