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The influence of *FKBP5* genotype on expression of *FKBP5* and other glucocorticoid-regulated genes, dependent on trauma exposure

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Abstract

FKBP5, an intrinsic regulator of the glucocorticoid receptor, has been associated with pathological behaviors particularly in the context of childhood trauma (CT), via a putatively regulatory polymorphism, rs1360780. However, *trans*- and *cis*-acting effects of this locus and its interaction with CT are incompletely understood. To study its effects on expression of glucocorticoid-regulated genes including *FKBP5*, we used lymphoblastoid cell lines (LCLs) derived from 16 CT-exposed patients with two substance dependence/suicidal behavior diagnoses (casesCT+) and 13 non CT-exposed controls (controlsCT-). This study in LCLs measures long-term trait-like differences attributable to genotype or lasting epigenetic modification. Through analysis of differential allelic expression (DAE) using an *FKBP5* 3'UTR reporter SNP, rs3800373, that is in strong linkage disequilibrium with rs1360780, we confirmed that the rs1360780 risk allele (A) (or conceivably that of a linked SNP) leads to higher *FKBP5* expression in controlsCT-. Intriguingly, casesCT+ did not show DAE, perhaps due to a genotype-predicted difference in *FKBP5* DNA methylation restricted to casesCT+. Furthermore, through correlation analyses on *FKBP5* expression at baseline and after induction by dexamethasone, we observed that casesCT+ had lower induction of *FKBP5* expression, indicating that overall they may have strong ultrashort negative-feedback. Only casesCT+ showed an effect of rs1360780 genotype on expression of *FKBP5* and other glucocorticoid-regulated genes. Together, these results confirm that the rs1360780 locus alters *FKBP5* expression and further that in *trans*-fashion this locus affects the expression of other glucocorticoid-regulated genes after a glucocorticoid challenge. Childhood trauma exposure appears to be essential for *trans*-effects of rs1360780 on glucocorticoid-regulated genes.

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DISCLOSURE / CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

Stress stimulates the hypothalamus to release corticotropin releasing factor (CRF). CRF induces pituitary gland secretion of adrenocorticotrophic hormone (ACTH), which stimulates the adrenal gland to release cortisol. The principal target of cortisol is the glucocorticoid receptor (GR). The activity of this receptor is regulated by an ultra-short negative feedback loop (Sapolsky *et al.*, 2000). Prolonged or excessive activation of the GR, which can occur when this feedback loop is impaired and following stress, has been implicated in the pathogenesis of mood and anxiety disorders (Holsboer, 2000, Pariante & Miller, 2001).

The FK506 binding protein 51 (FKBP5) is a critical negative regulator of GR function. Binding of FKBP5 to the GR reduces its cortisol-binding capacity and prevents nuclear translocation (Binder, 2009, Denny *et al.*, 2000, Jaaskelainen *et al.*, 2011, Wochnik *et al.*, 2005). GR activation rapidly enhances *FKBP5* gene expression, reducing GR sensitivity (Binder, 2009). There is strong linkage disequilibrium (LD) across the *FKBP5* gene including in individuals of African descent as shown in HapMap and in our earlier study (Roy *et al.*, 2010). Haplotypes including the single nucleotide polymorphisms (SNPs) rs1360780, rs9296158, rs3800373 and rs9470080 have been shown to interact with childhood trauma (CT) to predict post-traumatic stress disorder (PTSD), suicide attempts and major depression (Appel *et al.*, 2011, Binder *et al.*, 2008, Binder *et al.*, 2004, Koenen *et al.*, 2005, Mehta *et al.*, 2011, Roy *et al.*, 2010, Zimmermann *et al.*, 2011). The rs1360780 (A/G) SNP is adjacent to a functional glucocorticoid response element (GRE). It has been reported to be a cis-acting variant affecting *FKBP5* expression and GR sensitivity (Klengel *et al.*, 2013). In healthy individuals, the risk allele (A) has been associated with higher *FKBP5* expression and relatively reduced GR sensitivity as well as impaired recovery of cortisol levels in response to stress (Binder *et al.*, 2008, Binder *et al.*, 2004). Moreover, risk allele carriers exposed to CT showed less DNA methylation near and at functional GREs of the *FKBP5* gene (Klengel *et al.*, 2013). Nevertheless, the functionality of rs1360780 and its interaction with stress exposure is not yet fully understood.

Our study was performed in lymphoblastoid cell lines (LCLs) derived from an African American male sample of substance dependent patients exposed to CT (casesCT+) and healthy controls who had not experienced CT (controlsCT-). We investigated the way in which *cis*- or *trans*- acting functionality of rs1360780 genotypes affects not only *FKBP5* expression, but also expression of other glucocorticoid-regulated genes. We analyzed differential allelic expression (DAE) in casesCT+ and CT-controls who were heterozygous for both rs1360780 (A/G) in intron2 and rs3800373 (G/T) in the 3'UTR. As mentioned earlier, rs3800373 is strongly linked with rs1360780 in African ancestry individuals ($r^2 = 0.88$, $D' = 1.0$) in the HapMap project database. Moreover, in our previous study of these African Americans, the haplotypes including SNPs rs1360780 (A/G) and rs3800373 (G/T) are AG and GT with high frequencies ≈ 0.38 and ≈ 0.57 , respectively (Roy *et al.*, 2010). Therefore, the effect of rs1360780 on *FKBP5* expression would be easily detected by allelic difference at the reporter SNP rs3800373.

Furthermore, we investigated whether genotypes and CT influence expression of glucocorticoid-regulated genes after GR stimulation to identify genotype-induced GR

resistance and to dissociate allele-specific effects from long-lasting changes in *FKBP5* expression due to trauma-induced methylation of regulatory CpGs. In summary, in this study we investigated DAE, the combined influences of genotype and CT on *FKBP5* expression at baseline and after GR stimulation, the influence of *FKBP5* rs1360780 on other glucocorticoid-regulated genes after GR stimulation and finally the influence of genotype and CT on DNA methylation at the intron 2 and intron 7 regulatory regions of *FKBP5*, as previously described (Klengel *et al.*, 2013).

MATERIALS AND METHODS

Participants

A detailed description of this sample has previously been provided (Roy *et al.*, 2010). All participants gave written informed consent to the study that was approved by the Institutional Review Boards of the Department of Veteran Affairs New Jersey Healthcare System (VANJHCS) and the University of Medicine and Dentistry, New Jersey Medical School. Patients (90% men) were recruited from the Substance Abuse Treatment Program at the VANJHCS and were interviewed using the substance abuse section of the Structured Clinical Interview for DSM-IV to establish lifetime diagnoses. Patients had single or comorbid diagnoses of alcohol, cocaine or heroin addiction and a significant proportion had attempted suicide. Controls (40% men) were recruited from the same geographical area as the patients and were free of any lifetime alcohol or drug use disorder or suicide attempt. Participants completed the 28 item version of the Childhood Trauma Questionnaire (CTQ). The total CTQ score ranges from 25 to 125. Because of sample size issues, only LCLs from male participants were included in the current analysis. A total of 278 male patients and 72 controls had both DNA and CTQ data available. The total CTQ score ranged from 25 to 110; the mean (SE) score was 48.6 (1.0) for patients and 38.0 (2.0) for controls. Low and high CT groups were selected out of the total samples: a low CT group of controls (controlsCT-); (n = 13) with total CTQ score < 40 (mean: 31.5 ± 4.9); a high CT group of cases (casesCT+) defined as patients (n = 16) with total CTQ > 60 (mean: 74.1 ± 11.2) and with an average of two psychiatric disorders (alcohol, cocaine, heroin addiction, suicide attempt) (Table 1). The pyrosequencing study was done at a later date and at that time four more suitable LCLs became available: one controlCT-, rs1360780 GG, CTQ score = 33; three casesCT+: rs1360780 GG, CTQ scores = 65, 91; rs1360780 AA, CTQ score = 67. The participants whose LCLs were included in the current analysis had been genotyped for ancestry informative markers from which ethnic factor scores had been derived (Roy *et al.*, 2010). There was no difference between casesCT+ and controlsCT- in African ancestry: mean (SE) = 0.79 (0.03) vs. 0.80 (0.03), respectively, p = 0.74.

TaqMan genotyping of samples for *FKBP5* rs1360780 and rs3800373

The rs1360780 and rs3800373 SNPs were obtained as a TaqMan Assays-on-Demand (C__8852038_10; C__27489960_10) respectively (Applied Biosystems, Foster City, USA). Genotyping was performed according to the manufacturer's protocol and allelic discrimination genotype determined at end-point using ABI 7900HT Sequence Detection System 2.4. Genotyping accuracy was determined empirically by duplicate genotyping of

25% of the samples selected randomly. The error rate was <0.005, and the completion rate was >0.95.

Lymphoblastoid cell lines (LCLs)

LCLs derived from peripheral blood B cells transformed with Epstein Barr Virus and were maintained in culture for a minimum of fifty passages. LCLs were grown at 37 °C and 5 % CO₂ in RPMI-1640 steroid-free medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 IU/ml penicillin.

Quantitative RT-PCR

To study the expression of *FKBP5*, *FKBP4* and other glucocorticoid-regulated genes, we performed quantitative RT-PCR with primers (Supplementary Table S1) for *FKBP5*, *FKBP4*, three other known glucocorticoid-regulated genes; *TSC22D3* (TSC22 domain family member 3), *BIM* (BCL-2-interacting mediator of cell death), and *RUNX2* (Runx-related transcription factor 2) (Frodl *et al.*, 2012, Heidari *et al.*, 2012), and four genes, *LTF* (lactotransferrin) *MSL3L1* male-specific lethal 3 homolog (Drosophila) *ARG1* (arginase 1) and *PHF21A* (PHD finger protein 21A), which have been proposed to be glucocorticoid-regulated (Klengel *et al.*, 2013, Menke *et al.*, 2012). RNA was extracted with TRIzol (Thermo Scientific, Waltham, MA, USA) from untreated cells or following treatment with dexamethasone (Dex, 100 nM) (D4902, Sigma-Aldrich Chemie, Steinheim, Germany) for 24 hours. Residual genomic DNA contamination was removed by incubation with DNaseI (Thermo Scientific, Waltham, MA, USA) at 37°C for 30 min. Total RNA was reverse-transcribed into cDNA with the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA) according to the protocols. For quantitative PCR, 1 µl of cDNA was mixed with gene-specific primers (0.25 µM final concentration), 2× SYBR Green master mix (Applied Biosystems, Foster City, USA) in a 10 µl volume. Reactions were carried out on the ABI 7900 real-time PCR system with the following protocol: one cycle of 95 °C for 3 min, 40 cycles of 95 °C for 15 sec and 60°C for 1 min. Relative gene expression levels for each sample were calculated using the C_t values (normalized detection threshold). Gene expression was analyzed using *GAPDH* as internal control. *FKBP5* baseline expression levels were calculated using C_t values and relative to an arbitrarily chosen sample. Results are reported in fold change using the C_t values and 2^{- C_t} method. All samples were run in duplicate.

Quantitative Sanger sequencing to detect allelic imbalance in cDNA

To detect DAE, genomic DNA and RNA were extracted from each of 12 LCLs that were heterozygous for rs1360780 and rs3800373. To extract genomic DNA, briefly, cells were lysed in 1 % SDS, 0.05 M EDTA, 50 mM Tris-Cl, pH 8.0, and 100 ng/ml proteinase K, and incubated at 55 °C for 16 h. Genomic DNA was purified by phenol/chloroform extraction. DNA quality and quantity was assessed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Methods for extraction of RNA and cDNA synthesis are the same as described above in the 'Quantitative RT-PCR' section. PCR amplification with primers (Supplementary Table S1) for *FKBP5* was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, USA), 1 µl of forward and reverse primer (5 µM each), 2 µl of H₂O at 94°C for 1 min, followed by 40 cycles each

at 94°C for 20 sec, 60°C for 30 sec, 68°C for 30 sec, and a final extension step of 72°C for 10 min. The PCR products were purified using the ExoSAP-IT reagent (Affymetrix, Santa Clara, CA, USA) according to the protocol and sequenced on an Applied Biosystems 3730 DNA Analyzer with Big Dye Terminator Sequencing Mix (Applied Biosystems, Foster City, USA). To measure DAE in Sanger sequencing electropherograms, the areas under each peak for the G allele and T allele at target positions were measured using ImageJ1.46r (National Institutes of Health, <http://imagej.nih.gov/ij/>). The allele signals from the Sanger cDNA sequencing results were normalized using the allele-specific peak ratios from the Sanger gDNA sequencing. To test if the G allele and T allele were equally expressed, Sanger sequencing data were evaluated by a one-group t-test with values of G allele area / (G allele area + T allele area). The analyses were undertaken using JMP11 software (SAS Institute).

DNA methylation analysis by bisulfite pyrosequencing

We analyzed DNA methylation of *FKBP5* intron 2 including 3 CpGs and intron 7 including 2 CpGs in 14 controlsCT- and 19 casesCT+ (Table 1). These regions were selected according to their spatial and functional relationship to consensus GREs or rs13160780. Genomic DNA was sodium bisulfite converted using the EZ DNA Methylation Kit according to manufacturer's protocol (Zymo Research, Irvine, USA). DNA methylation of *FKBP5* was analyzed by pyrosequencing (EpigenDX)(Hopkinton, MA, USA) using primer sets: intron2 including 3 CpGs (ADS3136-FS1) and intron 7 including 2 CpGs (ADS3828-FS2). Methylation specific pyrosequencing was performed in a single run with numerous internal controls. Each DNA methylation assay was validated for PCR bias and incomplete bisulfite conversion using serial dilutions of *in vitro* methylated DNA. The percent methylation obtained from the mixing study was highly correlated with the percent of *in vitro* methylated DNA with R^2 values of >0.95 in each of our assays.

Statistical methods

The rs1360780 AA and AG genotypes were grouped together because both genotypes are equivalent in risk and the AA genotype is at low frequency. The Student's *t*-test was used for two-group comparisons. The potential effects of covariates (genotype, childhood trauma and Dex treatment) on gene expression were tested by analysis of covariance (ANCOVAs). All statistical tests were performed JMP11.

RESULTS

Differential allelic expression of *FKBP5* reporter SNP rs3800373 (G/T)

As seen in Figure 1, Dex treatment had no effect on *FKBP5* allelic expression in casesCT+ or controlsCT-. ControlsCT- did show a consistently higher G:T allelic expression ratio at baseline ($p = 0.001$) and after Dex treatment ($p = 0.005$). However, there was no DAE in casesCT+ (baseline: $p = 0.93$, Dex treatment: $p = 0.69$).

Combined effects of rs1360780 and childhood trauma on *FKBP5* expression

A two-tailed *t* test revealed that there was a trend effect for increased *FKBP5* expression at baseline in casesCT+ relative to controlsCT- ($F = 8.9$, 27 df, $p = 0.056$) (Supplementary Figure S2). However, there was no genotype effect. There was a main effect of Dex

treatment on *FKBP5* expression ($F = 98.0$, 1 df, $P < 0.0001$) (Table 2); gene expression was increased more than 5 fold in both casesCT+ and controlsCT- after treatment with 100 nM of Dex for 24 hrs (Figure 3A). Also, there was trend effect of genotype ($F = 3.2$, 1df, $p = 0.077$). *Post-hoc* separate ANCOVAs for casesCT+ and controlsCT- found no effect in controlsCT- ($F = 0.06$, 1 df, $p = 0.81$), but a significant genotype effect in casesCT+; the AA/AG risk genotypes had lower *FKBP5* induction than GG homozygotes ($F = 5.4$, 1 df, $p = 0.036$) (Figure 2A). Moreover, among AA/AG genotypes, casesCT+ showed a trend for lower induction of *FKBP5* expression relative to controlsCT- ($F = 4.38$, 1 df, $p = 0.055$) (Figure 2A). Furthermore, regression analysis revealed a negative correlation ($r = -0.48$, $p = 0.005$) between baseline *FKBP5* expression and fold increase of *FKBP5* expression after Dex treatment. This finding was detected in casesCT+ ($r = -0.50$, $p = 0.029$), and not in controlsCT- ($r = -0.38$, $p = 0.12$) (Figure 2B).

***FKBP4* expression after Dex treatment**

FKBP4 has an essential role in nuclear translocation of GR to induce transcription of GR target genes including *FKBP5* (Davies *et al.*, 2005). If *FKBP4* expression is changed, expression of *FKBP5* could be altered. However, we found no effect of Dex treatment, CT, nor *FKBP5* rs1360780 genotype on *FKBP4* expression (Table 2), suggesting that the low induction of *FKBP5* expression in casesCT+ was independent of *FKBP4* expression.

Expression of other glucocorticoid-regulated genes after Dex treatment

***TSC22D3*, *BIM* and *RUNX2*, Known glucocorticoid responsive genes**—The expression of *TSC22D3* was increased 20 – 30 fold after Dex treatment ($F = 129.9$, 1 df, $p < 0.0001$) (Table 2, Supplementary Figure S3). *BIM* and *RUNX2* had lower rates of induction (*BIM*: $F = 33.5$, 1 df, $p < 0.0001$, *RUNX2*: $F = 52.1$, 1 df, $p < 0.0001$) (Table 2, Supplementary Figure S3). However, unlike with *FKBP5* there was no effect of rs1360780 genotype or caseCT+/controlCT- status on gene expression (Table 2).

Correlations between changes in *TSC22D3*, *BIM* and *RUNX2* after Dex treatment and *FKBP5* induction—There was a significant positive correlation between increased expression in glucocorticoid-regulated genes and *FKBP5* in casesCT+ (*TSC22D3*: $r = 0.6$, $p = 0.01$, *BIM*: $r = 0.79$, $p = 3.0e-4$, *RUNX2*: $r = 0.46$, $p = 0.07$) but not in controlsCT- (Figure 3). Further analysis showed that in casesCT+, this significant correlation originated from the GG homozygotes (*TSC22D3*: $r = 0.7$, $p = 0.03$, *BIM*: $r = 0.8$, $p = 0.009$, *RUNX2*: $r = 0.79$, $p = 0.01$) and not AA/AG genotypes (Figure 3). However, these changes were not correlated with baseline *FKBP5* expression (Supplementary Figure S4).

***LTF*, *MSL3L1*, *PHF21A* and *ARG1*, putative glucocorticoid-regulated genes**—The expression of *LTF*, *MSL3L1*, *ARG1* and *PHF21A* showed varying results in LCLs of controlsCT- and casesCT+ after Dex treatment (Table 2, Supplementary Figure S5). An ANCOVA revealed a 1.5 to 2 fold increase in *LTF* expression after Dex treatment ($F = 16.3$, 1 df, $p = 0.0002$) that was independent of genotype ($p = 0.65$) and caseCT+/controlCT- status ($p = 0.50$). In contrast, there was decreased *MSL3L1* expression after Dex treatment ($F = 6.7$, 1 df, $p = 0.012$) but only in controlsCT- ($F = 5.5$, 1 df, $p = 0.02$), with no change in casesCT+. *ARG1* showed a less than two-fold increase in expression ($F = 6.2$, 1 df, $p =$

0.016) that was independent of genotype ($p = 0.78$) and caseCT+/controlCT- status ($p = 0.54$). Finally, *PHF21A* showed only a trend change in expression ($F = 2.2$, 1 df, $p = 0.15$) and a trend caseCT+/controlCT- effect ($F = 3.0$, 1 df, $p = 0.09$). Similar to *MSL3L1*, this signal derived from controlsCT- that showed decreased expression after Dex treatment, with no change in casesCT+. Therefore, these genes are not highly glucocorticoid-regulated in LCLs.

DNA methylation of *FKBP5*

We analyzed DNA methylation of *FKBP5* intron 2, including 3 CpGs, and intron 7, including 2 CpGs. ANCOVAs including all covariates found no effect of Dex treatment, genotype or caseCT+/controlCT- status on DNA methylation at intron 2; CpG1 ($F = 0.94$, 3 df, $p = 0.43$), CpG2 ($F = 0.74$, 3 df, $p = 0.53$) or CpG3 ($F = 0.46$, 3 df, $p = 0.53$) (Supplementary Table S2, Figure 4A). There was a trend effect on DNA methylation in intron 7; CpG1: $F = 2.36$, 3 df, $p = 0.08$; CpG2: $F = 2.24$, 3 df, $p = 0.09$. This contribution was due to the effect of genotype: CpG1: $F = 4.6$, 1 df, $p = 0.04$; CpG2: $F = 3.5$, 1 df, $p = 0.07$. *Post-hoc* ANCOVAs showed that the CpG1 signal derived from the casesCT+: carriers of the risk AA/AG genotypes had lower methylation than GG homozygotes after Dex treatment ($F = 6.8$, 1 df, $p = 0.02$) and at baseline ($F = 4.2$, 1 df, $p = 0.06$); the mean differences in methylation were 3.1% and 2.6% respectively (Figure 4B).

DISCUSSION

Previous studies have shown that *FKBP5* rs1360780 interacts with CT to predict stress-related disorders such as PTSD and suicidal behavior, including in African Americans (Binder *et al.*, 2008, Roy *et al.*, 2010). In the current study we investigated different aspects of the potential functionality of *FKBP5* rs1360780 in LCLs from African American men (Roy *et al.*, 2010): a group of casesCT+ who were substance dependent patients who had experienced high CT and a group of controlsCT-, free of substance dependence and suicidal behavior, who had not been exposed to CT. Importantly, the casesCT+ had not only experienced trauma but also had suffered psychiatric disease, on average two substance addictions. Nearly all earlier studies have performed analyses in peripheral blood cells (PBCs). However, in this study we used LCLs derived from PBCs transformed with Epstein Barr virus and maintained in culture for a minimum of fifty passages. We investigated DAE, the combined influences of genotype and CT on *FKBP5* expression at baseline and after GR stimulation, the influence of *FKBP5* rs1360780 on other glucocorticoid-regulated genes after GR stimulation and the influence of genotype and CT on DNA methylation at the intron 2 and intron 7 regulatory regions of *FKBP5*. As discussed below, other than the results for DAE, all the significant findings were in casesCT+ and not controlsCT-.

Importantly, for the design and interpretation of DAE analyses, there is high LD across *FKBP5* including between rs1360780 (A/G) and rs3800373 (G/T) (Bevilacqua *et al.*, 2012, Binder *et al.*, 2004, Huang *et al.*, 2014, Roy *et al.*, 2010). This haplotype has been associated with differential induction of *FKBP5* mRNA and protein by GR activation (Binder *et al.*, 2004) and with GR resistance such that A allele carriers of healthy controls have a prolonged cortisol response even to minor stressors (Ising *et al.*, 2008). Potential

functionality of rs1360780 was revealed by an assay in HeLa cells with luciferase gene constructs encompassing the consensus GRE site together with either the rs1360780 A risk allele or the G protective allele; after Dex treatment the luciferase activity of the construct with the A allele was higher than that of the G allele (Klengel *et al.*, 2013). Our study has demonstrated allele-specific differences in *FKBP5* expression in the LCLs of controlsCT-, but not casesCT+. ControlsCT- had a higher A:G, risk:protective allelic expression ratio both at baseline and after Dex treatment. This suggests that the haplotype including the rs3800373 G allele and the rs1360780 A risk allele has a *cis*-acting functional locus affecting *FKBP5* expression and the functionality might be disturbed by cellular long term memory associated with CT. However, because of the high LD across the gene it remains possible that other unknown functional SNPs, other than rs1360780 might be responsible for the DAE.

The balance between *FKBP5* expression and GR activation is a key component of an ultra-short negative feedback mechanism. GR activation directly induces *FKBP5* transcription (Vermeer *et al.*, 2003). After rapid induction of *FKBP5* in response to GR activation *FKBP5* binds to the GR, reduces the affinity of GR for cortisol and decreases translocation of the GR to the nucleus (Vermeer *et al.*, 2003, Zannas & Binder, 2014). Our study investigated elements of this negative feedback loop, and the effects of CT and the *cis*-acting *FKBP5* locus on it. At baseline, there was a trend for casesCT+ to have higher *FKBP5* expression than controlsCT-. GR stimulation increased expression in both groups. However, A risk allele carriers, who were casesCT+, showed strong sensitivity to GR stimulation compared with GG homozygotes, whereas there was no genotype effect in controlsCT-. In casesCT+, there was a negative correlation between baseline *FKBP5* expression and fold change increase in expression after GR stimulation, suggesting a ceiling effect. This finding was not detected in controlsCT-, perhaps because they had lower baseline *FKBP5* expression. This suggests that casesCT+ may be more vulnerable to future stress because they have already maximal compensated elevation of *FKBP5* expression. The interactive effect of the *FKBP5* A risk allele and CT exposure appears to lead to lower *FKBP5* induction after GR stimulation and therefore a ‘dampening’ of the negative feedback mechanism. Moreover, a significant positive correlation was observed after GR stimulation between induction of *FKBP5* and induction of known glucocorticoid-regulated genes, *TSC22D3*, *BIM* and *RUNX2*, only in the GG homozygotes, who were casesCT+ and not in controlsCT-. These results suggest that CT interacts with the *FKBP5* risk genotype to alter the function of the ultrashort feedback and downstream expression of glucocorticoid-regulated genes.

Accumulating evidence indicates that CT may exert long-lasting effects through epigenetic changes (Lutz & Turecki, 2014, Szyf, 2011). An earlier study of *FKBP5* DNA methylation in PBCs of adults showed that CT was associated with hypomethylation in intron 7 (Klengel *et al.*, 2013). Moreover, there was a negative correlation between DNA methylation and CTQ scores in the rs1360780 A risk allele carriers, but not GG carriers.) PBCs differ from LCLs, nevertheless we observed a similar genotypic difference in DNA methylation at CpG1 of intron 7; Klengel’s group observed a 12% decrease in methylation of A allele carriers relative to GG carriers among casesCT+. Likewise, we observed an approximately 4 % decrease in methylation of A allele carriers in casesCT+. Also, the percentages of DNA methylation in our study of CpG2 (85 ~ 90 %) of intron 7 and CpGs (CpG1: 78 ~ 82%;

CpG2: $\approx 70\%$) of intron 2 were consistent with methylation percentages in the previous report (Klengel *et al.*, 2013). This commonality suggests that CT may lead to allele-specific epigenetic changes with a decrease in DNA methylation as measured in PBCs as well as LCLs. There have been concerns that several factors could potentially affect DNA methylation patterns in LCLs compared to the original B lymphocytes including EB virus infection, cell culture conditions and freezing cycles. Several studies examined global methylation levels and concluded that LCLs are non-equivalent to PBCs (Sugawara *et al.*, 2011, Thompson *et al.*, 2013). However, most of the promoter methylation regions in PBCs were maintained in LCLs (Sugawara *et al.*, 2011) and studies in individual genes have shown limited variability (Brennan *et al.*, 2009, Saferali *et al.*, 2010). For example, Brennan and colleagues looked at 318 genes in LCL and PBC sample pairs and identified only 8% of genes with different methylation profiles. It is possible that our finding of a smaller difference in DNA methylation of intron 7 relative to the previous study (Klengel *et al.*, 2013) may be due to the effect of the EBV transformation or long term culture.

There are several limitations to our study. The sample sizes of LCLs from casesCT+ and controlsCT- were small, and therefore some of the negative results may be false. Nevertheless, our significant data reproduced results from other studies e.g. intron 7 methylation results (Klengel *et al.*, 2013). However, it is not clear whether the small difference in DNA methylation (12% in blood and 4% in LCLs) between A allele carriers and GG carriers affects gene expression. It may be that LCLs have a different array of potential factors affecting gene regulation than is found in PBCs. Although exposing cells to 100nM dex for 24 hours is a commonly used protocol for treatment of cell lines, it is possible that by this time protein levels of FKBP5 may already feedback on RNA expression. In our study, we have compared casesCT+ with healthy controlsCT-. It should be noted that the casesCT+ who have a minimum of two diagnoses of substance dependence and suicidal behavior, may have had many other sources of trauma or other significant environmental exposures.. The casesCT+ might also have been more likely to share other liability alleles, but it was impractical for us to evaluate this because of the size of the sample. Our study included only men and regulation of stress response genes may differ in women.

In conclusion, our study clearly showed *cis* and *trans*-effects of rs1360780 on gene regulation and combined effects with exposure to childhood trauma. The overall conclusion of our study and the previous study (Klengel *et al.*, 2013) is that childhood trauma might be imprinted in cellular memory and, in combination with genetic effects, might induce abnormal GR responses to stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Differential *cis*-action of *FKBP5* rs1360780 alleles, as detected by DAE, on rs3800373 expression between controlsCT- and casesCT+

A. The schematic representation depicts haplotypes between rs3800373 and rs1360780.

B. Differential allelic expression within LCLs, i.e. within each heterozygote, expression of G + T alleles = 1. Data are shown as mean $\pm$ SEM. **: p-value = 0.005. controlsCT-: n = 7; casesCT+ (patients exposed to childhood trauma): n = 5. Dex - = baseline; Dex + = treatment with dexamethasone; LCL = lymphoblastoid cell line.

Figure 2. Combined effects of *FKBP5* rs1360780 AA/AG genotypes and CT predict low *FKBP5* induction after GR stimulation.

A. *FKBP5* expression was measured after Dex treatment (0.1 uM) for 24hrs. ANCOVAs identified main effect of Dex treatment ($F = 97.95$, $p < 0.0001$) and trend effect of genotypes ($F = 3.24$, $p = 0.077$) in casesCT+ and controlsCT- combined. Dex - = baseline; Dex + = treatment with dexamethasone. Data are shown as mean $\pm$ SEM. **: $p\text{-value} = 0.005$, * $p\text{-value} < 0.05$. P-values were from ANCOVAs followed by post hoc test.

B. A negative correlation between baseline *FKBP5* expression and *FKBP5* induction in casesCT+. A left graph shows combined results of casesCT+ and controlsCT- and a right graph shows separate results of casesCT+ and controlsCT-. ControlsCT-: $n = 13$ (AA/AG, $n = 9$; GG, $n = 4$) CasesCT+ (patients exposed to childhood trauma): $n = 16$ (AA/AG, $n = 7$; GG, $n = 9$).

Figure 3. Correlation between expression of known glucocorticoid-regulated genes (*TSC22D3*, *BIM*, *RUNX2*) and *FKBP5* expression is dependent on childhood trauma exposure and rs1360780 genotype.

CasesCT+ n = 16; AA/AG n = 7, GG n = 9; ControlsCT- n = 13; AA/AG n = 9, GG n = 4.

Figure 4. DNA methylation of regulatory CpGs in (A) intron2 and (B) intron 7 of *FKBP5* in LCLs

ControlsCT-: n = 14 (AA/AG, n=9; GG, n=5) CasesCT+ (patients exposed to childhood trauma): n = 19 (AA/AG, n = 8; GG, n = 11). Dex - = baseline; Dex + = treatment with dexamethasone. Data are shown as mean $\pm$ SEM. *p < 0.05. P-values were from ANCOVAs followed by post hoc test.

Table 1:

Description of participants from whom lymphoblastoid cell lines were derived

African American men, N = 29	Controls (Low CTQ), N = 13		Cases (High CTQ), N = 16	
rs1360780 genotypes	AA/AG	GG	AA/AG	GG
rs3800373 genotypes	GG/GT	TT	GG/GT	TT
Number of samples	2/7	4	2/5	9
Age: mean (SD)	35.4 (7.8)	30.8 (6.5)	36.6 (11)	43.4 (6.9)
CTQ total score: mean (SD)	32.2 (5.5)	29.8 (3.4)	71.1 (6.1)	76.3 (13.9)

CTQ: Childhood Trauma Questionnaire, possible range 25 – 125 ---- Diagnoses of substance dependence and suicide attempts (SA) in cases: cocaine only (1); alcohol only (1); cocaine + alcohol (2); alcohol + heroin (1); cocaine + heroin (3); cocaine + SA (2); cocaine + alcohol + SA (4); alcohol + heroin + SA (1); alcohol + cocaine + heroin + SA (1) - For pyrosequencing, four more suitable LCLs became available: one control, rs1360780 GG, CTQ score=33; three cases: rs1360780 GG, CTQ scores = 65, 91; rs1360780 AA, CTQ score = 67

TABLE 2:

Effect of rs1360780 genotypes, childhood trauma, and Dex treatment on expression of *FKBP5* and other glucocorticoid-regulated genes

Gene	Independent variable	DF	F Ratio	p value
FKBP5	Genotypes	1	3.24	0.077
	controls/cases	1	1.80	0.186
	Dex -/+	1	97.95	<.0001
	Total model:F = 34.01, 3 df, p <.0001, r2= 0.65			
FKBP4	Genotypes	1	0.60	0.444
	controls/cases	1	0.24	0.624
	Dex -/+	1	0.06	0.808
	Total model:F = 0.39, 3 df, p = 0.760, r2= 0.02			
TSC22D3	Genotypes	1	0.01	0.924
	controls/cases	1	0.003	0.959
	Dex -/+	1	129.92	<.0001
	Total model:F = 43.31, 3 df, p <.0001, r2= 0.71			
BIM	Genotypes	1	0.73	0.396
	controls/cases	1	0.96	0.331
	Dex -/+	1	33.48	<.0001
	Total model:F = 11.92, 3 df, p <.0001, r2= 0.40			
RUNX2	Genotypes	1	0.51	0.477
	controls/cases	1	0.47	0.495
	Dex -/+	1	52.10	<.0001
	Total model:F = 17.63, 3 df, p <.0001, r2= 0.49			
LTF	Genotypes	1	0.21	0.649
	controls/cases	1	0.46	0.503
	Dex -/+	1	16.35	0.0002
	Total model:F = 5.63, 3 df, p = 0.002, r2= 0.24			
MSL3L1	Genotypes	1	0.03	0.858
	controls/cases	1	5.46	0.023
	Dex -/+	1	6.73	0.012
	Total model:F = 4.12, 3 df, p = 0.011, r2= 0.19			
ARG1	Genotypes	1	0.08	0.779
	controls/cases	1	0.37	0.544
	Dex -/+	1	6.23	0.016
	Total model:F = 2.27, 3 df, p = 0.091, r2= 0.11			
PHF21A	Genotypes	1	0.43	0.516
	controls/cases	1	3.04	0.087
	Dex -/+	1	2.16	0.147
	Total model:F = 1.75, 3 df, p = 0.168, r2= 0.09			