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Citation
Mercer, K B, B Dias, D Shafer, S A Maddox, J G Mulle, P Hu, J Walton, and K J Ressler. 2016. “Functional evaluation of a PTSD-associated genetic variant: estradiol regulation and ADCYAP1R1.” Translational Psychiatry 6 (12): e978. doi:10.1038/tp.2016.241. http://dx.doi.org/10.1038/tp.2016.241.

Published Version
doi:10.1038/tp.2016.241

Citable link
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Functional evaluation of a PTSD-associated genetic variant: estradiol regulation and ADCYAP1R1

KB Mercer1, B Dias1, D Shafer1,2, SA Maddox3, JG Mulle4,5, PH u 2, J Walton3 and KJ Ressler1,3

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 ADYC1R1 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 ovariolectomized after which a 2 mm pellet containing either sesame oil or 17β-estradiol (E2) dissolved in sesame oil at 1 μg μl$^{-1}$ (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., 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. mRNA was extracted using the RNaseasy 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 the TaqMan Gene Expression assays for Adcyap1r1 (Mm01326453_m1; catalog no. 4351372, Thermo Fisher Scientific, Waltham, MA, USA) and Gapdh, as an endogenous control (catalog no. 4352932, ThermoFisher Scientific). The 2$^{-ΔΔCT}$ method was used to compare fold change in expression between the condition and treatment groups. 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 ERα 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 (ERα; 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 ADCYAPIR1 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-hERα$^{GR2}$), and treated with E2 or ethanol only. mRNA was extracted and gene expression of ADCYAPIR1 was measured with commercially available assays. The 2$^{-ΔΔCT}$ method was used to compare fold change in expression between each condition and treatment group. See Supplementary Materials and Methods for more experimental details.

Crosslinking chromatin immunoprecipitation

HEK293T cells were transfected with pCMV-hERα and treated with E2. Following treatment, the cells were exposed briefly to formaldehyde to crosslink DNA-protein complexes, and then lysed and sonicated. Monoclonal antibody to ERα was used to isolate DNA bound by the receptor. After reversing the crosslinking, the ERα bound DNA was precipitated using standard phenol/chloroform extraction followed by ethanol precipitation. The ERE of interest within ADCYAPIR1 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 = 954). Consenting participants were asked to provide saliva sample or DNA collection and genotyping. They were also asked to complete questionnaires including the following: (1) the PTSD Symptom Scale to measure current PTSD symptoms and (2) the traumatic events inventory to assess the type and frequency of lifetime, traumatic events. 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. KE201, 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. A comparison of study specific genotypes between the larger study population and the subset used in this study are provided in Supplementary Table 2.

Detection of ADCYAPIR1 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 ml$^{-1}$, 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$^{-1}$). 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).

Total mRNA was extracted from whole-blood samples collected in Tempus Blood RNA tubes (catalog no. 432792; ThermoFisher Scientific) using the PurePure RNA 96 Cell kit (catalog no. 2900296; S PRIME, Gaithersburg, MD, USA). The expression of ADCYAPIR1 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 antiprobes. Reverse transcriptase PCR reactions were performed with 2 μl of total RNA and GoScript Reverse Transcription (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× HotStart-IT Probe qPCR Master Mix (catalog no. 75766, Affymetrix, Santa Clara, CA, USA); MgCl₂ and dNTPs (final concentrations of 5 and 0.1 μmol, respectively). 3 μl of complementary DNA, internal primers, and an internal DNA Detection Switch probe and antiprobe (GeneTAG Technology, Atlanta, GA, USA) were added. ADCYAPIR1 expression levels were normalized to GADPH using the 2$^{-ΔΔCT}$ method. The sequences and final concentrations for the primer, probe and antiprobe concentrations are provided in Supplementary Table 1.

**RESULTS**

Expression of ADCYAPIR1 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. Expression of Adcyap1r1 in the BNST is also activated by estradiol. We have shown previously in ovarioectomized female rats that treatment
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 4.4-fold increase in expression when compared to non-fear-conditioned, home-caged animals (P = 1.6 x 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.

Era-dependent activation of ADCYP1R1 expression

Estradiol regulates gene expression by binding to ERα or ERβ, activating a conformational change that allows the estradiol receptor complex to bind to chromatin and induce transcription. ERα 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 ADCYP1R1 is likely occurring via interaction with ERα. To address this hypothesis, we sought to determine whether introducing ERα into a cell line that expresses a detectable level of ADCYP1R1 but does not endogenously express ERα would result in enhanced expression of ADCYP1R1 in the presence of estradiol. Full-length hERα 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 ADCYP1R1 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 ERα. Among HEK293T cells treated with estradiol, there is a 3.9-fold increase in ADCYP1R1 expression for cells transfected with hERα compared with those transfected with GFP (P = 5.8 x 10^-4; Figure 2). These data reveal that ERα is sufficient to induce the expression of ADCYP1R1. 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 further support the role of estrogen-activated ERα, specifically, in estradiol-induced expression of ADCYP1R1.

In vivo binding of ERα to an ADCYP1R1 intronic ERE

E2/ERα activation of transcription occurs by binding to EREs, which are specific DNA sequences located throughout the genome.9 Within a particular intron of ADCYP1R1, 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 ADCYP1R1 in the cortex of human brain.13 To test whether or not this specific ERE is capable of binding ligand-activated ERα in vivo, consistent with that of a functional ERE, we performed crosslinking chromatin immunoprecipitation. HEK293T cells, which do not endogenously express ERα, were transiently transfected with full-length human ERα-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 ERα to ‘pull-down’ ERα-bound DNA. DNA that bound ERα 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 ERα to this particular ADCYP1R1 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 ERα is indeed able to bind to the predicted ERE containing a PTSD-associated SNP within ADCYP1R1.

Differential binding of ERα based on the SNP variant present within the ERE

With evidence of ERα 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 ERα, the efficiency of binding is correlated with enhancer activity.19 Thus, we hypothesized that the C allele, for which we observed lower ADCYP1R1 expression among homozygous carriers in our previous work,13 would have
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 ERα 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 ERα 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 ERα 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 ERα (P = 0.0005) in comparison with the canonical ERE (Figure 3b). Compared with binding of ERα 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/ERα 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. 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 mRNA were correlated 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 ΔCt value (corresponding to lower expression) if they have the CC risk genotype (range: 5.70–32.74 pg ml⁻¹) compared with women with either the GG or GC genotype (ΔCt = 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 ΔCt = 21.27 and 21.39, respectively; data not shown). We examined the expression of ADCYAP1R1 (by median split of ΔCt) 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 rs2267725.

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 ADCYAP1R1 expression | Low ADCYAP1R1 expression |
|---|---|---------------------------|--------------------------|
| All genotypes | | | |
| 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 χ², P = 0.031 | | | |
| B | N | Mean ADCYAP1R1 ΔCt | s.d. |
| Lower quartile of estradiol (E2) | | | |
| 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 ADCYAP1R1 expression | Low ADCYAP1R1 expression |
| CC genotypes only | | | |
| 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 χ², P = 0.034 | | | |
| D | N | Mean PTSD symptoms | s.d. |
| High ADCYAP1R1 expression | 48 | 7.97 | 9.22 |
| Low ADCYAP1R1 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 ERα on the expression of ADCYAP1R1 in the presence of a canonical, non-variant ERE sequence within the ADCYAP1R1 gene. In this model, ERα 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 ERα 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, ERα is not able to bind normally to the ERE, resulting in less expression of ADCYAP1R1, a dysregulated stress response, and increased PTSD symptoms. ERα, 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 ERα 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 ERα binding in a neuronal cell line that likely contains transcription factors that contribute to the activation of ADCYAP1R1 expression by facilitating E2/ERα binding to the ERE. The neuronal cell line we chose to utilize, SH-SYSY, was difficult to grow and we therefore discontinued working with these cells. However, we were able to detect low but convincing levels of ERα 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 ERE/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 ERα 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/ERα 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 such that the cellular mechanisms involving PAC1 and its ligand PACAP, which are a normal process of the stress regulation require binding of the PACAP ligand to the PAC1 G-protein-linked 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 ($r = 0.497$, $P < 0.005$).33 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. Research from Glover et al.43 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.33 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 D-cycloserine and psychotherapy, targeting PAC1 receptor for extinction, targeting tachykinin 2 for the prevention of fear and targeting angstosmin to improve the extinction of fear. Dr Ressler is also founding member of Extinction Pharmaceuticals to develop D-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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25.7 Translational Psychiatry (2016), 1 – 7

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

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