

ORIGINAL ARTICLE

Functional evaluation of a PTSD-associated genetic variant: estradiol regulation and *ADCYAP1R1*KB Mercer¹, B Dias¹, D Shafer^{1,2}, SA Maddox³, JG Mulle^{4,5}, P Hu², J Walton³ and KJ Ressler^{1,3}

Posttraumatic stress disorder (PTSD) affects 5–10% percent of the US adult population with a higher prevalence among women compared with men. Although it remains unclear how biological sex associates with susceptibility to PTSD, one mechanism may involve a role for estrogen in a gene by environment interaction. We previously demonstrated a sex-dependent association between the pituitary adenylate cyclase-activating polypeptide type 1 receptor (PAC1) and PTSD, where carriers of a C allele at single-nucleotide polymorphism (SNP) rs2267735 within the PAC1 receptor gene (*ADCYAP1R1*) have increased symptoms of PTSD. This SNP is located within a predicted estrogen response element (ERE), which regulates gene transcription when bound to estradiol (E2) activated estrogen receptor alpha (ERα). In the current study, we examined E2 regulation of *ADCYAP1R1* *in vitro*, in cell culture, and *in vivo* in mice and humans. We find in mice that fear conditioning and E2 additively increase *ADCYAP1R1* expression. *In vitro*, we show that E2/ERα binds to the *ADCYAP1R1* ERE, with less efficient binding to an ERE containing the C allele of rs2267735. In women with low serum E2, the CC genotype associates with lower *ADCYAP1R1* expression, which further associates with higher PTSD symptoms. These findings lead to a model in which E2 induces the expression of *ADCYAP1R1* through binding of ERα at the ERE as an adaptive response to stress. Inhibition of E2/ERα binding to the ERE containing the rs2267735 risk allele results in reduced expression of *ADCYAP1R1*, diminishing estrogen regulation as an adaptive stress response and increasing risk for PTSD.

Translational Psychiatry (2016) 6, e978; doi:10.1038/tp.2016.241; published online 13 December 2016

INTRODUCTION

Posttraumatic stress disorder (PTSD) is a psychiatric disorder that can affect children and adults, as well as civilian and non-civilian populations. Although anyone is susceptible to developing PTSD when exposed to a traumatic event, a greater prevalence has been reported among women compared with men. In fact, within the United States, the percent of females who suffer from PTSD during their lifetime is nearly twice that of men.^{1,2} This observation has led to renewed interest in the contribution of hormones, particularly estrogen, in psychiatric outcomes among women. Several research studies support an association between low levels of estrogen and increased fear and anxiety-related behaviors.^{3–7} In line with the implications of these research findings, genetic variants that interfere with estrogen regulation of the stress pathway may have a role in neuropsychiatric disease.

One example of estrogen regulation on cellular processes involves sequence-specific enhancers known as estrogen response elements (EREs).⁸ The ligand receptor complex formed by binding of estradiol (E2) to estrogen receptors (ERs) alpha or beta can translocate to the nucleus, where it can then interact with EREs to activate gene transcription.^{9–11} Sequence alterations in the relatively conserved ERE can interfere with the binding efficiency of E2-activated ER and negatively affect its enhancer activity.¹² As such, genomic polymorphisms or other mutations within an ERE can result in the dysregulation of normal responses triggered by estradiol. We previously identified a single-nucleotide polymorphism (SNP), rs2267735, that is associated with the decreased expression of *ADCYAP1R1* cortex messenger RNA

(mRNA) in females only.^{13,14} Interestingly, this genomic variant is located within a putative ERE in an intron of *ADCYAP1R1*, the gene encoding pituitary adenylate cyclase-activating polypeptide type 1 receptor (PAC1).

The PAC1 receptor, when bound to its ligand pituitary adenylate cyclase-activating polypeptide (PACAP), contributes to the stress response system through the hypothalamic–pituitary–adrenal axis by activating the production of cortisol.^{15–17} Impairment of the hypothalamic–pituitary–adrenal axis and related signaling pathways has been attributed to several psychiatric conditions including PTSD.^{18–24} Consequently, irregularities in the mRNA expression of neuropeptide hormones and receptors involved in related stress pathways could contribute to neuropsychiatric disorders. Consistent with this paradigm, we have shown previously that carriers of the rs2267735 risk genotype CC, compared with those with the CG or GG genotype, have not only a statistically significant decrease in expression levels of *ADCYAP1R1* but also higher levels of PTSD symptoms.¹³

We hypothesize that the genotype of the PTSD-associated SNP, rs2267735, influences estradiol-activated binding of ERα to an ERE, affecting the regulation of *ADCYAP1R1* (PAC1) gene expression and a normal stress response that when dysregulated could lead to PTSD. To test this hypothesis, we sought to determine whether or not estradiol-activated ERα is sufficient to induce the transcription of *ADCYAP1R1*; if the putative *ADCYAP1R1* ERE binds ERα differentially dependent on genotype at rs2267735; if serum estradiol has an effect on the expression of *ADCYAP1R1* in humans; and if the expression levels of *ADCYAP1R1* correlate with PTSD

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Received 6 January 2016; revised 28 August 2016; accepted 31 August 2016

symptoms. The results presented in this manuscript provide insight into a mechanism that may partly explain the biological effects of estradiol on PTSD outcome and other stress- and trauma-related disorders.

MATERIALS AND METHODS

Expression analysis of *Adcyap1r1* in mouse brain

Eight-week old female mice were ovariectomized after which a 2 mm pellet containing either sesame oil or 17 β -estradiol (E2) dissolved in sesame oil at 1 $\mu\text{g } \mu\text{l}^{-1}$ (catalog no. E8875; Sigma Aldrich, St Louis, MO, USA) was placed subcutaneously into 16 mice per treatment group (32 total). Ten days later, half of these mice ($N=16$; 8 from each treatment group) were fear conditioned using the following conditions: 6 kHz tone for 30 s followed by 0.6 mA foot shock for 1 s and a 2 min interval before the next set of tone shock (5 total). The remaining 16 mice were kept in their home cages in the vivarium. To mimic conditions done previously by Ressler *et al.*¹³ in female rats, the brain of each mouse was removed and immediately frozen on dry ice within 2 h of exposure to fear conditioning. Tissue punches (1 mm) were collected from the bed nucleus of the stria terminalis (BNST); a brain region previously found to associate with estradiol-induced expression of *Adcyap1r1*.¹³ mRNA was extracted using the RNeasy Mini kit (catalog no. 74104; Qiagen, Valencia, CA, USA) and converted to complementary DNA using the RT² First Strand kit (catalog no. 330401, Qiagen). For gene expression analysis, we performed quantitative PCR (qPCR) using TaqMan Gene Expression assays for *Adcyap1r1* (Mm01326453_m1; catalog no. 4351372, ThermoFisher Scientific, Waltham, MA, USA) and *Gapdh*, as an endogenous control (catalog no. 4352932, ThermoFisher Scientific). The $2^{-\Delta\Delta\text{Ct}}$ method was used to compare fold change in expression between the condition and treatment groups.²⁵ Analysis of variance was used to determine statistical significance of fold differences.

Competitive enzyme-linked immunosorbent assay to determine ERE binding

To test the binding capabilities of ER α to the putative EREs containing either the C or G allele of rs2267735, we performed a competitive binding enzyme-linked immunosorbent assay using the TransAM ER kit (catalog no. 41396; Active Motif, Carlsbad, CA, USA). To each microwell coated with canonical ERE sequence oligonucleotide (oligo), we added estradiol-treated MCF-7 nuclear extract (5 μg ; catalog no. 36016, Active Motif) and 10–100 pmol of one of four double-stranded oligo DNAs (two competing/experimental sequences plus a negative and positive control). The sequences of the oligos used for this experiment are provided in Supplementary Table 1. Each experimental assay was performed in triplicate. The controls were performed in duplicate. Individual oligos and nuclear extract were added to each microwell and incubated for 1 h. Next, a primary antibody (ER α ; 1:1000) and then secondary antibody (horseradish peroxidase-conjugated IgG; 1:1000) were added for 1 h each. After several rinses, a colorimetric reaction to horseradish peroxidase was initiated and absorbance was measured at a wavelength of 450 nm to determine binding efficiencies. Antibodies and other reagents were provided in the TransAM ER kit.

Expression analysis of *ADCYAP1R1* in transfected HEK293T cells

Human embryonic kidney (HEK) 293T cells were transfected with a plasmid containing either green fluorescent protein (GFP) or the full-length human estrogen receptor (pCMV-hER α)^{26,27} and treated with E2 or ethanol only. mRNA was extracted and gene expression of *ADCYAP1R1* was measured with commercially available assays. The $2^{-\Delta\Delta\text{Ct}}$ method was used to compare fold change in expression between each condition and treatment group.²⁵ See Supplementary Materials and Methods for more experimental details.

Crosslinking chromatin immunoprecipitation

HEK293T cells were transfected with pCMV-hER α and treated with E2. Following treatment, the cells were exposed briefly to formaldehyde to crosslink DNA/protein complexes, and then lysed and sonicated. A monoclonal antibody to ER α was used to isolate DNA bound by the receptor. After reversing the crosslinking, the ER α bound DNA was precipitated using standard phenol/chloroform extraction followed by ethanol precipitation. The ERE of interest within *ADCYAP1R1* was measured

using region-specific primers and qPCR. The qPCR measurement for a transcriptionally inactive region of the genome was used as a negative control. See Supplementary Materials and Methods for more experimental details.

Collection of phenotype and genotype data from study participants

Patients waiting for an appointment with their primary care or OB/GYN doctor at the Grady Memorial Hospital in Atlanta, GA, were recruited to participate in a research study aimed at identifying genetic factors that contribute to PTSD ($N=9554$). Consenting participants were asked to provide a saliva sample for DNA collection and genotyping. They were also asked to complete questionnaires including the following: (1) the PTSD Symptom Scale to measure current PTSD symptoms²⁸ and (2) the traumatic events inventory to assess the type and frequency of lifetime, traumatic events.²⁹ From some participants, whole blood was also drawn. Serum derived from a subset of these samples was used to measure estradiol (by RIA, catalog no. KE2D1, Siemens Healthcare Diagnostics, Malvern, PA, USA). Genotyping of rs2267735 was performed using either a TaqMan SNP genotyping assay (C_15872945_10, catalog no. 4351379, ThermoFisher Scientific) or the Sequenom MassArray iPLEX system (Agena Bioscience, San Diego, CA, USA) with custom-designed primers. The demographics of the recruited study population have been described in detail previously by Gillespie *et al.*³⁰ A comparison of study specific phenotypes between the larger study population and the subset used in this study are provided in Supplementary Table 2.

Detection of *ADCYAP1R1* mRNA in human whole blood by qPCR

Among 384 female study participants with a measure of estradiol, we selected 105 who met the following criteria: a measure of estradiol < 530 pg ml⁻¹, a genotype for rs2267735, mRNA with a RNA integrity number > 5.0 and a concentration > 100 ng μl^{-1} , and measures for both the PTSD Symptom Scale and traumatic events inventory. Samples with a gene expression threshold (Ct) value > 50 for duplicate reactions were excluded from further analysis, resulting in a final sample size of 95 (range of Ct values: 30.84–45.88). Among these 95 individuals, the majority had estradiol levels within the normal range for a non-pregnant female (< 20–443 pg ml⁻¹).³¹ Altogether, 92.6% of the individuals selected for these analyses had estradiol levels below 188 pg ml⁻¹, which is the lower limit of estradiol measured among pregnant women (Supplementary Table 2).³¹

Total mRNA was extracted from whole-blood samples collected in Tempus Blood RNA tubes (catalog no. 432792; ThermoFisher Scientific) using the PerfectPure RNA 96 Cell kit (catalog no. 2900296; 5 PRIME, Gaithersburg, MD, USA). The expression of *ADCYAP1R1* mRNA in blood was measured in duplicate by reverse transcriptase PCR reactions with target-specific primers, followed by qPCR with internal DNA Detection Switch probes and antiprobess.^{32,33} Reverse transcriptase PCR reactions were performed with 2 μl of total RNA and GoScript Reverse Transcriptase (catalog no. A5003, Promega, Madison, WI, USA), according to the manufacturer's protocol. qPCR reactions were performed in 20 μl volumes containing 10 μl of 2 \times HotStart-IT Probe qPCR Master Mix (catalog no. 75766, Affymetrix, Santa Clara, CA, USA); MgCl₂ and dNTPs (final concentrations of 5 and 0.1 mM, respectively), 3 μl of complementary DNA, internal primers, and an internal DNA Detection Switch probe and antiprobe (GeneTAG Technology, Atlanta, GA, USA) were added. *ADCYAP1R1* expression levels were normalized to *GAPDH* using the $2^{-\Delta\Delta\text{Ct}}$ method.²⁵ The sequences and final concentrations for the primer, probe and antiprobe concentrations are provided in Supplementary Table 1.

RESULTS

Expression of *ADCYAP1R1* in the brain of mice exposed to estradiol treatment and fear conditioning

The PAC1 receptor, encoded by *Adcyap1r1*, and its ligand PACAP have a significant role in stress regulation within the BNST. Within the BNST of rats, increased transcript levels of both PAC1 and PACAP are induced by chronic stress, which results in the release of corticosterone and anxiety-related behavior.^{34–36} Expression of *Adcyap1r1* in the BNST is also activated by estradiol. We have shown previously in ovariectomized female rats that treatment

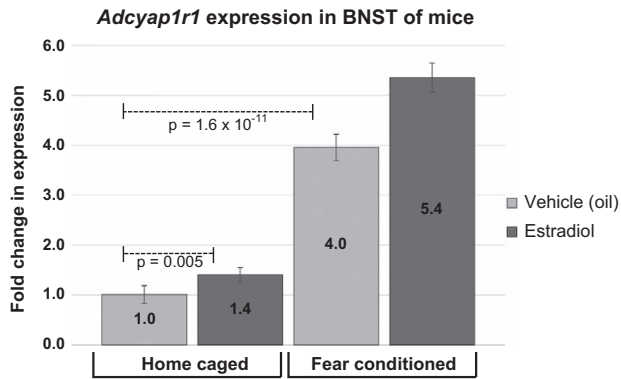


Figure 1. Ovariectomized female mice were given a subcutaneous pellet containing either estradiol or vehicle (sesame oil). The mice were then kept in their home cage in the vivarium (hc) or exposed to fear conditioning (fc) using a tone paired with foot shock. $N=7$ for each treatment group. Fold change in normalized expression was measured relative to normalized expression in vehicle plus home-caged mice. The data shown are the average fold change \pm s.d. in the expression of *ADCYAP1R1* in the bed nucleus of the stria terminalis (BNST) per treatment group.

with estradiol results in higher levels of *Adcyap1r1* expression in the BNST when compared with vehicle-only (oil)-treated controls.¹³ We replicated the *Adcyap1r1* expression response to estradiol in the BNST of ovariectomized female mice, revealing a slightly smaller but statistically significant 1.4-fold increase over that measured for vehicle-only exposed mice ($P=0.005$) (Figure 1). We also show that fear conditioning results in a 4-fold increase in expression when compared to non-fear conditioned, home-caged animals ($P=1.6 \times 10^{-11}$) (Figure 1). The combination of fear conditioning plus estradiol treatment results in a 5.4-fold increase in expression of *Adcyap1r1* that is greater than that exhibited by either condition separately (Figure 1). However, there is not a statistically significant interaction between estradiol and fear conditioning on fold increase ($P=0.1$), suggesting that the two may work independently to affect expression levels of *Adcyap1r1* in the BNST.

ER α -dependent activation of *ADCYAP1R1* expression

Estradiol regulates gene expression by binding to ER α or ER β , activating a conformational change that allows the estradiol receptor complex to bind to chromatin and induce transcription. ER α has been implicated in mood regulation and is also highly expressed in the BNST of the human brain.^{37,38} Therefore, we hypothesized that the estradiol effect on the expression of *ADCYAP1R1* is likely occurring via interaction with ER α . To address this hypothesis, we sought to determine whether introducing ER α into a cell line that expresses a detectable level of *ADCYAP1R1* but does not endogenously express ER α would result in enhanced expression of *ADCYAP1R1* in the presence of estradiol. Full-length hER α was transiently transfected into HEK293T cells, which were then treated with estradiol for several hours. The cells were also transfected with a GFP plasmid to control for the effects of transfection on changes in *ADCYAP1R1* expression. Expression was also examined in GFP transfected cells treated with estradiol to control for effects of estradiol on expression that do not involve ligand activation of ER α . Among HEK293T cells treated with estradiol, there is a 3.9-fold increase in *ADCYAP1R1* expression for cells transfected with hER α compared with those transfected with GFP ($P=5.8 \times 10^{-6}$; Figure 2). These data reveal that ER α is sufficient to induce the expression of *ADCYAP1R1*. Cells that were treated with estradiol and transfected with GFP show a minor and non-significant increase in fold change expression (1.0 versus 1.2) compared with vehicle-transfected cells (Figure 2). These data

ADCYAP1R1 expression induced by E2 & hER α in HEK293T cells

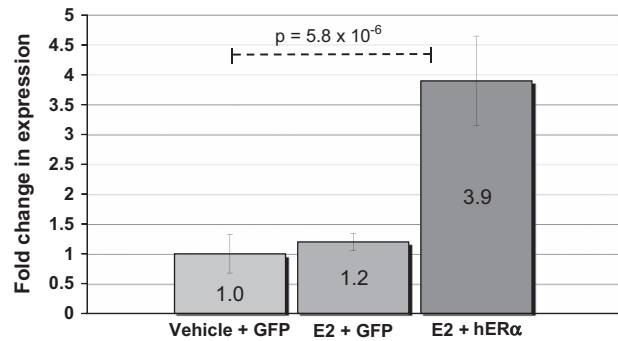


Figure 2. HEK293 cells were transiently transfected with full-length human estrogen receptor alpha (hER α) or green fluorescent protein (GFP) and treated with either estradiol (E2) or vehicle (ethanol) only. $N=6$ for each treatment group. Fold change in normalized expression is measured relative to normalized expression in cells transfected with GFP and treated with vehicle only. The data shown are the average fold change \pm s.d. in the expression of *ADCYAP1R1* per treatment group.

further support the role of estrogen-activated ER α , specifically, in estradiol-induced expression of *ADCYAP1R1*.

In vivo binding of ER α to an *ADCYAP1R1* intronic ERE

E2/ER α activation of transcription occurs by binding to EREs, which are specific DNA sequences located throughout the genome.⁹ Within a particular intron of *ADCYAP1R1*, there are several predicted EREs (purple boxes, Supplementary Figure 1). One of these EREs (underlined, Supplementary Figure 1) contains a SNP, rs2267735 (in red, Supplementary Figure 1), which correlates with allele-specific differential expression of *ADCYAP1R1* in the cortex of human brain.¹³ To test whether or not this specific ERE is capable of binding ligand-activated ER α *in vivo*, consistent with that of a functional ERE, we performed crosslinking chromatin immunoprecipitation. HEK293T cells, which do not endogenously express ER α , were transiently transfected with full-length human ER α -encoding complementary DNA. After the cells were treated with estradiol, then formaldehyde crosslinked to preserve DNA/protein complexes, the chromatin was incubated with a monoclonal antibody to ER α to 'pull-down' ER α -bound DNA. DNA that bound ER α was detected and measured using qPCR. Primers (in green, Supplementary Figure 1) were specifically designed to amplify a 55 bp region (chr7: 31 095 865–31 095 920) containing the predicted ERE sequence of interest. Relative to the percent input (non-immunoprecipitated DNA) observed for a transcriptionally inactive region of the genome (negative control). We found that binding of ER α to this particular *ADCYAP1R1* ERE is approximately ninefold greater (0.50% versus 0.06%) compared with a control DNA region ($P=0.002$; Figure 3a). These data suggest that the human ER α is indeed able to bind to the predicted ERE containing a PTSD-associated SNP within *ADCYAP1R1*.

Differential binding of ER α based on the SNP variant present within the ERE

With evidence of ER α binding to the ERE of interest, we then wanted to test whether there may be differential allele-specific binding efficiencies between the C versus G allele of rs2267735. As the ERE is a regulatory sequence that activates transcription when bound to ligand-activated ER α , the efficiency of binding is correlated with enhancer activity.³⁹ Thus, we hypothesized that the C allele, for which we observed lower *ADCYAP1R1* expression among homozygous carriers in our previous work,¹³ would have

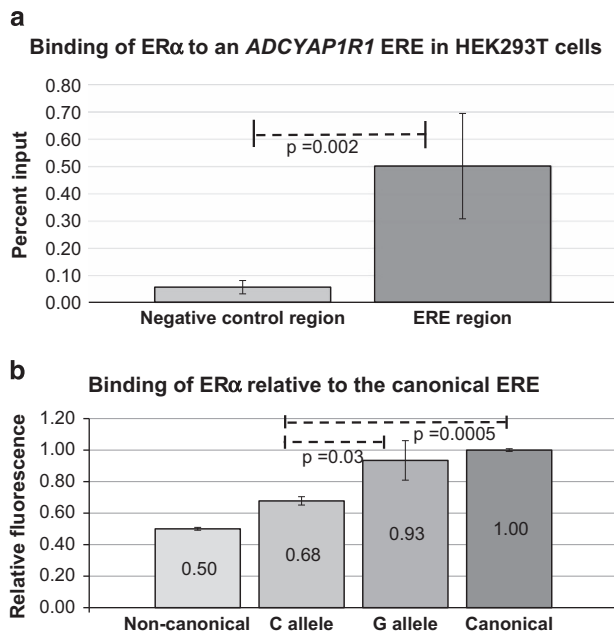


Figure 3. (a) Using chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR), we measured binding of estrogen receptor alpha (ER α) to two regions of the genome: an estrogen response element (ERE), which contains rs2267735 in an intron of *ADCYAP1R1* (ERE region), and a transcriptionally inactive region on chromosome 4 (negative control region). $N=6$ for each group. The qPCR measures obtained from the immunoprecipitated chromatin were divided by the measure obtained from the non-immunoprecipitated input sample (the amount of chromatin used in the ChIP experiment) using the same primers. The data represent the average percent of input \pm s.d. for the two regions. (b) A competitive enzyme-linked immunosorbent assay was used to measure the binding of ER α to double-stranded DNA sequences (oligos) relative to the canonical ERE-binding sequence. The fluorescent measures obtained for the competing oligos were transformed by dividing these values by the fluorescent measure obtained for the canonical oligo (positive control). The data represent the averaged inverse of these values \pm s.d. for each experimental oligo (C allele and G allele) and the non-canonical (negative control).

lower binding affinity compared with the G allele. Double-stranded oligos containing the genomic sequences of the ERE (in parenthesis, Supplementary Figure 1) with either the 'C' or 'G' allele were used in a competitive enzyme-linked immunosorbent assay to compare binding of ER α to that of the canonical ERE sequence. We used 20 pmol of oligo for four sequences (non-canonical, C allele, G allele and canonical; see Supplementary Table 1) to measure the ability of each oligo to 'out-compete' canonical ERE for ER α binding. The data for each oligo (non-canonical, C allele and G allele) were transformed to represent a measure of fluorescence relative to the positive control (canonical ERE; Figure 3b). As expected, the non-canonical oligo has the least affinity to ER α with a binding efficiency of 50% compared with that of the positive control. The second lowest binding was observed for the C allele oligo (68%). The *ADCYAP1R1* intronic ERE that contains the rs2267735 C allele shows a statistically significant reduction in binding to ER α ($P=0.0005$) in comparison with the canonical ERE (Figure 3b). Compared with binding of ER α to the G allele, which binds nearly as well as the canonical sequence, the C allele also binds with less efficiency (statistically significant; $P=0.03$). The directions of these results are consistent with the hypothesis that the C allele would bind least well of the two experimental conditions. The dosage effect of binding was also examined for a range of oligo concentrations (Supplementary

Figure 2). Interestingly, although all other oligo-binding measures remained relatively constant, the C allele oligo eventually reaches near normal binding at 100 pmol of oligo. This leads us to speculate that, regardless of having a C allele present at this particular *ADCYAP1R1* ERE, some binding may still occur in a dose-dependent manner.

ADCYAP1R1 expression relative to rs2267735 genotype and PTSD symptoms

The C allele of rs2267735 results in reduced binding of E2/ER α to the ERE with a binding efficiency that appears to be conditional. This reduced binding is presumably associated with lower levels of *ADCYAP1R1* transcript previously observed among study participants with the CC genotype.¹³ Although we have shown an estradiol-induced increase in the expression of *ADCYAP1R1* in mice, we have not yet observed this phenomenon in humans nor have we tested the effect of variable estradiol concentrations on expression levels. In addition to better understanding the effects of serum estradiol on *ADCYAP1R1* expression, we were also interested in testing the effect of estradiol on expression among those with the CC genotype at rs2267735. Assuming lower expression among women with the CC genotype, we wanted to determine whether having higher levels of estradiol could compensate, leading to the increased levels of *ADCYAP1R1* expression.

Serum estradiol and transcript levels of *ADCYAP1R1* were measured among 95 genotyped females from whom serum (for measuring estradiol) and mRNA were collected from whole-blood draws performed on the same day. In human whole blood, higher levels of serum estradiol correlate with increased *ADCYAP1R1* mRNA (Table 1A). Among the women with high estradiol (top of median split; range: 33.84–528.35 pg ml⁻¹), 61.7% have high expression. Correspondingly, 60.4% of women with low estradiol (bottom of median split; range: 5.70–32.74 pg ml⁻¹) have low expression. This association is statistically significant ($P=0.031$). Next, we tested the relationship between estradiol, genotype and *ADCYAP1R1* expression. Women with levels of serum estradiol in the lowest quartile (range: 5.7–15.0 pg ml⁻¹) have a higher, averaged *ADCYAP1R1* Δ Ct value (corresponding to lower expression) if they have the CC risk genotype (Δ Ct=22.02) compared with women with either the GG or GC genotype (Δ Ct=20.86; two-tailed t -test; $P=0.049$; Table 1B). Among women with estradiol measures in the top quartile (range: 101.13–528.35 pg ml⁻¹), we observe a more even distribution of *ADCYAP1R1* expression between the CC and GG or GC genotype groups (average Δ Ct=21.27 and 21.39, respectively; data not shown). We examined the expression of *ADCYAP1R1* (by median split of Δ Ct) among women with the CC genotype and either high (range: 41.91–269.31 pg ml⁻¹) or low (range: 5.70–32.74 pg ml⁻¹) estradiol measures and found that expression increases in the higher estradiol group ($P=0.034$; Table 1C). This same analysis was not statistically significant for those with the GG or GC genotype (data not shown). These latter results suggest that increased estradiol may act in a compensatory way to increase levels of *ADCYAP1R1* observed among women with the CC genotype at rs2267735.

We have shown an increase of *ADCYAP1R1* expression in response to acute stress in mice. However, it remained unclear if expression changes would also occur in humans, particularly in those with PTSD. In the current study, we found a negative relationship between blood mRNA *ADCYAP1R1* expression and PTSD symptoms among our study participants. The data reveal that individuals with low expression had greater PTSD symptom severity than those with high expression ($P=0.026$; Table 1D). We also tested the relationship between *ADCYAP1R1* expression and PTSD symptoms while using trauma exposure (quantitative variable derived from the traumatic events inventory measure) and measure of serum estradiol (pg ml⁻¹) as covariates in a linear

Table 1. The relationship between *ADCYAP1R1* expression and rs2267735 genotype, estradiol and PTSD symptoms in whole-blood mRNA from study participants

A	N	High <i>ADCYAP1R1</i> expression	Low <i>ADCYAP1R1</i> expression
<i>All genotypes</i>			
Low serum estradiol (range : 5.70–32.74 pg ml ⁻¹)	48	39.6% (N = 19)	60.4% (N = 29)
High serum estradiol (range: 33.84–528.35 pg ml ⁻¹)	47	61.7% (N = 29)	38.3% (N = 18)
		Pearson χ^2 , $P = 0.031$	
B	N	Mean <i>ADCYAP1R1</i> Δ Ct	s.d.
<i>Lower quartile of estradiol (E2)</i>			
rs2267735 genotype			
GG or GC (E2 range: 5.70–14.75 pg ml ⁻¹)	14	20.86	1.61
CC (E2 range: 5.70–15.0 pg ml ⁻¹)	10	22.02	0.86
		Two-tailed t -test, $P = 0.049$	
C	N	High <i>ADCYAP1R1</i> expression	Low <i>ADCYAP1R1</i> expression
<i>CC genotypes only</i>			
Low serum estradiol (range: 5.70–32.74 pg ml ⁻¹)	23	30.4% (N = 7)	69.6% (N = 16)
High serum estradiol (range: 41.91–269.31 pg ml ⁻¹)	19	63.2% (N = 12)	36.8% (N = 7)
		Pearson χ^2 , $P = 0.034$	
D	N	Mean PTSD symptoms	s.d.
High <i>ADCYAP1R1</i> expression	48	7.97	9.22
Low <i>ADCYAP1R1</i> expression	47	12.38	9.77
		Two-tailed t -test, $P = 0.026$	

Abbreviations: mRNA, messenger RNA; PTSD, posttraumatic stress disorder.

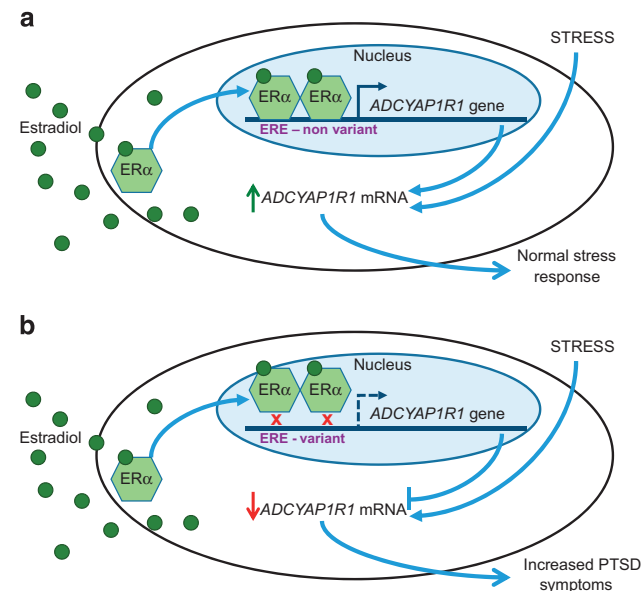


Figure 4. (a) A schematic of the hypothesized model for the role of estradiol and ER α on the expression of *ADCYAP1R1* in the presence of a canonical, non-variant ERE sequence within the *ADCYAP1R1* gene. In this model, ER α is able to bind normally to the ERE, initiate transcription and increase the expression of *ADCYAP1R1*, which allows for a normal biological response as result of stress. (b) A schematic of the hypothesized model for the role of estradiol and ER α on the expression of *ADCYAP1R1* in the presence of a non-canonical, variant ERE sequence containing the rs2267735 PTSD risk allele within the *ADCYAP1R1* gene. In this model, ER α is not able to bind normally to the ERE, resulting in less expression of *ADCYAP1R1*, a dysregulated stress response, and increased PTSD symptoms. ER α , estrogen receptor alpha; ERE, estrogen response element.

regression model. When accounting for the effects of these variables on PTSD symptoms, the association between *ADCYAP1R1* expression and PTSD remained significant ($P = 0.030$; Supplementary Table 3). Although there was a moderate increase for current PTSD symptoms among those in the lower range of serum estradiol (median split) compared with those in the upper range (mean PTSD Symptom Scale of 11.05 versus 9.23), these findings were not statistically significant ($P > 0.05$; data not shown).

Limitations

There are several limitations to the current study. In the course of these experiments, we found that *ADCYAP1R1* is not detectably expressed in all cell types. Lymphoblast cell lines with the CC and GG genotype of rs2267735 were originally obtained to measure the association between genotype and expression as a factor of variable E2 treatment, and to assess allele-specific binding efficiency of ER α to ERE *in vivo* with increasing exposure of E2. Unfortunately, we were unable to measure *ADCYAP1R1* expression in lymphoblast cell lines. In addition, it would have been preferable to measure ER α binding in a neuronal cell line that likely contains transcription factors that contribute to the activation of *ADCYAP1R1* expression by facilitating E2/ER α binding to the ERE. The neuronal cell line we chose to utilize, SH-SY5Y, was difficult to grow and we therefore discontinued working with these cells. However, we were able to detect low but convincing levels of ER α binding to the *ADCYAP1R1* ERE in HEK293T cells, but more accurate measures of binding may have been obtained using cells with greater similarity to those present in brain tissue where *ADCYAP1R1* is highly expressed. The relationship between PTSD and *ADCYAP1R1* expression remains speculative given that it was tested using whole blood and should be explored further by examining expression in post-mortem brain tissue from individuals with and without PTSD. The amount of receptor protein

produced in human brain tissue, particularly in the BNST, should also be measured and compared among individuals with differing genotypes at rs2267735. Although we have measured the levels of *ADCYAP1R1* mRNA in our experimental models, we cannot make any conclusions about the amount of receptor protein being produced. As mentioned above, the amount of receptor is likely key to the functionality of stress-related PACAP/PAC1 signaling.

DISCUSSION

We previously reported a genetic association between a SNP (rs2267735) within the gene *ADCYAP1R1* (PAC1) and PTSD in females. Because the rs2267735 SNP lies within a putative ERE, we hypothesized a role for estrogen in regulating *ADCYAP1R1* expression. We now report evidence of a functional role for rs2267735 in the dysregulation of ER α /ERE transcriptional activation of *ADCYAP1R1* and provide further insight and rationale for the sex-specific effects of this polymorphism on PTSD risk.

On the basis of our current data, we have developed a model for the putative cellular mechanisms through which the C allele of rs2267735 results in differential expression of *ADCYAP1R1* and increased susceptibility to PTSD (Figure 4). Similar to that previously reported among female rats, here we confirm that induced stress in female mice also results in an increased expression of *Adcyap1r1* mRNA in the BNST (Figure 4). We have now shown that estradiol can also increase the expression of *ADCYAP1R1*. Estradiol regulates the expression of *ADCYAP1R1* through ligand activation of ER α and binding to an ERE (ERE—non-variant) within the gene (Figure 4a). When the risk allele ('C') is present within the ERE sequence (ERE—variant), binding of E2/ER α is compromised (represented by red X's, Figure 4b). As such, reduced binding likely inhibits the activation of *ADCYAP1R1* transcription, resulting in less *ADCYAP1R1* mRNA (Figure 4b). Finally, as a result of these altered mechanisms of regulation, lower expression of *ADCYAP1R1* is associated with PTSD (Figure 4b). We suspect that lower mRNA results in less receptor protein (PAC1) such that the cellular mechanisms involving PAC1 and its ligand PACAP, which are a normal process of the stress regulation pathway,⁴⁰ are disrupted. We speculate that this dysregulation could be the reason for increased symptoms of PTSD.

The findings we present here are consistent with and help clarify the results of our original PAC1 association with PTSD finding.¹³ We previously found that increased methylation at a CpG within the promoter of *ADCYAP1R1* correlated with PTSD symptoms ($r=0.354$; $P<0.0005$).¹³ Typically, promoter methylation results in reduced transcription of a gene.⁴¹ Although this is not conclusive evidence of an inverse relationship between *ADCYAP1R1* expression and PTSD symptoms, it was the first piece of evidence to suggest this direction of association. Interestingly, new research has also revealed a negative correlation between ER α binding and DNA methylation (within various regions of the same gene), providing a potential, alternative mechanism through which ER α may positively affect the expression of *ADCYAP1R1*.⁴² The relationship between DNA methylation, ER α binding and expression of *ADCYAP1R1* would be an interesting focus of future investigation.

We have also previously shown that females with the PTSD risk genotype at rs2267735 ('CC') have a statistically significant reduction in cortex *ADCYAP1R1* mRNA compared with females with the CG or GG genotype and males with the CC genotype.¹³ Deductively, these data further insinuate that the degree of *ADCYAP1R1* expression and PTSD symptoms are inversely correlated. In support of this reasoning, results presented in this manuscript reveal that either those with high PTSD symptoms have lower expression levels of *ADCYAP1R1* or that low expression levels increases risk for having greater symptoms of PTSD.

The signaling pathway associated with *ADCYAP1R1* (PAC1) and stress regulation requires binding of the PACAP ligand to the PAC1

G protein-coupled receptor. Thus, the association between PACAP and PTSD must also be considered when trying to understand the relationship between PAC1 and PTSD. An increase in PACAP is regularly observed as a biological response to stress.⁴⁰ We have previously shown that high levels of serum PACAP38 correlate with increased symptoms of PTSD in our study participants ($r=0.497$, $P\leq 0.005$).¹³ However, despite increased levels of PACAP, the rate-limiting factor for signaling activation is the number of receptors available to bind. Thus, for PTSD, in which the expression of *ADCYAP1R1* and presumably levels of PAC1 are reduced, PACAP would be insufficient to compensate for the outcome associated with decreased receptor levels. One explanation would be that decreased PAC1 might lead to an inability to downregulate PACAP release in a compensatory manner in response to stress.

The effect of estradiol on stress and anxiety behaviors has been well established in the scientific literature. Recent findings show differences in brain activity in response to psychosocial stress depending on whether women are in the low or high estrogen phase of their menstrual cycle.⁷ Research from Glover *et al.*³ shows that women in the low estrogen phase of their cycle have impaired fear inhibition. This was supported by another study by Wegerer *et al.*⁴³ that showed that women with lower levels of estradiol have stronger intrusive memories—one of the symptoms of PTSD.⁴³ If low levels of estradiol increase risk for negative psychiatric outcomes, it is possible that the administration of estradiol during a time of trauma or anxiety might provide protective effects against the development of stress-related disorders. Data supporting this hypothesis come from Ferree *et al.*,⁴⁴ where the effects of being on oral contraceptives or receiving emergency contraception immediately after being sexual assaulted resulted in fewer PTSD-type symptoms. These data favor the hypothesis that susceptibility to developing PTSD may be related to levels of estradiol among women at the time of trauma.

As genetic variants associated with neuropsychiatric disorders continue to be identified, functional analyses into the consequences of such variants will be necessary to provide insight into the molecular mechanism of disease. Uncovering these mechanisms can provide invaluable clues that may result in prevention or more effective treatment options. In this manuscript, we provide one of the first analyses into the molecular effects of a PTSD genetic risk variant. In doing so, we also identify a mechanism of estrogen regulation that may partially explain increased risk of PTSD among females. Our study illuminates the sex-dependent relationship between *ADCYAP1R1* polymorphisms, trauma and estrogen on risk for PTSD. It also provides additional support for estradiol as a potential therapeutic treatment in the prevention of PTSD, particularly for females with the *ADCYAP1R1* risk genotype.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was primarily supported by the National Institutes of Mental Health (MH096764 and MH071537 to KJR). Support was also received from Emory and Grady Memorial Hospital General Clinical Research Center, National Institutes of Health National Centers for Research Resources (M01RR00039). Dr Ressler is also on the Scientific Advisory Boards for Resilience Therapeutics, Sheppard Pratt-Lieber Research Institute, Laureate Institute for Brain Research, The Army STARRS Project and the Anxiety and Depression Association of America. He holds patents for the use of α -cycloserine and psychotherapy, targeting PAC1 receptor for extinction, targeting tachykinin 2 for the prevention of fear and targeting angiotensin to improve the extinction of fear. Dr Ressler is also founding member of Extinction Pharmaceuticals to develop α -cycloserine to augment the effectiveness of psychotherapy, for which he has received no equity or income within the past 3 years. He receives or has received research funding from NIMH, HHMI, NARSAD and the Burroughs Wellcome Foundation.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (<http://www.nature.com/tp>)