Dissertation zur Erlangung des Doktorgrades
der Fakultät für Biologie
der Ludwig-Maximilians-Universität München

Epigenetic Signature of Childhood Trauma

Max Planck Institut für Psychiatrie
Abteilung für Translationale Forschung in der Psychiatrie
und
Ludwig-Maximilians-Universität München
Fakultät für Biologie

Vorgelegt von

Torsten Klengel
aus
München, Deutschland

2015

Tag der Einreichung: 12.06.2015

Tag der mündlichen Prüfung: 15.01.2016

Erster Gutachter: PD Dr. rer. nat. habil. Carsten T. Wotjak

Zweiter Gutachter: Prof. Dr. rer. nat. Wolfgang Enard

Dritter Gutachter: Prof. Dr. rer. nat. Heinrich Leonhardt

Vierter Gutachter: PD Dr. rer. nat. Bettina Bölter

Fünfter Gutachter: Prof. Dr. rer. nat. Elisabeth Weiß

Sechster Gutachter: Prof. Dr. rer. nat. Jürgen Soll

Contents Page

Statutory declaration and statement / Eidesstattliche Versicherung und Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 12.06.2015

Torsten Klengel

Hiermit erkläre ich,

dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

dass ich mich anderweitig einer Doktorprüfung ohne Erfolg nicht unterzogen habe.

dass ich mich im Jahr 2009 mit Erfolg der Doktorprüfung im Hauptfach Medizin bei der Medizinischen Fakultät der Universität Würzburg unterzogen habe.

München, den 12.06.2015

Torsten Klengel

List of Abbreviations

%	percent
3'UTR	3 Prime Untranslated Region
3C	Chromatin Conformation Capture
5-HTTLPR	serotonin-transporter-linked polymorphic region
Avp	arginine vasopressin gene
BDNF	Brain Derived Neurotrophic Factor
CNV	Copy Number Variation
CpG	CG dinucleotide
Crh	Corticotrophin Releasing Hormone
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/CRISPR associated 9
CTQ	Childhood Trauma Questionnaire
DALY	Disability Adjusted Life Year
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
FACS	Fluorescence-Activated Cell Sorting
FKBP5	FK506 binding protein 5 (gene name)
FKBP51	FK506 binding protein 51 kDa (protein name)
FKBP52	FK506 binding protein 52 kDa (protein name)
GR	Glucocorticoid Receptor (protein name)
GRE	Glucocorticoid Response Element
GWAS	Genome Wide Association Study
GxE	Gene by Environment Interaction
HDAC	Histone deacetylase
HPA-axis	Hypothalamus-Pituitary-Adrenal axis
Hsp90	Heat shock protein 90
IGF2	Insulin-Like Growth Factor 2
kb	kilobase

lncRNA	long-noncoding RNA
MAO-A	Monoamine Oxidase A
MDD	Major Depressive Disorder or Major Depression
MeDIP-chip	Methylated DNA immunoprecipitation coupled with array hybridization
miRNA	micro RNA
mRNA	messenger RNA
N	Number
N-terminal	Amine-terminus, start of a protein
NGFI-A	Nerve growth factor-induced protein A
Nr3c1	Nuclear receptor subfamily 3, group C, member 1, Gene name fort the GR
p	p-value
piRNA	piwi interacting RNA
PPI	peptidylprolylisomerase (PPI) domain
PTSD	Post-Traumatic Stress Disorder
RNA	Ribonucleic acid
rs	Reference SNP cluster ID.
SLC6A4	Solute carrier family 6 (gene name for serotonin transporter)
SNP	Single Nucleotide Polymorphism
TALEs	Transcription activator-like effectors
TATA-box	cis regulatory DNA sequence, often found in promoters
TBP	TATA-binding protein
TPR	tetratricopeptide repeat protein domain
TSS	Transcription start site

List of Figures

List of Tables

List of Publications

The work presented in this cumulative thesis was conducted under supervision and mentorship of Elisabeth B. Binder MD, PhD at the Department for Translational Research in Psychiatry at the Max Planck Institute for Psychiatry in Munich, Germany. The thesis presented here contains the following publications:

Klengel T, Mehta D, Anacker C, Rex-Haffner M, Pruessner JC, Pariante CM, Pace TWW, Mercer KB, Mayberg HS, Bradley B, Nemeroff CB, Holsboer F, Heim CM, Ressler KJ, Rein T, Binder EB. Allele–specific DNA *FKBP5* demethylation: a molecular mediator of gene–childhood trauma interactions. Nature Neuroscience. 16(1), 33-41, 2013 doi:10.1038/nn.3275.

Mehta D, **Klengel T**, Conneely KN, Smith AK, Rex-Haffner M, Loeschner A, Gonik M, Mercer KB, Bradley B, Müller-Myhsok B, Ressler KJ, Binder EB. Childhood maltreatment is associated with distinct genomic and epigenetic profiles in posttraumatic stress disorder. Proc. Natl. Acad. Sci. USA. 110(20), 8302-8307, 2013 doi: 10.1073/pnas.1217750110

Declaration/Declaration of contribution as co-author

Paper I

Klengel T, Mehta D, Anacker C, Rex-Haffner M, Pruessner JC, Pariante CM, Pace TWW, Mercer KB, Mayberg HS, Bradley B, Nemeroff CB, Holsboer F, Heim CM, Ressler KJ, Rein T, Binder EB. Allele–specific DNA *FKBP5* demethylation*:* a molecular mediator of gene–childhood trauma interactions. Nature Neuroscience. 16(1), 33-41, 2013 doi:10.1038/nn.3275.

T.K. and E.B.B. designed the experiments, performed the luciferase assays and the genetic, methylation and expression analyses, analyzed the data, and wrote the initial version of the paper. D.M. performed the RNA expression experiments and data analyses and revised the paper. M.R.-H. performed the chromatin conformation capture experiments. C.A. and C.M.P. performed the cell-culture experiments with human hippocampal progenitor cells and revised the paper. T.W.W.P. performed *ex vivo* glucocorticoid receptor sensitivity experiments and revised the paper. J.C.P. analyzed magnetic resonance imaging data and revised the paper. E.B.B., F.H., K.J.R., K.B.M., H.S.M., B.B., C.B.N., C.M.H. and T.R. organized sample collection and collaborations, obtained funding, supervised data analyses, and revised the paper.

Paper II

Mehta D, **Klengel T**, Conneely KN, Smith AK, Rex-Haffner M, Loeschner A, Gonik M, Mercer KB, Bradley B, Müller-Myhsok B, Ressler KJ, Binder EB. Childhood maltreatment is associated with distinct genomic and epigenetic profiles in posttraumatic stress disorder. Proc. Natl. Acad. Sci. USA. 110(20), 8302-8307, 2013 doi: 10.1073/pnas.1217750110

T.K. extracted DNA for methylation arrays, measured and controlled quality. TK implemented 450k methylation array procedures and hybridized 450k methylation arrays as well as designed Sequenom EpiTYPER validation experiments and revised the paper.

Torsten Klengel Elisabeth B Binder

English Summary

Childhood abuse is one of the major risk factors for the development of adult psychopathology though the response to childhood abuse and other types of early life adversities is not uniform. Genetic predisposition modulates the exposure to environmental factors in form of gene by environment interaction. This has been shown for FKBP5, a modulator of the stress hormone axis, with certain alleles in *FKBP5* conferring a higher risk towards PTSD in adulthood in response to childhood abuse. This thesis investigates the potential molecular mechanism behind this gene by environment interaction and delineates an allele-specific demethylation mechanism in response to childhood abuse. In addition, data on genome-wide gene expression and DNA methylation profiles in peripheral blood in response to childhood abuse is presented providing evidence for the hypothesis that childhood trauma leads to a different molecular trajectory towards adult psychopathology compared to adult traumatization. The data presented here contribute to our understanding of the molecular mechanisms underlying gene by environment interactions in psychiatry and the pathophysiology of trauma- and stress-induced psychiatric disorders.

Deutsche Zusammenfassung

Missbrauch und Misshandlung im Kindesalter ist einer der stärksten Risikofaktoren für psychiatrische Erkrankungen im Erwachsenenalter. Jedoch entwickelt nur ein Teil der exponierten Kinder im Erwachsenenalter eine psychiatrische Erkrankung. Dabei moduliert die individuelle Genetik das Verhältnis von Umweltfaktoren und späteren Erkrankungen in Form von Gen-Umwelt-Interaktionen. Ein Beispiel dafür ist die Gen-Umwelt-Interaktion von FKBP5, einem Regler der Stress-Hormon-Achse, und Traumatisierung im Kindesalter auf das Risiko an einer post-traumatischen Belastungsstörung im Erwachsenenalter zu erkranken. Sogenannte Risikoallele in Form von Einzelnukleotid-Polymorphismen in *FKBP5* erhöhen dabei die Wahrscheinlichkeit, an einer PTBS im Erwachsenenalter zu erkranken. Die vorliegende Arbeit untersucht die zu Grunde liegenden molekularen Mechanismen dieser Gen-Umweltinteraktion und zeigt, dass Kindesmissbrauch und Misshandlung eine lang-anhaltende epigenetische Veränderung im *FKBP5* Gen in Abhängigkeit von der genetischen Prädisposition bewirkt. Darüber hinaus zeigen genom-weite Genexpression und DNA Methylierungsprofile aus peripherem Blut eine spezifische molekulare Antwort auf Kindesmissbrauch und Kindesmisshandlung und stützen damit die Hypothese, dass Traumatisierungen im Kindesalter zu einer von Traumatisierung im Erwachsenenalter unterscheidbaren molekularen Antwort führen. Die vorliegende Arbeit zeigt damit erstmals einen Mechanismus für eine Gen-Umweltinteraktion in der Psychiatrie und führt Genetik und Umweltfaktoren als bestimmende Risikofaktoren in einem molekularen Modell zusammen. Darüber hinaus stützen die präsentierten Daten die These, dass Traumatisierung im Kindesalter im Gegensatz zu Traumata im Erwachsenalter zu unterscheidbaren molekularen Veränderungen führt, die einerseits eine individualisierte Diagnostik zulassen und auf der anderen Seite therapeutische Möglichkeiten eröffnen die auf die individuelle Krankheitsgeschichte Rücksicht nehmen und so die Erfolgswahrscheinlichkeit einer Therapie erhöhen.

Aims of the thesis

The first goal of this thesis is to investigate the molecular mechanisms underlying the statistical gene by environment interaction (GxE) of *FKBP5* with childhood abuse on the risk to develop psychiatric disorders, in particular post-traumatic stress disorder (PTSD) in adulthood. Therefore, the molecular function of the single nucleotide polymorphism (SNP) rs1360780, an intronic variant that has been described in multiple GxE and gene association studies, will be investigated. In addition, this thesis will investigate the molecular response to childhood abuse in humans on the level of DNA methylation as one major epigenetic factor and if this response is genotype dependent. This thesis will also test the hypothesis that peripheral tissue such as whole blood may be informative for the detection of environmentally induced epigenetic alterations and mechanisms which may also overlap with alterations in neuronal tissue, potentially contributing to psychiatric disorders. The second part of this thesis will investigate the genome wide gene expression and DNA methylation profile of childhood abuse in humans. Therefore, adult-trauma exposed, PTSD positive individuals with and without the history of childhood abuse will be compared to adult-trauma exposed PTSD negative individuals and differences in the gene expression and DNA methylation profile are presented.

Introduction

Stress-Related Psychiatric Disorders – Major Depression and Post-Traumatic Stress Disorder as Examples

Psychiatric disorders such as major depression (MDD) or post-traumatic stress disorder (PTSD) are highly prevalent in the general population with an estimated lifetime prevalence for MDD of 17.1% (12.7% for males and 21.3% for females) [1] and a lifetime prevalence for PTSD of 7.8% (5.0% for males and 10.4% for females) [2] with considerable variations in prevalence depending on geographic location, ethnicity, gender, socioeconomic status and other factors. For MDD, the burden of disease as measured by disability adjusted life years (DALYs) was estimated to account for 2.5% of the global DALYs and therefore identified as the leading cause of burden worldwide [3].

Despite considerable efforts to delineate the underlying pathomechanisms during the last decades, our understanding of the molecular mechanisms underlying multifactorial and complex disorders such as schizophrenia, MDD and PTSD remain elusive and currently available treatment options are often ineffective leading to prolonged treatment and often chronic illness.

Pathophysiology – Genetics

Twin, family and more recently genetic association studies support the notion that genetic factors contribute to the risk of developing psychiatric disorders. In contrast to monogenic diseases, psychiatric disorders are predominantly complex disorders most likely with multiple interacting genetic variations contributing to the phenotype. The heritability for MDD, i.e. the proportion of variance in MDD risk attributed to genetic factors, has been estimated to be 37% and between 30% and

40% for PTSD indicating a moderate contribution of genetic factors to the disease [4-6]. Numerous candidate gene association studies have been performed in the past decades indicating associations between specific genetic variation, most often represented by common single nucleotide polymorphisms (SNPs), and disease phenotypes. Although these studies helped gain more insight into the genetic liability to disease risk, many candidate association studies failed replication or showed only minimal contribution to disease risk. More recently, genome wide association studies (GWAS) have been performed for several psychiatric disorders. These studies represent an unbiased approach towards identification of genetic variants associated with specific traits on a genome wide level but require large sample sizes to comply with statistical testing needs especially in the field of psychiatry where effect sizes are small. To date, the largest GWAS performed collected genetic data of about 40,000 schizophrenia cases and over 113,000 controls yielding over 100 distinct genetic loci potentially contributing to the risk to develop schizophrenia (Figure 1) [7].

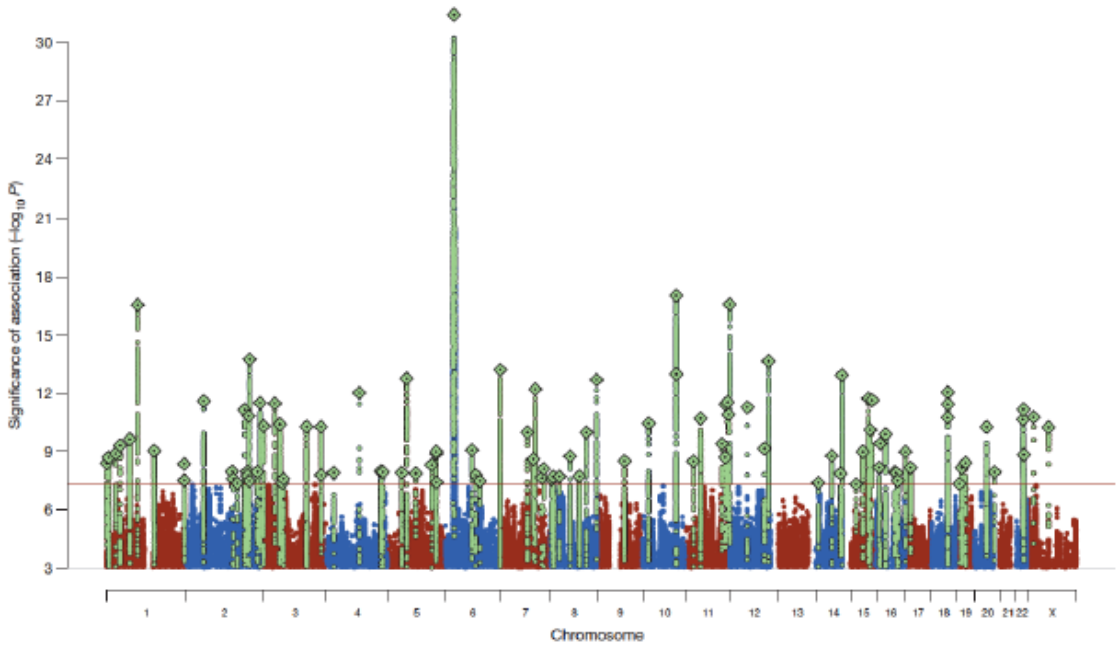


Figure 1. Manhattan plot of the most recent GWAS on schizophrenia. The red line represents the statistical threshold of $p=5\times10^{-8}$ for genome wide significance. In total 108 loci were found to be associated with schizophrenia suggesting genes i.e. involved in immune function but also unrelated

to previous findings to influence the genetic disposition to develop schizophrenia. Figure taken from Schizophrenia Working Group of the Psychiatric Genomics Consortium, Nature, 2014 [7].

This most successful study so far supports the ability to detect genetic variants that contribute –even marginally- to the risk for disease by increasing the overall N in psychiatric GWAS. In contrast, GWAS on MDD or PTSD have so far not yielded robust genome-wide significant results [8-14]. The most current GWAS on MDD comprises about 10,000 cases and 10,000 controls but failed to detect a genome wide significant signal at a sample size at which previous schizophrenia GWAS already detected 5 significant signals (Figure 2).

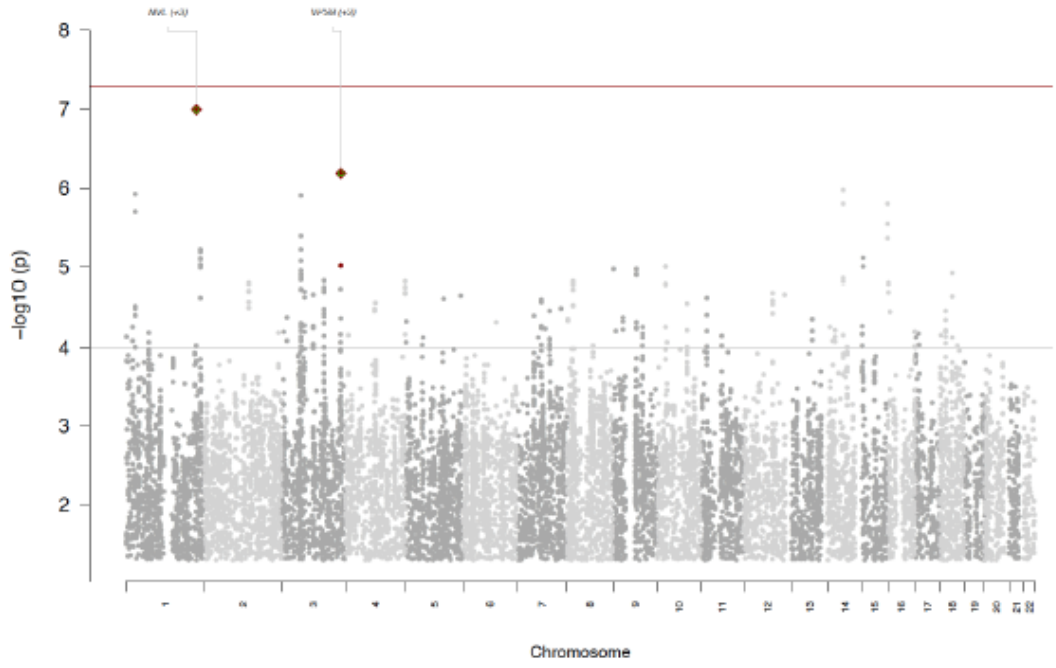


Figure 2. Manhattan plot similar to Figure 1 representing genome wide significant association of genetic variants with MDD. No SNP reached genome wide significance investigating 9240 cases vs. 9519 controls. Figure taken from Major Depressive Disorder Working Group of the Psychiatric GWAS, Mol. Psychiatry, 2012 [8].

For PTSD, the current GWAS' performed were considerably limited in their sample size and also failed to detect a robust genome wide significant signal [12-14]. For both entities, MDD and PTSD, large collaborative efforts led by the Psychiatric Genomics

Consortium are underway to collect more data leading to larger overall sample sizes [8].

Although the schizophrenia studies showed that increasing the overall sample size is a justified strategy, disorders with lower genetic contribution or higher genetic heterogeneity such as MDD and PTSD might require samples sizes that are unfeasible to achieve. Moreover, although worldwide collaborative efforts can collect an enormous amount of genetic and phenotypic data, an independent replication of such huge efforts is difficult to achieve. In addition, MDD and PTSD exhibit in contrast to schizophrenia not only a stronger genetic heterogeneity but also a much stronger phenotypic heterogeneity with potentially very distinct disorders combined under the clinical terms MDD and PTSD thus making the identification of robust genetic signals challenging. Finally, in contrast to schizophrenia, environmental factors contributing to the overall risk for disease are thought to be more pronounced in MDD and PTSD [15-20].

Pathophysiology – Environmental factors

The strong influence of negative environmental factors on the development of MDD has been shown for childhood abuse and stress exposure through so-called life events, highlighting the profound influence of the environment on disease risk [21-23]. Moreover, PTSD is regarded as the prototype disorder with respect to environmental impact illustrating the influence of the environment through the exposure to a qualifying trauma being necessary to trigger the disease [15]. Moreover, numerous studies employing animal models showed the profound effect of environmental stressors in particular in early life on behavioral and molecular phenotypes [24,25]. Nevertheless while most humans are exposed to stressful or even traumatic life events during their life time, only a minority of the affected individuals develop any disease. This leads to the concept of gene by environment interaction

(GxE) combining the effects of genetic predisposition and environmental factors [26]. In fact, GxE refers to the differential effect of a given environment on a phenotype depending on the individual's genotype. Although current research often focuses on negative life events, trauma and severe stress, the presence of positive environmental conditions such a supportive social network or an adequate socioeconomic status also influences mental health status. Thus, by definition, GxE studies should take positive environments into account potentially counterbalancing the impact of trauma exposure [27]. Finally, the exposure to stress does not form a linear relationship with negative mental health outcome but might also increase resilience depending on timing, duration and intensity of the stressor and subsequent environmental conditions [28].

Gene by Environment Interactions (GxE)

Although the first theoretical and experimental principles of GxE have been described since the early last century, groundbreaking studies by Avshalom Caspi and Terrie Moffitt first combined the influence of the environment and the influence of genetic factors towards stress-related disorders in a statistical framework of GxE. They described the interactive effects of genetic predisposition at the monoamine oxidase A (*MAO-A*) locus and the serotonin transporter locus (*SLC6A4*) with childhood adversities in human towards the risk to develop antisocial behavior and depression [29,30]. Thus, for the first time the authors showed that the influence of the environment represented by childhood maltreatment is not uniform towards the development of psychiatric disorders but moderated by the genetic disposition with so called risk alleles leading to a higher risk of disease compared to so called protective alleles associated with no or a reduced risk in response to maltreatment exposure. These results triggered subsequent studies showing that environmental factors differentially influence GxE depending on the type of environmental factor, moderating factors such as social context, timing of the trauma or environmental

factor, severity and how they are measured. Further studies investigated other candidate loci from the monoamine transmitter system, neurotropic factors and the hypothalamus-pituitary adrenal axis that were associated with stress-related phenotypes [31]. No genome-wide GxE studies have been published so far.

Although these studies highlighted the importance of the interaction of environment and genetic predispositions, criticism has been raised because of low sample sizes leading to a lack of power, tendency to yield false positive results and low replication rates [26,31-33]. In addition, the statistical framework of GxE studies has been challenged because of the lack of proper control over confounding factors such as gender, ethnicity and age [34]. A critical factor is the measurement of the environment varying from prospective third party records such as governmental records to retrospective self-reports by affected individuals and from very distinct environmental factors such as childhood sexual abuse to broader inventories such as stressful life events. In addition, the environment is a complex, multidimensional space with many environmental factors mutually influencing each other and specific GxEs. The GxE of the 5-HTTLPR and the exposure to a natural disaster on the risk to develop PTSD highlights this notion as the interaction was observable only in hurricane exposed individuals living in areas with a low socioeconomic status compared to exposed individuals living in a more advantageous context [35,36].

Besides the conceptual and statistical considerations mentioned above, knowledge about the molecular underpinnings of the statistical findings might provide a better understanding of GxE and of the pathophysiology of mental disorders.

FKBP5 influences the regulation of the hypothalamus-pituitary-adrenal axis

A prominent example of subsequent GxE studies is the interaction of the *FKBP5* (FK506 binding protein 5) gene with childhood abuse on the risk for PTSD,

depression, suicidal behavior and aggression [37-42]. In humans, FKBP5 functions as a regulator of the glucocorticoid receptor (GR) and the hypothalamus-pituitary-adrenal (HPA) axis. FKBP5 is involved in the regulation of this central stress hormone axis by negative feedback loops, which are critical for cortisol secretion. The dysregulation of the HPA axis and its feedback loops has been implicated in the pathophysiology of many stress-related psychiatric disorders [43]. First shown by Binder et al., childhood abuse exposure lead to higher PTSD symptom levels in adulthood in individuals carrying a risk haplotype of *FKBP5* including the SNPs rs3800373, rs9296158, rs9470080 and rs1360780 compared to individuals carrying the protective haplotype [37]. Of note, although this interaction appeared robust, no interaction of any SNP in *FKBP5* with adult trauma on adult psychopathology was observed (Figure 3). This GxE was later replicated [38] and further studies suggested that *FKBP5* SNPs interact with childhood abuse not only on PTSD but also on the risk to develop MDD, aggressive behavior, suicidal ideation, general health and intermediate phenotypes such as amygdala reactivity [44].

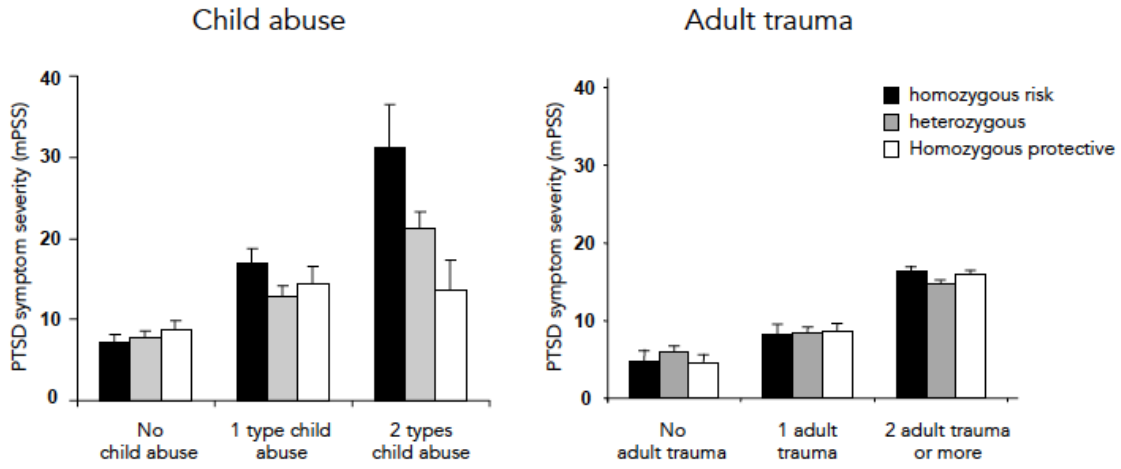


Figure 3. Interaction of *FKBP5* with exposure to childhood abuse on risk for PTSD in adulthood. The left panel shows that increasing exposure to severe childhood abuse lead to an increasing severity of PTSD symptoms in childhood. This relationship is moderated by *FKBP5* with the "risk" allele leading to higher symptom levels in adulthood compared to the "protective" allele. In contrast, the right panel shows the relationship of exposure to adult trauma and PTSD symptom severity, which is not moderated by genetic variation in *FKBP5*. Figure adapted from Binder et al., 2008 [37].

FKBP5 as a molecular hub for gene by environment interactions

FKBP51 (gene name *FKBP5*, located on the short arm of chromosome 6) was discovered in 1985 as part of the steroid hormone receptor complex [45]. The name FKBP5 or FKBP51 is derived from the ability of FKBP51 to bind FK506 (Tacrolimus), an immunosuppressive drug that inhibits the peptidylprolylisomerase (PPI) domain [46]. Apart from the PPI domain, FKBP5 also contains a binding domain (TPR – tetratricopeptide repeat protein domain) for Heat shock protein 90 (Hsp90), a chaperone protein [47]. Studies in Squirrel monkeys showed that although these animals exhibited high levels of cortisol, they did not develop signs of hypercortisolaemia as expected [48]. The underlying mechanism for this discrepancy was identified later showing that the glucocorticoid receptor (GR) of squirrel monkeys has a low affinity for cortisol without exhibiting causative sequence differences [49]. Moreover, subsequent studies showed that squirrel monkeys have high levels of FKBP51 that were shown to result in inhibitory effects on GR function leading to GR resistance [50-53]. FKBP51 reduces the affinity of the GR to the activating hormone cortisol thereby reducing the translocation of the GR to the nucleus and inhibiting the transcriptional activation and repression of GR dependent genes [53]. It has been speculated that the inhibitory effect of FKBP51 is mediated by replacing other proteins from the GR-complex such as the homologue FKBP52 that interacts with the dynein/dynactin motor complex and facilitates the translocation of the GR into the nucleus [53-56] (Figure 4).

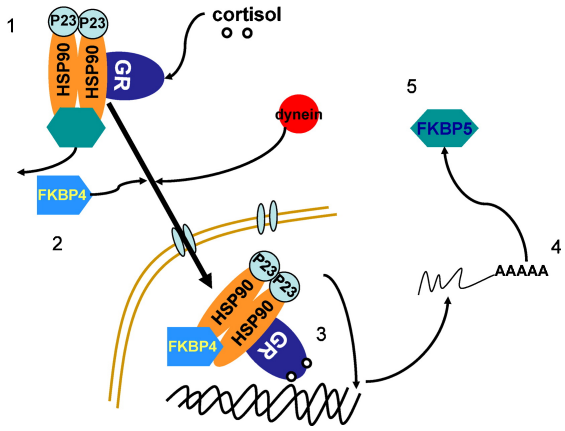


Figure 4. FKBP51 interaction with the GR complex. *FKBP5* is transcriptionally activated by the GR through binding to predominantly intronic GREs leading to an increased FKBP51 protein translation. FKBP51 then provides a negative feedback to the GR through binding via hsp90 thus lowering the affinity of cortisol to the GR. Cortisol binding to the GR leads to an exchange of FKBP51 with FKBP52 (transcribed from the *FKBP4* gene) allowing the translocation of the GR to the nucleus. Figure taken from Binder, PNEC, 2008 [57].

FKBP51 as part of the negative feedback loop limiting the sensitivity of the GR to cortisol is transcriptionally activated by the GR itself through binding of the GR to several functional glucocorticoid response elements (GREs) located in the promoter region but also in downstream intronic regions that might form a three-dimensional loop structure to establish contact between the downstream enhancer elements and the promoter region [58,59]. Dynamic *FKBP5* expression is present in many tissues including peripheral tissues such as whole blood and saliva but is also present in the human central nervous system and strong expression is observed at baseline in the rodent hippocampus as well as after transcriptional induction in the amygdala and the paraventricular nucleus [60-64].

The ability of the GR to transcriptionally activate the *FKBP5* gene and the ability of FKBP5 as a protein to regulate GR function suggested that mRNA/protein expression levels could be used as a marker for GR sensitivity. Although this consideration is valid, high FKBP5 levels could in fact represent a highly active GR (thereby activating *FKBP5*) or a less active GR (through inhibition by FKBP5), a problem that can be solved by analyzing stimulated expression data, for example before and after GR activation by a synthetic agonist representing a sensitive readout of the GR status [61,65].

Based on the role of *FKBP5* in regulating the HPA axis, an early candidate gene association study of *FKBP5* on antidepressant treatment response in depressed individuals showed *FKBP5* SNPs were associated with HPA axis responsiveness as

well as to antidepressant treatment response [66]. Additional studies linked 'risk' *FKBP5* polymorphisms with the delayed recovery from psychosocial stress in healthy individuals [67], increased salivary cortisol release in infants in response to mild stress [68], non-suppression of the stress hormone axis in response to dexamethasone administration [37] and activation of stress-related brain regions [69]. In clinical cohorts, the association of 'risk' haplotypes or polymorphisms is less straightforward with risk allele carriers showing GR resistance through dexamethasone suppression and a reduced *FKBP5* mRNA induction in MDD [61,70] but enhanced GR sensitivity in PTSD [37,71].

The SNPs previously implicated in gene association and GxE studies mentioned above are in high linkage disequilibrium and distributed across the locus from the promoter area to the 3'UTR in Caucasians and intron 1 to the 3'UTR in Africans thus making the identification of a causal variant difficult.

Epigenetic Modifications and Gene Regulation

Epigenetics or epigenetic modifications subsume molecular modifications and mechanisms of chromatin organization and gene regulation that act without changing the underlying DNA sequence. In a strict definition, these changes are stably inherited through either mitosis and/or meiosis, although many current examples of epigenetic mechanisms do not fulfill this requirement. This has lead to a broad definition of epigenetics that includes mechanisms of gene regulation without alterations of the genetic code. Epigenetics initially focused on the question of how diverse cellular phenotypes can arise from a singular genotype blueprint, i.e. how cell lineage identity is achieved and maintained by non-genetic factors and only more recently has the term epigenetics been adopted to describe non-genetic mechanisms influencing a phenotype.

Epigenetic mechanisms include the post-translational modification of histone proteins, modifications of single nucleotides most commonly in form of DNA methylation or hydroxymethylation at cytosine residues altering the chromatin structure and the accessibility of the DNA to transcriptional regulators and the regulation of transcription and translation by non-coding RNAs. For a comprehensive description of these epigenetic mechanisms, see [72-78]. Here, I only briefly summarize the main features of these modifications.

Histone-modification: The DNA strand needs to be highly condensed into chromosomes, which is facilitated by wrapping the DNA around core histone proteins forming the nucleosome and thereby allowing the DNA to fit into the nucleus. To regulate the accessibility of important functional regions of the DNA strand to transcriptional regulators, histone proteins can comprise a plethora of modifications predominantly at the N-terminal tails that alter the spatial structure of the chromatin and the interaction of DNA binding proteins leading to an increase or decrease of transcription.

DNA methylation and related chemical modifications: DNA methylation is the addition of a methyl group to nucleotide residues, where cytosine is the most common. These modifications influence how proteins bind to the DNA leading to increased or decreased transcription depending on their genomic location. More recent work shows that there are modifications beyond the classical 5-methyl cytosine modification with 5-hydroxymethyl cytosine as potentially important modification in neuronal tissue [79]. The major determinant of DNA methylation patterns is the nucleotide sequences itself i.e. though the distribution of GC content and CpG density but also repetitive elements and transcription factor binding sites. Additional evidence has been shown for a genetic determination of DNA methylation pattern by DNA sequence and sequence variants [80].

Non-coding RNAs: Aside from protein coding RNAs, non-coding RNAs play a major role in the regulation of gene transcription [81,82]. Non-coding RNAs include i.e.

micro RNAs (miRNAs), piwi-interacting RNAs (piRNAs) or long non-coding RNAs (lncRNAs) that can interfere with transcriptional regulation and translation on multiple levels.

For a long time, epigenetic modifications, especially DNA methylation, have been considered irreversible defining cellular identity in a multicellular organism. By now it has been shown that even stable chemical modifications such as DNA methylation can show a highly dynamic regulation with the potential of reversibility making these mechanisms suitable for encoding the long-term impact of the environment also in post-mitotic tissue such as neurons. Although depicted here separately for clarity, epigenetic mechanisms form a complex interactive network with joint activities of different mechanisms contributing to a molecular outcome [83]. The epigenetic state comprising all epigenetic mechanisms within the cell is called the epigenome and varies from cell to cell. Epigenetic mechanisms and epigenetic modifications are associated with a broad range of medical conditions spanning all fields of medicine from cancer, metabolic disorders to neurological and psychiatric disorders. Due to the availability of cost-effective methods that allow the high-throughput investigation of methylation marks, DNA methylation became the best-investigated epigenetic mark in psychiatry especially in human cohorts. Therefore I will focus on DNA methylation in the next chapters not excluding the contribution of other epigenetic mechanisms towards stress-related psychiatric disorders.

Environmental Epigenetics - Focus on Stress and Behavior

Epigenetic mechanisms as described above shape genomic architecture and function inside the nucleus and are considered as a molecular bridge between the genome and the surrounding environment. Thus, epigenetics are thought to be able to facilitate the interaction and adaptation of the organism to varying environmental conditions. This concept raised great interest in many biomedical fields including

psychiatry and neuroscience [84-87]. Moreover, it has been shown that the many epigenetic marks remain plastic i.e. changeable in post-mitotic neuronal tissue throughout the lifespan and that epigenetic drugs such as HDAC inhibitors exert behavioral effects in animal models fueling the hope of epigenetic drugs potentially influencing psychiatric or neurological disorders [88-90].

Epigenetic studies, in particular with regard to stress-related disorders, have focused on the HPA axis as the most important stress response system in mammals and the first studies in animal models support the hypothesis of a differential influence of the environment on the epigenome especially in HPA related genes. In addition, most of the studies focused on DNA methylation, in part due to the availability of cost effective and readily available analytical methods. One of the most influential examples is the nurturing behavior in rats influencing the DNA methylation at the glucocorticoid receptor (*Nr3c1*) and thus interfering with the stress response of the offspring [91]. Here, the quality of maternal care of the rat dam altered the histone acetylation and methylation pattern of the *Nr3c1* 1_7 promoter region in the hippocampus of the offspring. Decreased levels of maternal care as expressed by reduced licking and grooming led to increased methylation at the NGFI-A binding site of the *Nr3c1* promoter and decreased GR mRNA expression compared to offspring from a high-licking and grooming mother providing good maternal care. Offspring of low licking and grooming animals subsequently showed an altered behavioral but also endocrine response to stress in adulthood. Another example is the effect of maternal separation (a form of early life stress in rodents models) on DNA methylation and gene expression of the mouse *Avp* gene [92]. Here, separation of the offspring from the mother animal at post-natal day 1-10 induced an increase in *Avp* mRNA expression and in turn a long-term decrease in DNA methylation at the *Avp* gene enhancer region in the offspring paraventricular nucleus potentially contributing to the increased secretion of corticosterone in adulthood and impaired behavioral response to stress after early life stress. In line with the studies on the GR

and *Avp*, Elliott et al. used a social defeat paradigm to investigate the effect of adult stress on DNA methylation of the corticotrophin releasing hormone gene (*Crh*) in mice and found demethylation of the *Crh* promoter region in stress-susceptible compared to resilient animals [93]. Finally, *Fkbp5* has also been investigated with respect to epigenetic modifications. Although no direct evidence for a stress-dependent epigenetic regulation of *Fkbp5* in rodent models has been observed, in-vivo GR activation by corticosterone is sufficient to induce an intermediate demethylation in *Fkbp5* suggesting that *Fkbp5* methylation may be an indicator for corticosterone release and thus measure of stress exposure in mice [65,94]. The mechanism by which GR activation can lead to a local demethylation in and around GREs remains elusive although GR induced demethylation has been described before and DNA strand break repair mechanisms might be involved in this process [95,96]. Although no direct evidence exist, active demethylation at GREs can also involve intermediate steps such as hydroxymethylation, a form of DNA methylation that has been described to be an intermediate step of DNA demethylation by other transcription factors [97,98].

Parallel to studies in animal models, first investigations in monozygotic (i.e. genetically identical) twins showed that even at birth detectable gene expression differences exist, potentially linking the intrauterine environment to epigenetic factors influencing gene transcription [99]. Further studies on monozygotic twins also showed that even while no epigenetic differences at birth were detectable, monozygotic twins develop divergent DNA methylation and histone acetylation profiles over their lifetime suggesting a differential influence of the environment [100]. In line with these observations, more recent studies have stratified monozygotic twins according to the presence of psychiatric traits or disorders in one twin and compared the epigenetic profile between twin pairs suggesting varying epigenetic pattern according to disease state [101-104]. Nevertheless, a stochastic drift in DNA methylation and other factors such as allelic expression contribute to the differences

observed as well. Other examples supporting the influence of the environment on the epigenome in humans are epidemiological studies on individuals exposed to severe food restriction [105-107]. Here, periconceptional caloric restriction during the so-called Dutch hunger winter during World War II was associated with DNA methylation differences at the *IGF2* locus [106] but also on a global scale leading to metabolic differences in exposed vs. unexposed individuals in later life potentially influencing the risk for subsequent disease [105].

Similar to the studies by Weaver et al on the rat *Nr3c1* 1_7 promoter methylation in response to maternal care [91], McGowan et al showed that childhood abuse in humans is associated with increased DNA methylation at the human 1_F GR promoter site (the orthologue of the rat 1_7 promoter) in postmortem hippocampal tissue [108]. The overall expression of the glucocorticoid receptor was lower in individuals with the history of childhood abuse compared to non-abused individuals. Suderman et al. expanded these focused studies and compared the DNA methylation profile of 6.5 million nucleotides around the *NR3C1* locus in human samples of individuals exposed to childhood abuse and controls with samples of the rat maternal care paradigm [109]. The gene promoter methylation levels were comparable between the two species suggesting a similar cross-species response to early life stress and the overall DNA methylation changes included both hypomethylated regions as well as hypermethylated regions suggesting a differential and region specific response. Moreover, the human differential methylated regions were enriched in promoter as well as last exon regions containing regulatory elements linked to gene expression regulation. Interestingly, the most prominent differences were not observed at the *NR3C1* locus but at the protocadherin gene locus with increased DNA methylation in abused human samples paralleled by an increased methylation in low maternal care samples in rat.

Besides the prominent example of glucocorticoid receptor DNA methylation with regard to maternal behavior as well as childhood abuse, numerous studies have used predominantly case-control designs to investigate DNA methylation differences in human cohorts. Here, neuronal tissue is largely unavailable in large cross-sectional studies. Therefore peripheral tissue such as whole blood, FACS sorted PBMCs; spit or buccal cells are leveraged to determine an overall methylation pattern between case and control subjects. Because of the strong influence of stress in early childhood, most studies investigate the effect of childhood trauma and early stressful life events. Other studies investigated the epigenetic signature of psychiatric phenotypes such as MDD or PTSD compared to age- and sex-matched controls. The initial studies based on hypothesis driven candidate genes have been recently replaced and extended by genome wide approaches using array technology or next generation sequencing. Examples for these studies are summarized in Table 1.

Study	Demographic details	Environmental exposure or phenotype	Outcome phenotype	Tissue investigated	Gene(s) analyzed	Findings
McGowan et al. 2009[108]	N=36, males only, N=12 for abuse+suicide, N=12 for non-abused+suicide, N=12 controls	Childhood Experience of Care and Abuse Questionnaire	Suicide	post-mortem hippocampal brain tissue	Promoter region 1F of NR3C1 by bisulfite mapping	Increased Methylation of 1F Promoter, leading to decreased NGFI-A binding and reduced hippocampal glucocorticoid receptor (NR3C1) expression in suicide victims with childhood abuse
Alt et al., 2010[110]	N=12, N=6 MDD, N=6 controls, male=7, female=5		MDD	Samples of different brain tissues	Several GR promoter regions including 1F by pyrosequencing	No differences in GR promoter methylation observed although expression of GR isoforms and NGFI-A transcription factor was altered in MDD vs. controls
Uddin et al., 2010[111]	N=100, N=23 for PTSD+, N=77 for PTSD-, male=40, female=60	Different trauma types from a list of 19 diverse events	PTSD	Peripheral blood	14495 genes by Illumina 27k BeadChip	Results were stratified according to uniquely methylated/unmethylated gene in each group. The number of uniquely methylated genes was significantly higher in PTSD affected patients with an enrichment of immune related genes
Smith et al., 2011[112]	N=110, N=25 for PTSD+childhood trauma, N=26 for controls+childhood trauma, N=34 for controls with no childhood trauma	Childhood Trauma Questionaire (CTQ)	PTSD	Peripheral blood	Global and site-specific methylation by Illumina 27k BeadChip	Increased global methylation in subjects with PTSD. CpG sites in five genes (TPR, CLEC9A, APC5, ANXA2, and TLR8) were differentially methylated in subjects with PTSD compared to controls. Enrichment of immune related genes.
Uddin et al., 2011[113]	N=100, N=33 for MDD, N=67 for controls		Depression	Peripheral blood	14495 genes by Illumina 27k BeadChip	Similar to Uddin et al., 2010, individuals were stratified according to disease status. Individuals with depression showed less uniquely methylated and unmethylated genes. An enrichment of inflammatory genes was detected.

Fuchikami et al., 2011[114]	N=38, N=20 for MDD, N=18 for controls		MDD	Peripheral blood	BDNF by Sequenom EpiTYPER	Patients with MDD and healthy controls showed significantly different methylation profile at the exon I of BDNF (hyper- and hypomethylation) suggesting a disease specific methylation profile
Rusiecki et al., 2012[115]	N=150 US soldiers, N=75 post deployment PTSD, N=74 no PTSD, males=100, females=50	Afghanistan war, Deployment as a proxy for trauma exposure	PTSD	Peripheral blood	LINE-1 and Alu by pyrosequencing	LINE-1: hypermethylated in controls post- versus pre-deployment and hypomethylated in cases versus controls postdeployment, Alu: hypermethylated for cases versus controls predeployment, genomic location of individual retrotransposons not determined
Sabunciyan et al., 2012[116]	N=65, N=39 for MDD, N=26 for controls		MDD	Postmortem frontal cortex brain tissue	Genome-wide DNA methylation by CHARM	224 candidate regions with DNA methylation differences >10%. These regions are highly enriched for neuronal growth and development genes
Alasaari et al., 2012[117]	N=49, N=24 with high stress levels, N=25 with low stress levels		Burnout/ Depression	Peripheral blood	SLC6A4 by bisulfite sanger sequencing	Nurses with stress had significantly lower promoter methylation levels at all five CpG residues compared to nurses with low stress. No association of Burnout/Depression with methylation levels
Labonte et al., 2012[118]	N=61, N=61 for suicide+abuse, N=20 for suicide-abuse, N=16 for controls, all males	CECA.Q Childhood Experience of Care and Abuse Questionnaire	Suicide	Hippo-campal samples	Genome-wide DNA methylation by MeDIP-chip	362 differentially methylated promoters in individuals with a history of abuse compared with controls. Genes involved in cellular/neuronal plasticity were significantly enriched
Yang et al., 2013[119]	N=192, N=96 for abuse, N=96 for	Childhood trauma		Saliva	Genome-wide DNA	Significantly different methylation at 2868 CpG

	controls, 42% males				methylation by Illumina 450k Array	sites in numerous genes related to health problems between abused vs control individuals
Melas et al., 2013[120]	N=1668, N=392 for MDD, N=1276 for controls, males=675, females=993		Depression	Saliva	MAOA and NR3C1 by Sequenom EpiTYPER	MAOA methylation levels were decreased in depressed females compared to controls
Kang et al., 2013[121]	N=108		MDD	Peripheral blood	SLC6A4 by pyrosequencing	Higher SLC6A4 promoter methylation significantly associated with childhood adversities, family history of depression, higher perceived stress, and more severe psychopathology
Zhao et al., 2013[122]	n=168, Caucasian, 84 twim pairs discordant for MDD	MDD	Depressive symptoms	Peripheral blood	SLC6A4 by pyrosequencing	Variation in DNA methylation level of the serotonin transporter gene promoter region is associated with variation in depressive symptoms with increasd methylation in response to increased depression symptoms

Table 1. Examples for candidate gene and genome-wide studies on DNA methylation on stress-related psychiatric disorders and early life trauma in humans. Table taken and adapted from Klengel et al., Neuropharmacology, 2014[123].

A major limitation of these representative examples is that none of these studies account for the genetic heterogeneity present in human cohorts, a confounder that has been already shown to influence the DNA methylation pattern [124,125]. In addition, the studies highlighted above are predominantly descriptive and allow only minimal insight into the molecular mechanisms of environmentally induced epigenetic changes with regard to the underlying pathomechanisms of psychiatric disorders.

The first paper in the following chapter investigates the molecular mechanism of the GxE of *FKBP5* and childhood abuse and provides evidence for the functional SNP mediating the genetic effect of this GxE. This paper shows for the first time that a common SNP can influence the three-dimensional structure of the *FKBP5* locus leading to an enhanced transcriptional response to stress. In addition, this paper provides evidence for genotype-dependent epigenetic modifications in response to environmental factors.

The second paper investigates the gene expression signature as well as the corresponding DNA methylation profile on a genome wide level in individuals suffering from PTSD exposed to adult and childhood trauma versus individuals exposed to adult trauma only. This paper provides evidence for distinct epigenetic and transcriptional signatures in response to childhood abuse compared to adult trauma suggesting divergent molecular mechanisms involved in the pathogenesis of PTSD depending on the type and timing of the disease-causing traumatic event.

Paper I

Klengel T, Mehta D, Anacker C, Rex-Haffner M, Pruessner JC, Pariante CM, Pace TWW, Mercer KB, Mayberg HS, Bradley B, Nemeroff CB, Holsboer F, Heim CM, Ressler KJ, Rein T, Binder EB. Allele–specific DNA *FKBP5* demethylation*:* a molecular mediator of gene–childhood trauma interactions. Nature Neuroscience. 16(1), 33-41, 2013 doi:10.1038/nn.3275.

Allele-specific *FKBP5* DNA demethylation mediates gene–childhood trauma interactions

Torsten Klengel[1], Divya Mehta[1], Christoph Anacker[2], Monika Rex-Haffner[1], Jens C Pruessner[3], Carmine M Pariante[2], Thaddeus W W Pace[4], Kristina B Mercer[5], Helen S Mayberg[4], Bekh Bradley[4,6], Charles B Nemeroff[7], Florian Holsboer[1], Christine M Heim[4,8], Kerry J Ressler[4,5,9], Theo Rein[1] & Elisabeth B Binder[1,4]

Although the fact that genetic predisposition and environmental exposures interact to shape development and function of the human brain and, ultimately, the risk of psychiatric disorders has drawn wide interest, the corresponding molecular mechanisms have not yet been elucidated. We found that a functional polymorphism altering chromatin interaction between the transcription start site and long-range enhancers in the FK506 binding protein 5 (*FKBP5*) gene, an important regulator of the stress hormone system, increased the risk of developing stress-related psychiatric disorders in adulthood by allele-specific, childhood trauma–dependent DNA demethylation in functional glucocorticoid response elements of *FKBP5*. This demethylation was linked to increased stress-dependent gene transcription followed by a long-term dysregulation of the stress hormone system and a global effect on the function of immune cells and brain areas associated with stress regulation. This identification of molecular mechanisms of genotype-directed long-term environmental reactivity will be useful for designing more effective treatment strategies for stress-related disorders.

© 2013 Nature America, Inc. All rights reserved.

Epidemiological, family and molecular genetic studies have shown that genetic predisposition as well as stressful or traumatic life events, especially in childhood, are important risk factors for psychiatric disorders, including major depression and post-traumatic stress disorder (PTSD) and that these factors most likely have interactive, rather than additive, effects[1]. Although specific gene × environment interactions have been described[2–5], the molecular basis of gene × environment interaction in mood and anxiety disorders remains obscure. *FKBP5* is an important functional regulator of the glucocorticoid receptor complex[6]. The glucocorticoid receptor is a pivotal nuclear receptor of the stress hormone system mediating the negative feedback of this axis to terminate the stress response after the end of a threat[7]. Dysregulation in this system has been described in stress-related psychiatric disorders and as a long-term consequence of exposure to early life trauma[8,9]. FKBP5 alters glucocorticoid receptor function by decreasing ligand binding and impeding translocation of the receptor complex to the nucleus[10,11]. Furthermore, FKBP5 is part of an intracellular ultra-short negative feedback loop that regulates glucocorticoid receptor activity. Glucocorticoid receptor activation induces *FKBP5* transcription via activation at predominantly intronic steroid hormone response elements[12], leading to increased transcription of *FKBP5*, entailing restrained glucocorticoid receptor activity. We and others have shown that polymorphisms in *FKBP5* (haplotypes including rs1360780, rs9296158, rs3800373 and rs9470080) interact with early trauma or childhood abuse to predict adult PTSD, suicide attempts and major depression[3,13–17]. Here we identified a molecular mechanism for this gene × environment interaction by long-term epigenetic modifications.

RESULTS

rs1360780 moderates the risk for PTSD after early trauma

Previously, we found that the same *FKBP5* polymorphisms that interact with early trauma are also associated with altered induction of *FKBP5* mRNA by glucocorticoid receptor stimulation in peripheral blood[18]. We hypothesized that the associated functional variant lies in or close to glucocorticoid response elements (GREs) in the *FKBP5* locus. Given that the originally genotyped variants are in high linkage disequilibrium over the entire locus, we used genotype data from Illumina OmniExpress Single Nucleotide Polymorphism (SNP) arrays spanning the whole *FKBP5* locus in 192 individuals of the Grady trauma project and imputed all currently known variants using the 1,000 Genomes project data (http://www.1000genomes.org/)[19]. This resulted in 799 polymorphisms imputed with a quality greater than 0.6 and an average quality score of 0.93. Linkage disequilibrium with rs1360780, rs9296158, rs9470080 and rs3800373 was evaluated using the tagger software implemented in Haploview version 4.2. Of the imputed polymorphisms, 48 had an r^2 greater than 0.4 with at least one of the four polymorphisms mentioned above to interact

[1]Max Planck Institute of Psychiatry, Munich, Germany. [2]King's College London, Institute of Psychiatry, Section of Perinatal Psychiatry and Stress, Psychiatry and Immunology, Department of Psychological Medicine, London, UK. [3]McGill Centre for Studies in Aging, Faculty of Medicine, McGill University, Verdun, Quebec, Canada. [4]Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, Georgia, USA. [5]Howard Hughes Medical Institute, Chevy Chase, Maryland, USA. [6]Atlanta Veterans Affairs Medical Center, Decatur, Georgia, USA. [7]Department of Psychiatry and Behavioral Sciences, University of Miami Miller School of Medicine, Miami, Florida, USA. [8]Institute of Medical Psychology, Charité University Medicine Berlin, Berlin, Germany. [9]Yerkes National Primate Research Center, Emory University, Atlanta, Georgia, USA. Correspondence should be addressed to E.B.B. (binder@mpipsykl.mpg.de) or T.K. (klengel@mpipsykl.mpg.de).

Received 15 June; accepted 1 November; published online 2 December 2012; doi:10.1038/nn.3275

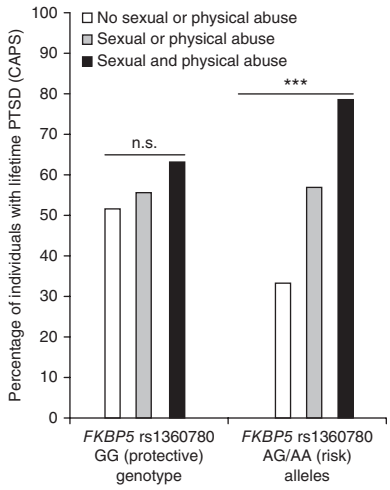


Figure 1 *FKBP5* × childhood abuse interaction on PTSD lifetime diagnosis. Shown is the interaction of child abuse and *FKBP5* rs1360780 protective genotype (left) or risk allele carrier status (right) on percentage lifetime PTSD (CAPS). *FKBP5* protective genotype: no abuse, $N = 133$; one type, $N = 27$; two types, $N = 16$; *FKBP5* risk allele carriers: no abuse; $N = 252$, one type, $N = 69$; two types, $N = 22$ (n.s. $P > 0.05$, $^{***}P < 0.001$).

with early trauma and these variants spanned 192 kb of the locus (**Supplementary Table 1**). Of all genotyped and imputed variants tagging this association, rs1360780, the variant originally identified as being associated with differential induction of *FKBP5* by glucocorticoid receptor activation[18], was the SNP in the tagging bin that was located closest to a functional GRE (see detailed description below).

In an expanded African-American cohort (N = 1,963) from the Grady trauma project (which includes 1,194 more individuals than were present in our original report[3]), we first confirmed and extended our previously reported interaction of *FKBP5* rs1360780 and early trauma on adult PTSD symptoms. We not only observed a significant interaction of child abuse, determined by the Childhood Trauma Questionnaire (CTQ) and *FKBP5* rs1360780, on current adult PTSD symptoms, as measured by the modified PTSD symptom scale (mPSS total score, $F_{1963,2} = 4.40$, $P = 0.012$), but also on lifetime PTSD, as assessed by the criterion standard diagnostic instrument, the clinician-administered PTSD scale (CAPS, $N = 519$). In a logistic regression analysis, both early trauma ($P < 0.001$) and the interaction of early trauma and the *FKBP5* rs1360780 risk allele carrier status ($P = 0.034$) were significant predictors for lifetime PTSD without main genetic effect of *FKBP5*. The risk of suffering from lifetime PTSD was significantly increased by exposure to early trauma in *FKBP5* risk allele carriers ($\chi^2 = 28.6$, degrees of freedom = 2, $P < 0.001$), but not in carriers of the protective genotype ($\chi^2 = 2.02$, degrees of freedom = 2, $P = 0.36$) (**Fig. 1**). These data lend further support to the robustness of the moderation of child abuse–related risk for adult PTSD by rs1360780.

rs1360780 affects *FKBP5* chromatin shape and transcription

The glucocorticoid receptor transcriptionally regulates *FKBP5* mainly by distal intronic GREs[20]. rs1360780 is located in a functional enhancer region and 488 bp from a GRE in intron 2. To test whether this SNP itself has the potential to alter *FKBP5* transcription, we cloned reporter gene constructs encompassing the consensus GRE site together with either the rs1360780 A/T risk allele or the C/G protective allele (**Fig. 2a**). The construct with the risk allele exhibited stronger activity than the construct with the protective allele, both in the absence of glucocorticoid receptor (1.4-fold, $P < 0.001$) and in the presence of activated glucocorticoid receptor (1.7-fold, $P = 0.036$) (**Fig. 2b**,**c**). These data are consistent with previously reported *in vivo* data showing a stronger correlation of *FKBP5* mRNA and plasma cortisol in peripheral blood cells from risk genotype carriers as compared with carriers of the protective allele[18]. In fact, the sequence containing the A/T risk allele of rs1360780 is predicted to form a putative TATA box, possibly enhancing gene transcription as compared to the C/G allele. The A allele indeed exhibited a stronger TATA box binding protein (TBP) binding than the G allele ($P = 0.009$; **Fig. 2d**). Data from chromatin immunoprecipitation experiments suggest that this distal GRE comes into contact with RNA polymerase and, likely, the transcription start site (TSS), supporting a functional consequence of altered TBP binding in intron 2 on *FKBP5* mRNA

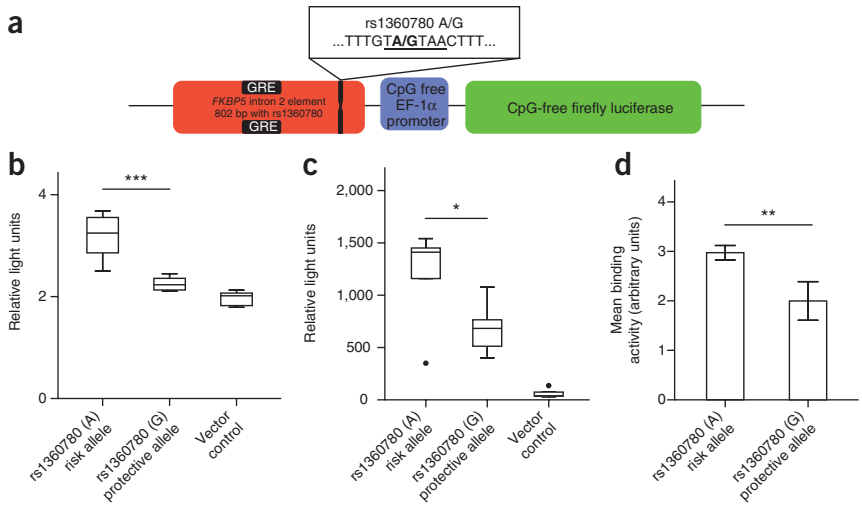


Figure 2 Genotype- and glucocorticoid receptor–dependent enhancer activities of intron 2 sequences of *FKBP5*. (**a**) Representation of the reporter construct. The luciferase gene is driven by a basal human elongation factor-1 alpha (EF-1α, *EEF1A1*) promoter with a 802-bp fragment of *FKBP5* intron 2 containing the putative GRE and rs1360780 cloned in front of the promoter. The rs1360780 risk allele A/T might form a TATA box–like sequence, which would enhance transcriptional activity. (**b**) Allele specificity of the reporter gene activity in glucocorticoid receptor–free HEK 293 cells. The reporter activity was reduced for the protective allele versus the risk allele ($^{***}P < 0.001$, Student's t test, unpaired, two sided). (**c**) Stimulation with 50 nM dexamethasone significantly enhanced intron 2–driven luciferase activity in glucocorticoid receptor–expressing HeLa cells in a genotype-dependent manner. Reduced activity was observed for the protective allele versus the risk allele ($^{*}P = 0.036$, Student's t test, unpaired, two sided). Baseline luciferase activity in HeLa cells without dexamethasone stimulation did not reveal significant differences (data not shown). In the box plots, the box extends indicate lower quartile and upper quartile, and the whiskers denote sample minimum and maximum. The line in the box represents the median and dots represent outliers. (**d**) Relative binding of recombinant TBP to a double-stranded 60-mer oligonucleotide containing either the A or G allele of rs1360780. The A allele exhibited a stronger binding to TBP than the G allele. ($^{**}P = 0.009$, Student's t test, unpaired, two sided). The assay was run in triplicates, background activity was subtracted from the measurement reading. Data are expressed as mean ± s.e.m.

© 2013 Nature America, Inc. All rights reserved.

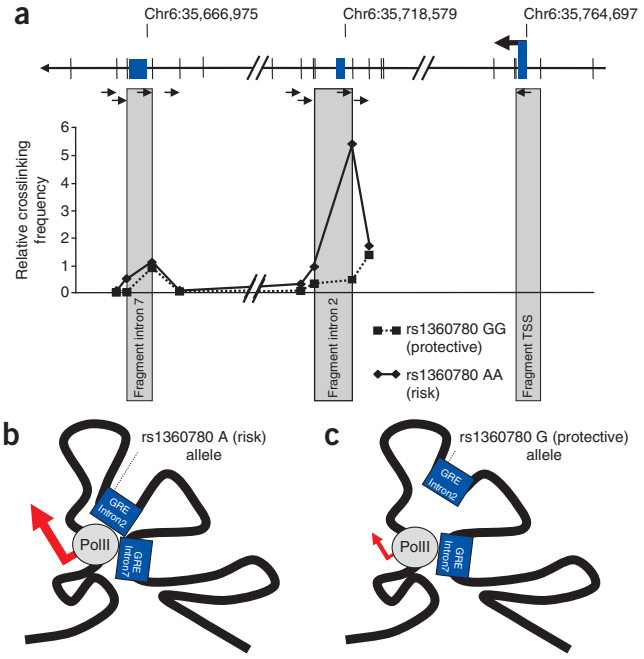


Figure 3 Long-distance interaction of GREs in *FKBP5*. Chromatin conformation capture confirmed a genotype-dependent interaction of the *FKBP5* TSS (TSS) with intron 2 and intron 7 in lymphoblastoid cell lines. (**a**) Top, *FKBP5* genomic locus with the TSS indicated by an arrow and intron 2 and intron 7 indicated by black boxes. The upright thin lines represent EcoRI cutting sites of genomic DNA. Bottom, chromatin conformation capture interaction data. Amplicon primers are indicated by small arrows, and the fragments containing the TSS, intron 2 and intron 7 are emphasized by gray boxes. Data from quantitative PCR are plotted as relative crosslinking frequency on the *y* axis. A specific interaction was detected as the local peak in interaction frequencies. Thus, intron 7 showed a stronger interaction with the TSS than with fragments upstream and downstream of the intron 7 fragment in both cell lines. Moreover, we observed a strong local peak for the interaction of the TSS with intron 2, but only in the cell line carrying the risk (AA) genotype. This confirmed the physical interaction of the TSS with intron 7 and intron 2 in a genotype-dependent manner. (**b**,**c**) Three-dimensional interaction of intronic GREs in intron 2 and 7 of *FKBP5* with the TSS. *FKBP5* mRNA transcription is induced by cortisol via a three-dimensional interaction and loop formation of predominantly distal enhancer regions (blue) harboring GREs with the core promoter site (PolII = RNA polymerase II). The interaction of intron 2 with the TSS in risk allele carriers leads to an increased *FKBP5* induction in response to glucocorticoid receptor activation (represented by a red arrow).

transcription[20]. Taking advantage of the chromatin conformation capture technique, we found an rs1360780 genotype–independent interaction of intron 7 with the TSS via three-dimensional loop formation (**Fig. 3** and **Supplementary Fig. 1a–d**) and a genotype-dependent interaction of intron 2 and the TSS in a homozygote risk (AA) allele carrier, but not a carrier of the protective (GG) genotype. These results support the theory that the functional effects reported for the *FKBP5* haplotype on *FKBP5* mRNA and protein levels could be mediated by the different chromatin conformation and, thus, transcriptional effects of the two opposite alleles of rs1360780 (ref. 18).

Consistent with the ultra-short negative feedback between FKBP5 and glucocorticoid receptor activation, this genotype-mediated increased *FKBP5* transcription has been shown to be associated with glucocorticoid receptor resistance in healthy controls[3,15] and with a decreased efficiency of negative feedback of the stress hormone axis accompanied by prolonged cortisol release[21]. We sought to unravel the molecular mechanism by which a genetic predisposition to react more strongly to a stressor in the short term interacts with exposure to childhood trauma to increase the risk of developing psychiatric disorders in the long term.

DNA demethylation in *FKBP5* in traumatized individuals

Epigenetic changes, especially changes in DNA methylation, have been reported as long-lasting consequences of early trauma[22–24], and glucocorticoid receptor activation has been shown to induce local changes in DNA methylation at GREs, including in the murine *Fkbp5* locus[25,26]. We hypothesized that excessive cortisol release following early life stress exposure in *FKBP5* risk allele carriers would lead to epigenetic changes in the GREs of *FKBP5*, resulting in lasting disruptions of the ultra-short feedback loop that balances FKBP5 and glucocorticoid receptor activity, entailing dysregulation of the stress hormone system, and ultimately increasing the risk for certain psychiatric disorders.

Table 1 Sample description of Grady trauma project cohort and Conte Center cohort

	Subjects with ≥2 childhood traumas (sexual and physical) according to TEI	Controls with no childhood trauma (sexual and physical) according to TEI	*P* value, χ^2 or ANOVA
Grady trauma project cohort	*N* = 30	*N* = 46	
Age (mean ± s.d.)	41.46 ± 11.67 years	40.97 ± 11.86 years	0.860
Age minimum	19 years	18 years	
Age maximum	59 years	61 years	
Gender (female/male)	22/8	36/10	0.783
Race (*N*)			
African American	27	45	0.150
Caucasian	2	0	
Mixed	1	0	
Other	0	1	
mPSS total (mean ± s.d.)	26.72 ± 13.56	3.35 ± 3.53	<0.001
BDI total score	21.27 ± 12.50	4.80 ± 4.63	<0.001
CAPS score current	75.21 ± 58.74	16.35 ± 22.72	<0.001
CTQ total score	76.97 ± 20.14	29.76 ± 4.94	<0.001
CTQ sexual abuse score	17.27 ± 6.18	5.11 ± 0.38	<0.001
CTQ emotional abuse score	17.07 ± 5.06	6.17 ± 2.25	<0.001
CTQ physical abuse score	15.83 ± 5.60	6.11 ± 1.22	<0.001
Number of adult trauma (TEI)	4.10 ± 2.15	1.12 ± 1.29	<0.001
Conte Center cohort	*N* = 56		
Age mean (mean ± s.d.)	28.45 ± 6.98		
Age minimum	18		
Age maximum	45		
Gender (female/male)	56/0		
Race (*N*)			
African American	31		
Caucasian	17		
Mixed	2		
Other	6		
BDI total score (mean ± s.d.)	9.13 ± 11.14		
CTQ total score	51.98 ± 22.90		
CTQ sexual abuse score	9.00 ± 5.86		
CTQ emotional abuse score	12.34 ± 7.01		
CTQ physical abuse score	10.23 ± 5.26		

BDI, Beck Depression Inventory; TEI, Traumatic Events Inventory.

© 2013 Nature America, Inc. All rights reserved.

To investigate the possibility that *FKBP5* × early trauma interactions are mediated by epigenetic modifications, we performed DNA methylation analysis by pyrosequencing of bisulfite-treated genomic DNA extracted from peripheral blood cells from individuals selected from the Grady trauma project. DNA from individuals having experienced both sexual and physical child abuse were compared with DNA from individuals without any childhood trauma (N = 76; high trauma cohort, N = 30; control cohort, N = 46; **Table 1**). We screened the CpG island in the vicinity of the TSS, as well as the regions around functional GREs[20] in the putative promoter region and introns 2, 5 and 7 for the extent of CpG methylation (**Fig. 4a** and **Supplementary Table 2**). We observed significant DNA methylation higher than 5% in regions close to the GREs in the promoter region (30–100%), intron 2 (30–93%) and intron 7 (29–100%), but not in the CpG island or the two GREs in intron 5 (<5%). In intron 7, but not in the two other regions, two methylated CpGs were located in consensus GRE sequences.

We next tested the effects of *FKBP5* genotype (rs1360780 risk allele carrier model) and exposure to childhood abuse (CTQ categorical scale), as well as their interaction, on the extent of DNA methylation in these three regions, summarizing the percentage methylation of these 16 CpGs into seven bins of one to three CpGs to a mean methylation score according to their spatial proximity to the consensus GRE sites (**Fig. 4b** and **Supplementary Fig. 2**). The analyses were corrected for age and gender as covariates and multiple testing. In one bin of intron 7 (bin 2), but not the other bins, significant early trauma ($F_{73,1}$ = 8.2, P = 0.006), genotype ($F_{73,1}$ = 34.33, P < 0.001) and interaction effects between genotype and early trauma ($F_{73,1}$ = 31.01, P_{corr} < 0.001) on methylation were observed. This bin contains three CpGs, with one CpG located in the consensus GRE in intron 7 (**Supplementary Fig. 2**). An average decrease of 12.3% in DNA methylation in these three CpGs was detected in child abuse–exposed risk allele carriers compared to the other three groups (**Supplementary Fig. 3**). When correlating levels of child abuse measured using the log-transformed total CTQ score and the subscores for physical, emotional and sexual abuse on this scale, with the mean methylation of this CpG bin in intron 7, we found significant differences in the correlation coefficients between the risk allele carriers (N = 55) and the protective genotype carriers (N = 19) (R = −0.646, P < 0.001 for risk allele and R = 0.414, P = 0.078 for protective genotype carriers and total CTQ with a Fisher z score of −4.23 and $P = 7.0 \times 10^{-5}$; **Fig. 5** and **Supplementary Table 3**). Heterozygous individuals carrying one risk allele did not differ from homozygous risk allele carriers (**Supplementary Fig. 4**). This emphasizes the effects of early trauma severity on *FKBP5* demethylation in risk allele carriers, but not in protective genotype carriers.

To test whether the observed effects of child abuse may have been confounded by the increased exposure to more recent types of trauma in the abused group (**Table 1**), we explored the correlation of intron 7 bin 2 DNA methylation and the number of exposures to adult trauma separately in risk alleles carriers with or without child abuse. We did not observe a correlation of exposure to adult trauma with intron 7 bin 2 DNA methylation in either the childhood abuse risk allele group (R = 0.020, P = 0.933, N = 20) or the control risk allele group (R = 0.018, P = 0.917, N = 35). This suggests that the observed effect of early trauma on DNA methylation is independent of subsequent exposure to trauma (**Supplementary Fig. 5**).

We next investigated the relationship of severity of early trauma and *FKBP5* DNA methylation in a second female-only cohort recruited as part of a separate study investigating the effects of early trauma on biological markers[4,27] (**Table 1**). In this cohort, we correlated the log-transformed total CTQ score as well as the subscores for physical, emotional and sexual abuse with the mean DNA methylation of the three CpGs in the second bin of intron 7. In *FKBP5* risk genotype carriers (N = 33), we observed a negative correlation between the methylation score and the total CTQ score (R = −0.273, P = 0.124), the physical abuse subscore (R = −0.397, P = 0.022) and the emotional abuse subscore (R = −0.397, P = 0.022), but not the subscore for sexual abuse (R = 0.118, P = 0.514) (**Fig. 5**). In carriers of the protective genotype (N = 23), these correlations were either absent or positive (**Supplementary Table 3**). The difference in correlations was significant for the physical abuse subscore (Fischer z score = −2.33, P = 0.019). These data from an independent sample support the notion of allele-specific demethylation of CpGs close to and in functional

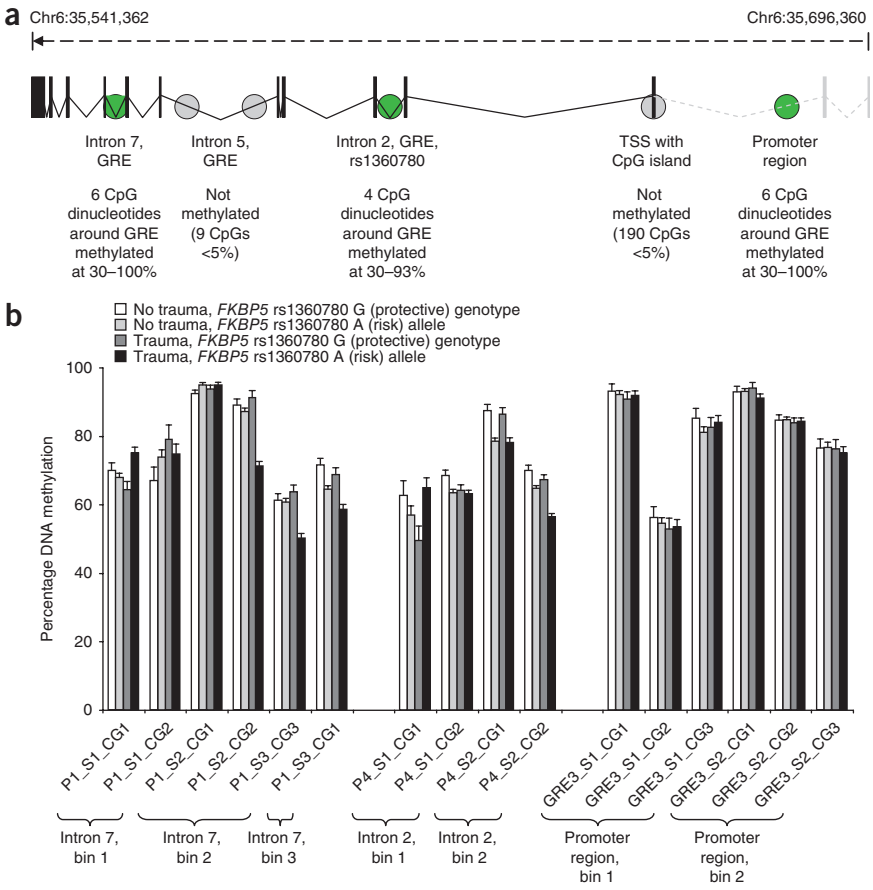


Figure 4 DNA methylation of the *FKBP5* locus. (**a**) Significant DNA methylation was observed in the promoter region, intron 2 and intron 7 of *FKBP5*, as indicated in green. (**b**) Single CpG site methylation in the four groups (early trauma × *FKBP5* rs1360780 carriers). Data are expressed as mean ± s.e.m.

© 2013 Nature America, Inc. All rights reserved.

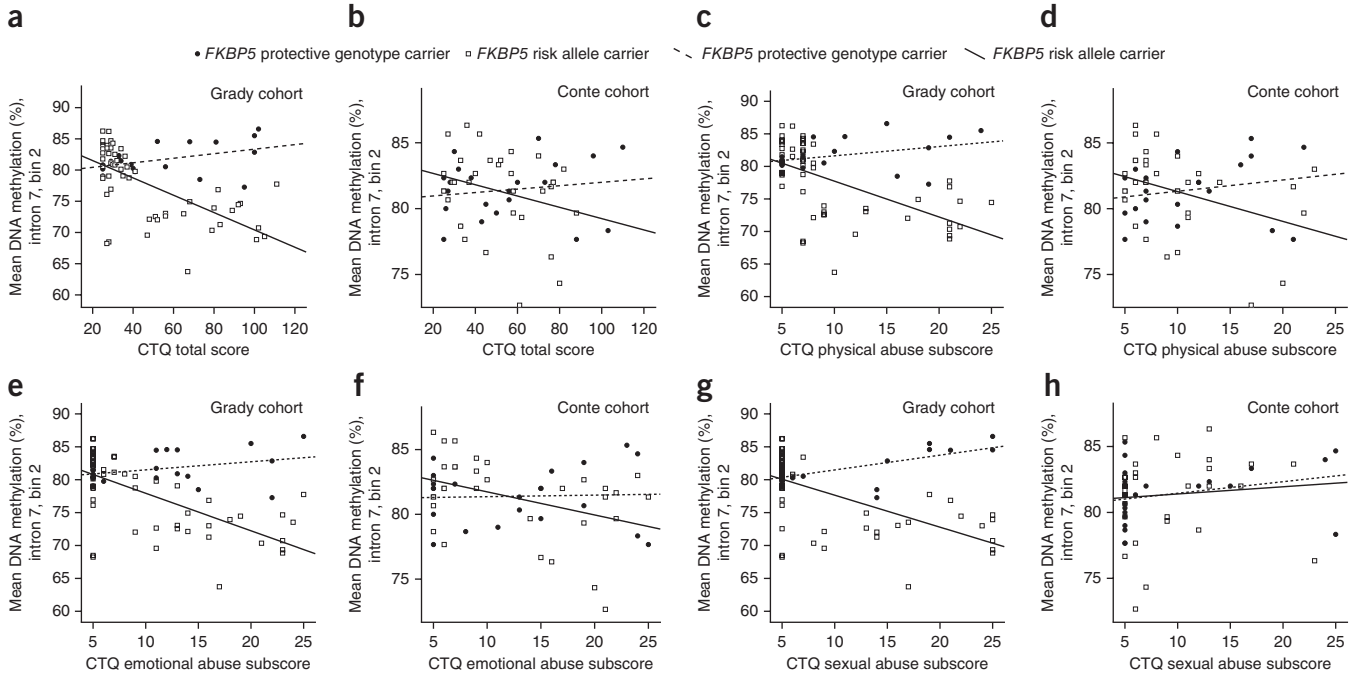


Figure 5 Differential *FKBP5* intron 7 DNA methylation depends on genotype and trauma exposure. Correlation between intron 7 bin 2, mean methylation and log-transformed CTQ scores by *FKBP5* rs1360780 genotype in the Grady and Conte cohort are shown. (**a**) Grady cohort. Risk allele carriers exhibited a strong negative correlation ($R = -0.646$, $P < 0.001$) between methylation and CTQ total load compared with carriers of the protective genotype ($R = 0.414$, $P = 0.078$) (Fisher z score = –4.23, $P < 0.001$). (**b**) Conte cohort. Correlation between methylation and total CTQ in risk allele carriers ($R = -0.273$, $P = 0.124$), and in carriers of the protective genotype ($R = 0.153$, $P = 0.485$) (Fisher z score = –1.5, $P = 0.133$). (**c**) Grady cohort. Negative correlation was found between methylation and the CTQ physical abuse subscore in risk allele carriers ($R = -0.586$, $P < 0.001$), but not in carriers of the protective genotype ($R = 0.360$, $P = 0.130$) (Fisher z score = –4.49, $P < 0.001$). (**d**) Conte cohort. Negative correlation was observed between methylation and the CTQ physical abuse subscore in risk allele carriers ($R = -0.397$, $P = 0.022$), but not in carriers of the protective genotype ($R = 0.246$, $P = 0.258$) (Fisher z score = –2.33, $P = 0.019$). (**e**) Grady cohort. Negative correlation was found between methylation and the CTQ emotional abuse subscore in risk allele carriers ($R = -0.685$, $P < 0.001$), but not in carriers of the protective genotype ($R = 0.321$, $P = 0.181$) (Fisher z score = –4.1, $P < 0.001$). (**f**) Conte cohort. Negative correlation was found between methylation and the CTQ emotional abuse subscore in risk allele carriers ($R = -0.397$, $P = 0.022$), but not in carriers of the protective genotype ($R = 0.022$, $P = 0.922$) (Fisher z score = –1.53, $P = 0.126$). (**g**) Grady cohort. Negative correlation was found between methylation and the CTQ sexual abuse subscore in risk allele carriers ($R = -0.656$, $P < 0.001$), but not in carriers of the protective genotype (R = 0.599, $P = 0.007$) (Fisher z score = –5.17, $P < 0.001$). (**h**) Conte cohort. Negative correlation was found between methylation and the CTQ sexual abuse subscore in risk allele carriers ($R = 0.118$, $P = 0.514$), and in carriers of the protective genotype ($R = 0.305$, $P = 0.922$) (Fisher z score = –0.68, $P = 0.496$).

© 2013 Nature America, Inc. All rights reserved.

GREs in intron 7 of the *FKBP5* gene with early trauma exposure. The difference in effect size and relative contributions of different trauma types is likely a result of the fact that this second cohort was exposed to less overall trauma, including childhood abuse, than the cohort from the Grady trauma project (**Table 1**). In addition, the replication cohort was ethnically diverse, but the effects of childhood abuse on DNA methylation were consistent across the two main ethnic groups, European and African Americans.

To rule out the possibility that trauma- or psychiatric disease–related differences in immune cell fractions confound the reported results[28], we reran the analysis in 41 women of the replication cohort (21 risk allele carriers, 20 protective genotype carriers) in whom we also had data on the relative amount of the main immune cell subtypes (CD14-positive monocytes and neutrophils, CD4- and CD8-positive lymphocytes, and CD16- and CD56-positive NK cells). Using these as covariates, we found that the differences in correlations between CTQ scores and intron 7 methylation in the two genotype groups remained substantial. For physical abuse, the corrected correlations were $R = -0.476$ in risk allele carriers and $R = 0.40$ in carriers of the protective genotype, with a significant z score of 2.78 ($P = 0.005$). In addition, we did not observe any correlations between the relative amount of immune cell subtypes and intron 7 DNA methylation, suggesting that possible trauma-related differences in these cell types are not likely to confound our results. However, we cannot exclude the effect of immune cell subtypes that were not specifically tested.

Demethylation of *FKBP5* in neuronal cells

Our data therefore point to an allele-specific, early trauma exposure–dependent demethylation of CpGs close to and in GREs in intron 7 of *FKBP5* (**Supplementary Fig. 1a–d**). To determine whether this demethylation could be related to excessive glucocorticoid receptor activation and might also be seen in tissues other than peripheral blood, including neuronal tissue, we used a multipotent human hippocampal progenitor cell line in which exposure to dexamethasone has been shown to be accompanied by a reduction in cell proliferation and neuronal differentiation[29]. Cells were exposed to dexamethasone during both the proliferation and differentiation phases, similar to previous studies[29]. The percentage of DNA methylation across CpGs in introns 2 and 7 in these neuronal progenitor cells was comparable to that in peripheral blood cells. Exposure to dexamethasone led to a highly significant ($P < 0.001$) DNA demethylation in CpGs in intron 7, but not intron 2 (**Fig. 6a**). In fact, the CpG bin in intron 7 that was affected by early trauma in *FKBP5* risk allele carriers showed the strongest DNA demethylation following glucocorticoid receptor

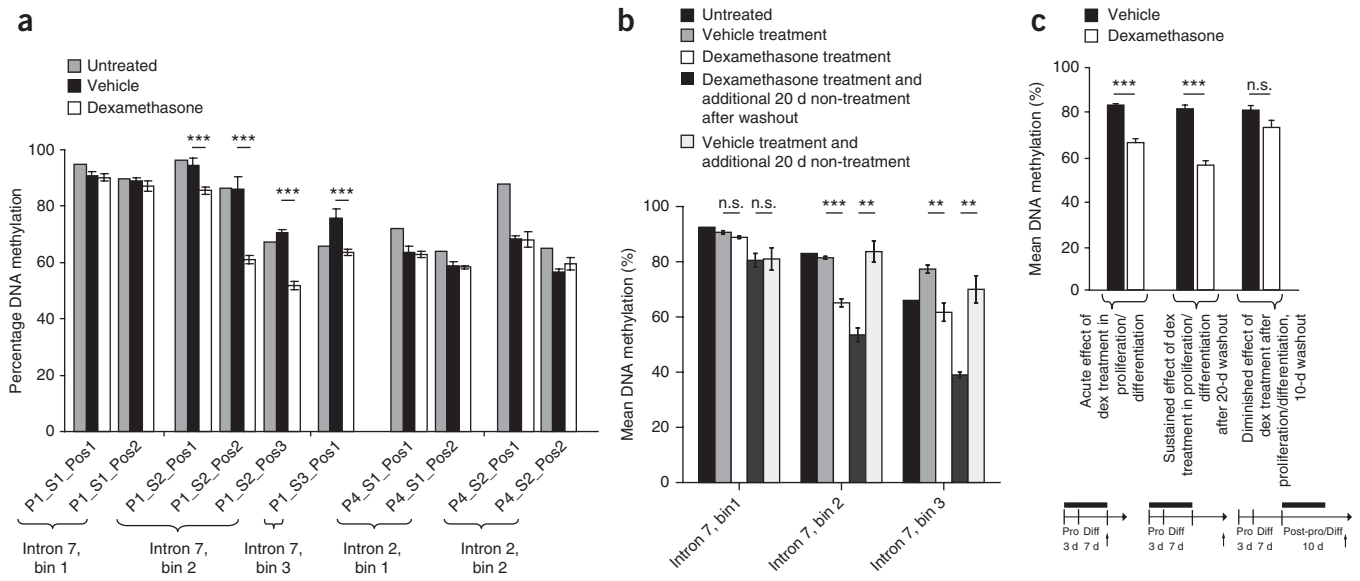


Figure 6 Effects of glucocorticoid receptor agonist treatment on *FKBP5* DNA methylation in hippocampal progenitor cells. (**a**) Dexamethasone treatment induced demethylation in the human hippocampal progenitor cell line HPC03A/07 in the proliferation and differentiation phase in intron 7 (***$P < 0.001$), but not intron 2. Data are expressed as mean ± s.e.m. (**b**) Dexamethasone treatment of human hippocampal progenitor cells in proliferation and differentiation phase resulted in significant demethylation of CpGs in *FKBP5* intron 7, bins 2 and 3 (***$P < 0.001$, **$P = 0.01$, n.s. $P > 0.05$, Student's *t* test, unpaired, two-sided). Dexamethasone treatment in the proliferation phase only resulted in similar demethylation of CpGs in *FKBP5* intron 7 in comparison with the differentiation phase (data not shown). A subsequent washout of dexamethasone and an additional 20-d incubation revealed similar results, supporting a long-lasting demethylation in bins 2 and 3. Data are expressed as mean ± s.e.m. (**c**) Treatment in proliferation and differentiation phase resulted in an immediate demethylation of intron 7 bin 2 (***$P < 0.001$, Student's *t* test, unpaired, two-sided). Treatment in the proliferation and differentiation phase and subsequent washout phase in steriod-devoid medium for 20 d revealed a stable demethylation by glucocorticoid receptor activation. In contrast, dexamethasone treatment after proliferation and differentiation did not induce a long-lasting demethylation in intron 7 bin 2 (n.s. $P = 0.063$, Student's *t* test, unpaired, two-sided), suggesting a sensitive period of time for glucocorticoids-mediated epigenetic changes. Data are expressed as mean ± s.e.m.

stimulation in hippocampal progenitor cells (average of 17.1% demethylation in these three CpGs, $P < 0.001$ for differentiation phase, which includes proliferation). The observed demethylation in intron 7 remained unchanged after 20 days in culture in steroid-devoid medium, supporting a stable epigenetic memory of glucocorticoid-induced demethylation (**Fig. 6b**). Moreover, glucocorticoid receptor activation by dexamethasone treatment after proliferation and differentiation did not result in demethylation in intron 7 bin 2, suggesting a sensitive period of time for glucocorticoids-mediated epigenetic changes (**Fig. 6c**).

The existence of a sensitive time period for these epigenetic effects was also apparent from data from human blood cells, as only exposure to child abuse, but not later trauma, predicted *FKBP5* demethylation (see above), and there was a lack of correlation of serum cortisol levels and DNA methylation in blood samples from both human cohorts taken at the same time point (Grady trauma project, $R = 0.083$, $P = 0.489$, $N = 72$; Conte replication cohort, $R = 0.069$, $P = 0.603$, $N = 59$; **Supplementary Fig. 6**). These data further substantiate the conclusion that these specific CpGs in intron 7 are sensitive to stable, glucocorticoid receptor–mediated demethylation during a vulnerable time period and that our findings of early trauma–and genotype-dependent demethylation also pertain to tissues beyond peripheral blood.

Functional effects of intron 7 demethylation

We then investigated whether the observed changes in DNA methylation in intron 7 alter the glucocorticoid responsiveness of *FKBP5* *in vitro* and *in vivo*. We inserted 515 bp of intron 7, encompassing the three functional GREs, into a CpG-free reporter plasmid[30] (**Fig. 7a**) and assessed its activity in HeLa cells as a function of insert methylation. No effect of methylation was observed in the absence of glucocorticoid receptor activation, which is consistent with the absence of correlation between intron 7 methylation and baseline *FKBP5* expression in peripheral blood cells (data not shown). However, methylation significantly attenuated the response to 50 nM dexamethasone (relative induction = 19-fold in unmethylated versus 13-fold in methylated construct, $P = 0.035$; **Fig. 7b**). These data indicate that methylation of CpGs surrounding the functional GREs in intron 7 decreases the induction of *FKBP5* by glucocorticoid receptor activation without affecting baseline activity.

To test whether intron 7 DNA methylation alters the ultra-short feedback loop between glucocorticoid receptor and *FKBP5*, and thereby alters glucocorticoid receptor sensitivity, we correlated the mean methylation score of intron 7 bin 2 with glucocorticoid receptor sensitivity measured *ex vivo* in peripheral blood monocytes in 32 individuals from the replication cohort. We observed a strong negative correlation between the half-maximal inhibitory concentration (IC_{50}) for dexamethasone-mediated inhibition of lipopolysaccharide-induced interleukin 6 production in these cells ($N = 32$, $R = -0.531$, $P = 0.002$), which is a measure of glucocorticoid receptor sensitivity. This suggests that, as expected, less methylation of intron 7 CpGs is associated with higher induction of *FKBP5* by glucocorticoid receptor activation, especially in risk allele carriers, representing an enhancement of the ultra-short feedback loop leading to increased glucocorticoid receptor resistance (**Fig. 7c**).

Global effect on gene transcription and brain structure

FKBP5 risk allele carrier status and early trauma exposure lead to demethylation of intron 7 CpGs in *FKBP5*, which further amplifies

© 2013 Nature America, Inc. All rights reserved.

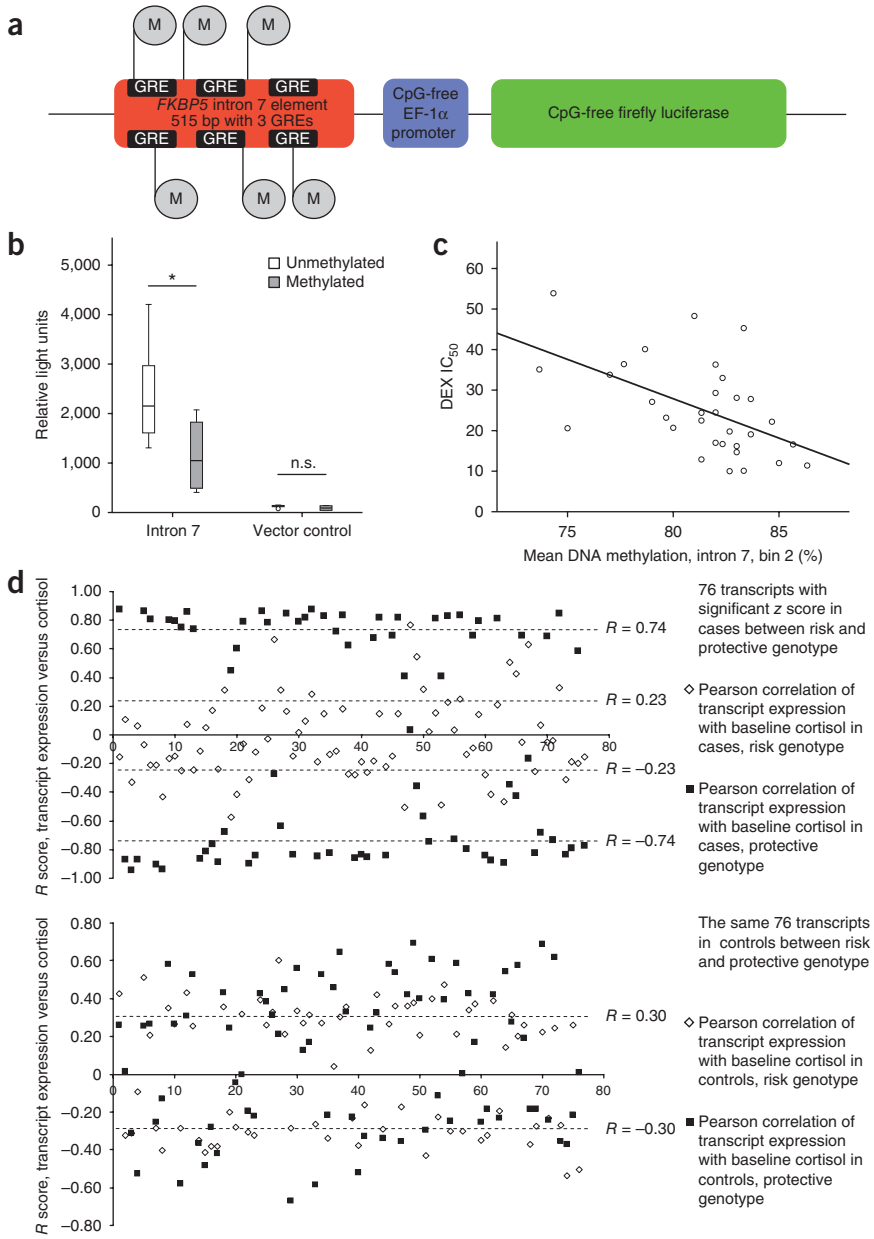


Figure 7 Functional effects of *FKBP5* intron 7 methylation. (**a**) Representation of the reporter construct. A 514-bp fragment of *FKBP5* intron 7 containing three putative GREs was cloned in front of the EF-1α promoter and the luciferase gene, acting as transcriptional regulator. Only the *FKBP5* insert can be methylated *in vitro* by MSssI, as the vector is CpG free. Circled M denotes putative methylation sites. Two of them are located in consensus GRE sequences. (**b**) Stimulation by 50 nM dexamethasone was attenuated by DNA methylation (*$P = 0.035$, n.s. $P = 0.146$, Student's *t* test, unpaired, two-sided). Baseline luciferase activity without dexamethasone stimulation did not reveal significant differences ($P > 0.05$; data not shown). In the box plots, the box extends indicate lower quartile and upper quartile, and the whiskers denote sample minimum and maximum. The line in the box represents the median and dots represent outliers. (**c**) Methylation in intron 7 was correlated with dexamethasone-mediated inhibition of lipopolysaccharide-induced interleukin 6 production (DEX IC_{50}) in peripheral blood monocytes *ex vivo* ($R = –0.531$, $P = 0.002$). This correlation was stronger in rs1360780 risk allele carriers ($N = 19$, $R = –0.691$, $P = 0.001$) than carriers of the protective genotype ($N = 13$, $R = –0.119$, $P = 0.698$). This suggests that lower DNA methylation in intron 7 leads to stronger *FKBP5* induction and to glucocorticoid receptor resistance. (**d**) Exposure to early trauma enhanced *FKBP5* genotype–dependent differences in glucocorticoid receptor–dependent gene expression in peripheral blood. Top, *R* scores of the correlation of peripheral blood gene expression and cortisol for the 76 transcripts with significant genotype-dependent correlation differences in individuals with high levels of early trauma (cases, $N = 55$) and stratified by *FKBP5* genotype (risk allele: $N = 40$, CTQ total score = 67.88 ± 14.71; protective genotype: $N = 15$, CTQ total = 72.44 ± 17.68). For all transcripts, the correlation with cortisol, and therefore the presumable responsiveness to the glucocorticoid receptor, was higher in *FKBP5* protective genotype carriers (black squares) with a mean *R* of 0.74 than in carriers of the risk allele (open squares), with a mean *R* of 0.23. Bottom, correlation between gene expression levels and cortisol of the same transcripts, but in individuals that were not exposed to early trauma (controls, $N = 74$; risk allele: $N = 60$, CTQ total = 28.42 ± 2.96; protective genotype: $N = 14$, CTQ total = 28.79 ± 3.21). In this group, no genotype-dependent differences of the correlation were observed, with mean *R* scores of 0.31 and 0.35 for risk and protective genotypes, respectively. *x* axis represents individual transcripts 1 to 76.

genotype-dependent differences in the FKBP5 and glucocorticoid receptor ultra-short feedback loop and, thus, glucocorticoid receptor sensitivity. *FKBP5* genotype–dependent differences in the global regulation of glucocorticoid receptor–sensitive genes should therefore be amplified in trauma-exposed risk allele carriers compared with protective genotype carriers. To test this hypothesis, we examined the effects of *FKBP5* rs1360780 genotype × environment interaction on peripheral blood mRNA expression of glucocorticoid receptor–responsive genes, as measured by gene expression arrays, in 129 individuals (child abuse + risk allele, $N = 40$; child abuse + protective genotype, $N = 15$; no child abuse + risk allele, $N = 60$; no child abuse + protective genotype, $N = 14$)[15]. In all 129 individuals, 1,627 transcripts showed a significant correlation ($P < 0.05$) with plasma cortisol concentrations, suggesting that they are responsive to glucocorticoid receptor activation. We found significant differences in the correlation of 76 of these transcripts with cortisol plasma levels when stratifying by *FKBP5* genotype in individuals with child abuse (Fisher *z* score ≥ 1.96; **Supplementary Table 4**). For these 76 transcripts, the mean absolute correlation coefficient with plasma cortisol was $R = 0.23$ in the risk allele carriers with child abuse (that is, those exhibiting a demethylation of *FKBP5* intron 7) as compared with $R = 0.74$ in the carriers of the protective genotype with child abuse (in which intron 7 methylation remained largely stable). This indicates a relative glucocorticoid receptor resistance in the trauma-exposed *FKBP5* risk allele versus protective genotype carriers. These 76 transcripts did not show a genotype-dependent difference in

© 2013 Nature America, Inc. All rights reserved.

correlation coefficients in individuals that were not exposed to child abuse (**Fig. 7d**), suggesting that exposure to early trauma enhances the *FKBP5* genotype–dependent effect of glucocorticoid receptor sensitivity, most likely by epigenetic mechanisms. The genes most strongly affected by *FKBP5* genotype for their correlation with plasma cortisol levels in trauma-exposed individuals showed a significant over-representation (WikiPathways, $P < 0.01$) of transcripts in the T cell receptor signaling pathway ($P_{adjusted} = 5.15 \times 10^{-7}$), the TGF-β signaling pathway ($P_{adjusted} = 0.0007$), the Wnt signaling pathway and pluripotency ($P_{adjusted} = 0.0044$), and the inflammatory response pathway ($P_{adjusted} = 0.0099$)[31]. These findings suggest that the combination of *FKBP5* risk allele carrier status and early trauma exposure alters the stress hormone–dependent regulation of several genes in peripheral blood cells, and might thereby enhance the reported association of early trauma with immune and inflammatory dysregulation, further promoting system-wide symptoms of stress-related disorders[32,33].

Structural imaging data from a subset of the replication cohort revealed that peripheral blood *FKBP5* methylation significantly correlated with the volume of the right hippocampal head ($N = 34$, $R = -0.484$, $P_{corr} = 0.014$; **Supplementary Fig. 7**). Despite the smaller N values for the structural imaging data in the less-traumatized replication cohort, these preliminary data suggest that *FKBP5* demethylation is not only associated with an altered sensitivity of peripheral glucocorticoid receptors and more global changes in immune cell gene expression, but also with morphological changes in the brain indicative of a higher stress hormone system reactivity, thereby bridging the gap between abuse-related peripheral methylation changes and effects in the CNS.

DISCUSSION

Here we provide evidence for an epigenetic mechanism that mediates the combined effects of environmental exposure in early life and a genetic polymorphism on the risk of developing stress-related psychiatric disorders. According to this mechanism, genetic variations shaped by evolution can determine the environmental adaption during the lifetime of an individual via epigenetic processes. Our data suggest the following scenario for an *FKBP5* × child abuse interaction (**Supplementary Fig. 1a–d**): genetic differences lead to divergent chromatin conformations and interactions of long-range enhancers with the TSS, resulting in a differential transcriptional activation of *FKBP5* by glucocorticoid receptor activation in response to childhood abuse. These changes in chromatin structure include regions around distal GREs and, together with increased cortisol levels and thus glucocorticoid receptor binding, lead to changes in DNA methylation in intron 7, further increasing the differential responsiveness of *FKBP5* to glucocorticoid receptor activation. When installed during developmentally critical periods, these epigenetic changes remain stable over time. This model describes how epigenetic mechanisms stabilize and, in an environment-dependent manner, further amplify differential activities that were originally bestowed on the enhancer complex by genetic differences.

On a systems level, these molecular changes further tighten the ultra-short feedback loop between FKBP5 and the glucocorticoid receptor leading to glucocorticoid receptor resistance. Enhanced *FKBP5* responsiveness would not only lead to long-term changes in stress hormone system regulation, but also to alterations of neuronal circuits, as reflected by structural changes in the hippocampus, and other glucocorticoid receptor responsive systems, as reflected by global gene expression changes in the immune system, resulting in a higher risk for trauma-associated psychiatric, immune and metabolic disorders in exposed adults. The fact that the interaction of *FKBP5* genotype and early trauma can predispose an individual to both PTSD and depression indicates that this interaction is likely associated with stress sensitivity in general, crossing current diagnostic borders. Our findings appear to be of particular relevance for the developing organism, as the effects on DNA methylation seemed to be restricted to exposure to childhood trauma and were not influenced by traumatic experiences in adulthood or current cortisol levels, suggesting that there is a sensitive period in early development for these epigenetic effects. The importance of disinhibition of *FKBP5* for mood and anxiety disorders is supported by rodent models showing that enhanced transcription of *Fkbp5* in the amygdala is required for stress-, neuropsin- and EphB2-dependent induction of anxiety-like behavior in mice[34]. In addition, stress responsiveness is impaired in *Fkbp5* knockout animal[35,36].

In our studies, we focused on adverse environmental exposures. It is, however, possible that the described polymorphisms define not only risk versus resilience, but possibly environmentally reactive versus less reactive individuals. This would imply that the so-called risk-allele carriers may also profit more from positive environmental change. Our results provide insight into the molecular mechanisms and consequences of this gene × environment interaction, facilitating a better understanding of the pathophysiology of stress-related psychiatric disorders by including genetic and environmental information in the diagnosis, and potentially aiding in the development of new treatments targeting this mechanism.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession numbers. Data from the microarray experiment are deposited at the GEO repository, GSE42002.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We thank T. Mletzko, K. Hafner and N.C. Gassen for their help in acquiring and interpreting data, and P. Weber for data visualization. Special thanks to A.W. Graham, A. Brown, D. Crain and D. Cross for their assistance in recruiting subjects and managing the Grady Trauma Project. This work was supported by a European Research Council starting grant (grant #281338, GxE molmech), a grant from the National Alliance for Research in Schizophrenia and Affective Disorders and a grant from the Behrens Weise Stiftung to E.B.B., a grant from the National Institute of Mental Health (MH071538) to K.J.R., and a grant from the National Institute of Mental Health (MH58922) to C.B.N., C.M.P. and C.A. have received funding from the National Institute for Health Research Biomedical Research Centre for Mental Health, Institute of Psychiatry and South London and Maudsley National Health System Foundation Trust, a Clinician Scientist Fellowship from the UK Medical Research Council (G108/603) to C.M.P., and the Commission of European Communities 7th Framework Programme Collaborative Project Grant Agreement n 22963 (Mood Inflame), also to C.M.P., C.M.H. is supported in part by Public Health Service Grant UL1 RR025008 from the Clinical and Translational Science Award program, the US National Institutes of Health, the National Center for Research Resources and by a K Award (K01 MH073698–01, Neural Substrates of Depression Risk after Child Abuse).

AUTHOR CONTRIBUTIONS

T.K. and E.B.B. designed the experiments, performed the luciferase assays and the genetic, methylation and expression analyses, analyzed the data, and wrote the initial version of the paper. D.M. performed the RNA expression experiments and data analyses and revised the paper. M.R.-H. performed the chromatin conformation capture experiments. C.A. and C.M.P. performed the cell-culture experiments with human hippocampal progenitor cells and revised the paper. T.W.W.P. performed *ex vivo* glucocorticoid receptor sensitivity experiments and revised the paper. J.C.P. analyzed magnetic resonance imaging data and revised the paper. E.B.B., F.H., K.J.R., K.B.M., H.S.M., B.B., C.B.N., C.M.H. and T.R. organized sample collection and collaborations, obtained funding, supervised data analyses, and revised the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the online version of the paper.

© 2013 Nature America, Inc. All rights reserved.

Published online at http://www.nature.com/doifinder/10.1038/nn.3275.
Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Caspi, A. & Moffitt, T.E. Gene-environment interactions in psychiatry: joining forces with neuroscience. *Nat. Rev. Neurosci.* **7**, 583–590 (2006).
2. Caspi, A., Hariri, A.R., Holmes, A., Uher, R. & Moffitt, T.E. Genetic sensitivity to the environment: the case of the serotonin transporter gene and its implications for studying complex diseases and traits. *Am. J. Psychiatry* **167**, 509–527 (2010).
3. Binder, E.B. *et al.* Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. *J. Am. Med. Assoc.* **299**, 1291–1305 (2008).
4. Heim, C. *et al.* Effect of childhood trauma on adult depression and neuroendocrine function: sex-specific moderation by CRH receptor 1 gene. *Front. Behav. Neurosci.* **3**, 41 (2009).
5. Xie, P. *et al.* Interaction of FKBP5 with childhood adversity on risk for post-traumatic stress disorder 3. *Neuropsychopharmacology* **35**, 1684–1692 (2010).
6. Pratt, W.B. & Toft, D.O. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.* **18**, 306–360 (1997).
7. Binder, E.B. The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. *Psychoneuroendocrinology* **34** (suppl. 1), S186–S195 (2009).
8. Holsboer, F. The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology* **23**, 477–501 (2000).
9. Heim, C., Newport, D.J., Mletzko, T., Miller, A.H. & Nemeroff, C.B. The link between childhood trauma and depression: insights from HPA axis studies in humans. *Psychoneuroendocrinology* **33**, 693–710 (2008).
10. Scammell, J.G., Denny, W.B., Valentine, D.L. & Smith, D.F. Overexpression of the FK506-binding immunophilin FKBP51 is the common cause of glucocorticoid resistance in three New World primates. *Gen. Comp. Endocrinol.* **124**, 152–165 (2001).
11. Wochnik, G.M. *et al.* FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. *J. Biol. Chem.* **280**, 4609–4616 (2005).
12. Jääskeläinen, T., Makkonen, H. & Palvimo, J.J. Steroid up-regulation of FKBP51 and its role in hormone signaling 4. *Curr. Opin. Pharmacol.* **11**, 326–331 (2011).
13. Appel, K. *et al.* Moderation of adult depression by a polymorphism in the *FKBP5* gene and childhood physical abuse in the general population. *Neuropsychopharmacology* **36**, 1982–1991 (2011).
14. Koenen, K.C. *et al.* Polymorphisms in FKBP5 are associated with peritraumatic dissociation in medically injured children. *Mol. Psychiatry* **10**, 1058–1059 (2005).
15. Mehta, D. *et al.* Using polymorphisms in FKBP5 to define biologically distinct subtypes of post-traumatic stress disorder: evidence from endocrine and gene expression studies. *Arch. Gen. Psychiatry* **68**, 901–910 (2011).
16. Roy, A., Gorodetsky, E., Yuan, Q., Goldman, D. & Enoch, M.A. Interaction of FKBP5, a stress-related gene, with childhood trauma increases the risk for attempting suicide. *Neuropsychopharmacology* **35**, 1674–1683 (2010).
17. Zimmermann, P. *et al.* Interaction of FKBP5 gene variants and adverse life events in predicting depression onset: results from a 10-year prospective community study. *Am. J. Psychiatry* **168**, 1107–1116 (2011).
18. Binder, E.B. *et al.* Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment 45. *Nat. Genet.* **36**, 1319–1325 (2004).
19. 1000 Genomes Project Consortium. A map of human genome variation from population-scale sequencing. *Nature* **467**, 1061–1073 (2010).
20. Paakinaho, V., Makkonen, H., Jaaskelainen, T. & Palvimo, J.J. Glucocorticoid receptor activates poised FKBP51 locus through long-distance interactions. *Mol. Endocrinol.* **24**, 511–525 (2010).
21. Ising, M. *et al.* Polymorphisms in the *FKBP5* gene region modulate recovery from psychosocial stress in healthy controls. *Eur. J. Neurosci.* **28**, 389–398 (2008).
22. McGowan, P.O. *et al.* Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat. Neurosci.* **12**, 342–348 (2009).
23. Murgatroyd, C. *et al.* Dynamic DNA methylation programs persistent adverse effects of early-life stress 8. *Nat. Neurosci.* **12**, 1559–1566 (2009).
24. Weaver, I.C. *et al.* Epigenetic programming by maternal behavior. *Nat. Neurosci.* **7**, 847–854 (2004).
25. Thomassin, H., Flavin, M., Espinas, M.L. & Grange, T. Glucocorticoid-induced DNA demethylation and gene memory during development. *EMBO J.* **20**, 1974–1983 (2001).
26. Lee, R.S. *et al.* Chronic corticosterone exposure increases expression and decreases deoxyribonucleic acid methylation of *Fkbp5* in mice. *Endocrinology* **151**, 4332–4343 (2010).
27. Pace, T.W. *et al.* Increased peripheral NF-kappaB pathway activity in women with childhood abuse-related post-traumatic stress disorder. *Brain Behav. Immun.* **26**, 13–17 (2011).
28. Sommershof, A. *et al.* Substantial reduction of naive and regulatory T cells following traumatic stress 1. *Brain Behav. Immun.* **23**, 1117–1124 (2009).
29. Anacker, C. *et al.* Antidepressants increase human hippocampal neurogenesis by activating the glucocorticoid receptor. *Mol. Psychiatry* **16**, 738–750 (2011).
30. Klug, M. & Rehli, M. Functional analysis of promoter CpG methylation using a CpG-free luciferase reporter vector. *Epigenetics* **1**, 127–130 (2006).
31. Kelder, T. *et al.* Mining biological pathways using WikiPathways web services 3. *PLoS ONE* **4**, e6447 (2009).
32. Danese, A. *et al.* Elevated inflammation levels in depressed adults with a history of childhood maltreatment 6. *Arch. Gen. Psychiatry* **65**, 409–415 (2008).
33. Pace, T.W. *et al.* Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress. *Am. J. Psychiatry* **163**, 1630–1633 (2006).
34. Attwood, B.K. *et al.* Neuropsin cleaves EphB2 in the amygdala to control anxiety. *Nature* **473**, 372–375 (2011).
35. Touma, C. *et al.* FK506 binding protein 5 shapes stress responsiveness: modulation of neuroendocrine reactivity and coping behavior 1. *Biol. Psychiatry* **70**, 928–936 (2011).
36. O'Leary, J.C. III *et al.* A new anti-depressive strategy for the elderly: ablation of FKBP5/FKBP51. *PLoS ONE* **6**, e24840 (2011).

© 2013 Nature America, Inc. All rights reserved.

ONLINE METHODS

Human samples. The initial patient cohort that we investigated is part of a larger study investigating the roles of genetic and environmental factors in predicting response to stressful life events in a predominantly African-American, highly traumatized, urban population of low socioeconomic status and now counts over 4,000 participants[3,37–39]. All procedures in this study were approved by the Institutional Review Boards of the Emory University School of Medicine and Grady Memorial Hospital. The samples for the replication study of 56 female subjects were recruited as part of the larger Conte Center Study for the Psychobiology of Early-Life Trauma[4,27]. The study was approved by the Institutional Review Board of Emory University School of Medicine.

Psychometric instruments. PTSD symptomatology was assessed by the mPSS as described previously[3]. The CAPS was used to assess current and lifetime PTSD diagnosis[3]. BDI was used to assess depressed mood in all individuals with the 21-item self report[3]. The CTQ was used as a continuous measure of childhood abuse and neglect in all individuals using the 28-item version of the CTQ[40]. For child abuse stratification, we used the subscales for sexual and physical abuse.

For the gene x environment interaction analyses, participants were dichotomized into two groups for each of the two categories of abuse (presence or absence of severe physical or sexual abuse according to CTQ). Cut-off scores of ≥13 for sexual abuse and ≥13 for physical abuse were used to define exposure to severe abuse in each of the categorized subjects. Finally, we created a 3-level categorical composite variable across the two abuse types, grouping participants into those with no severe exposure to any childhood abuse (scores less than cut-off for both types of abuse) and those with one or two exposures.

For the DNA methylation analyses, we divided participants into two groups for each of the three categories of abuse according to the presence or absence of moderate-to-severe physical, emotional or sexual abuse. Cut-off scores of ≥8 for sexual abuse, ≥13 for emotional abuse and ≥10 for physical abuse were used to define exposure to moderate to severe abuse in each of the categorized subjects. Finally, we created a categorical composite variable across the three abuse types, grouping participants into those with no moderate to severe exposure to any childhood abuse (scores less than cut-off for all types of abuse) and those with moderate to severe exposure to at least two types of abuse.

To allow for comparisons with previous studies[3], we also used the TEI, which assesses lifetime history of trauma exposure to a range of traumatic events, including childhood sexual and physical abuse, and was assessed in all individuals. The TEI child abuse variable was highly concordant with the categorizations using the CTQ. To summarize the level of exposure to trauma other than child abuse, we summed the total number of different types of non-child abuse trauma exposure (TEI) reported by each subject. In this cohort, the different types of non–child abuse trauma were experience at an average of 22.6 ± 10.6 years (mean ± s.d.) of age, and we refer to this type of trauma as adult trauma.

DNA and RNA collection and extraction. For genotyping, DNA was extracted either from saliva (Oragene DNA, DNA Genotek) or whole blood as described previously for the Grady trauma project[3], and from whole blood for the Conte center sample[4]. For pyrosequencing, genomic DNA was extracted from peripheral blood using the Gentra Puregene Blood Kit (Qiagen) as described previously[18]. DNA quality and quantity was assessed by a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and Quant-iT Picogreen (Invitrogen). For peripheral blood mRNA, blood was drawn between 8:00 and 9:00 a.m. into Tempus RNA tubes (Applied Biosystems) and stored at −20 °C until RNA extraction. RNA was isolated using the Versagene kit (Gentra Systems) and quantified using the Nanophotometer, and quality checks were performed on the Agilent Bioanalyzer as described previously[15].

Genotyping. In the Grady trauma cohort, genotype of rs1360780 was evaluated using TaqMan SNP genotyping assays (Applied Biosystems) and Sequenom i-plex assays (Sequenom). For imputation of SNPs in the *FKBP5* locus, we used genotypes from the Illumina OmniExpress array. In the Conte center cohort and the human hippocampal progenitor cell line, rs1360780 was evaluated using a TaqMan SNP genotyping assay[3].

Methylation analysis by bisulfite pyrosequencing. Methylation analysis by pyrosequencing of bisulfite-treated genomic DNA was performed by varionostic GmbH (http://www.varionostic.de/). Genomic DNA was bisulfite converted using the EZ DNA Methylation Gold Kit (Zymo Research). Amplicons were generated from bisulfite DNA covering four intronic glucocorticoids-responsive elements, the CpG island around the TSS and one region upstream of the TSS (**Fig. 4a** and **Supplementary Table 5**). Sequencing was performed on the Q24 System with PyroMark Q24 analysis software in CpG (Qiagen). Data were analyzed using IBM SPSS Statistics, Version 17, as indicated. For subsequent analysis of DNA, we combined single CpG measures to bins, assuming that the DNA methylation around a transcription factor binding site is a functional unit. We divided the CpGs in the sequenced regions of *FKBP5* into putative functional units according to their relative location to consensus GRE sites and chose the pyrosequencing primers accordingly. For example, in intron 7, three consensus GRE sites are described and their spatial relations to the CpG sites, the amplicon and sequenced regions are depicted in **Supplementary Figure 2**. CpGs in bin 1 are upstream of the 3 GREs, CpGs in bin 2 are surrounding and contained in the first consensus GRE, and the CpG in bin 3 is located in the third GRE. No CpGs were close to the second GRE.

Statistical methods. The gene × environment interaction results with the mPSS score as outcome were obtained from 1,963 African-American subjects with complete *FKBP5* genotype (rs1360780), CTQ and mPSS data and include individuals for which a gene × environment interaction had already been reported[3], adding 1,194 new individuals. In this analysis, we restricted ethnicity to African American only, whereas, in the previous study, individuals from other ethnic backgrounds were also included (less than 10%). In addition, 519 individuals were examined using the CAPS for lifetime PTSD diagnosis, with 368 individuals not included in the previous study[3]. Child abuse was defined using the categorical CTQ child abuse variable as described above. The *FKBP5* SNP rs1360780 was analyzed using a risk allele carrier model. A general linear model was used to analyze the effects on mPSS total score, with the 3-level categorical CTQ sexual and physical abuse variable described above, rs1360780, their interaction term, as well as age and sex as predictors. Controlling for current depressive symptom severity (using the BDI) and total types of trauma exposure (TEI) did not change the results. A logistic regression was used to analyze the effects of lifetime PTSD as assessed by the CAPS, with the categorical CTQ child abuse variable, rs1360780, in a risk allele carrier model, their interaction term, as well as age and sex as predictors. For *post hoc* analysis, a contingency table in the two risk genotype carrier groups tested for effects of severity of child abuse (CTQ child abuse 3 level variable) on the presence of lifetime PTSD.

SNPs within 304-kb segment containing the *FKBP5* locus (Chr6, 35553493–35858276, version hg18) were selected from the OmniExpress Human SNP array (Illumina). Genotypes of these SNPs in 192 subjects from the Grady trauma projects were used to impute all variants in this locus detected in the 1000 Genomes projects[19] version release June 2010 in Yorubans using Impute v.2 (ref. 41). The proximity of SNPs to GREs was assessed using data presented previously[20], as well as ChIP-Seq data for the region of imputation from the ENCODE project (http://genome.ucsc.edu/cgi-bin/hgTrackUi?g=wgEncodeTfBindingSuper)[42].

To examine DNA methylation of the *FKBP5* locus, we selected an extreme subset of individuals from the Grady trauma project that had experienced at least both physical and sexual child abuse ($N = 30$) versus individuals that had not experienced any child abuse according to the TEI and CTQ and with no current or lifetime PTSD or depression ($N = 45$). For data analysis, the percentage of DNA methylation was summed for CGs lying in the same sequencing primer (**Fig. 4a**,**b** and **Supplementary Table 5**) and used as the dependent variable in a general linear model that included trauma exposure, *FKBP5* risk allele carrier status, their interaction term, and age and sex as predictors. We corrected for multiple testing using a Bonferroni correction for the seven CG bins as well as three tests (main effect for trauma status, *FKBP5* risk allele carrier status and their interaction) with an alpha level of 0.05/21 ($P < 0.0023$) considered as statistically significant. In a second type of analysis, DNA methylation was correlated to the severity of child abuse using the $\log_{10}$-transformed continuous CTQ score in both the Grady Trauma Project and the Conte center project.

Bonferroni corrections were applied three times. First, when testing the DNA methylation in the seven CpG bins as outcome for main SNP effects, main environment effect and interaction effects that is a total of 21 tests, second when testing correlations between DNA methylation of bin 2 in intron 7 with volumetric magnetic resonance imaging data. Here the correction was for the four brain

© 2013 Nature America, Inc. All rights reserved.
npg

doi:10.1038/nn.3275

regions (hippocampal head, body, tail and amygdala), but not each hemisphere, as the volumes across hemispheres are highly correlated. Third, we corrected for multiple testing when performing the pathway analyses for gene–expression where WikiPathways automatically corrects for the number of pathways tested. For these analyses, the corrected *P* values – P_{corr} are presented.

RNA expression microarray and data analysis. RNA was amplified and hybridized onto Illumina HT-12 v3.0 arrays (Illumina) as described previously[15]. Raw microarray scan files from the Illumina BeadScan confocal laser scanner were exported using the Illumina Beadstudio program and loaded into R. The data were transformed and normalized using the variance stabilizing normalization[43]. A total of 15,877 probes passing the filter criteria of Illumina probe detection $P < 0.01$ in 5% of the individuals were used for subsequent analysis. The expression profiles were further normalized using ComBat, an empirical Bayes method for batch correction. Association analysis was performed using general linear models in R. The significance of association was estimated by two-tailed *P* values using the ANOVA *F* test. Correlations were calculated using the Pearson correlations functions in R. Pathway analysis was done using WebGestalt, a WikiPathways tool with Bonferroni correction for multiple testing. Global mRNA expression data were assessed in individuals from the Grady trauma project. From a total of 396 with mRNA expression measures, we selected cases ($N = 55$, physical and sexual abuse, mPSS score > 20) and controls ($N = 74$, no childhood abuse, mPSS < 20). These groups were subsequently stratified by *FKBP5* genotype into risk allele carrier status. To determine which transcripts are regulated by glucocorticoids, we regressed the gene expression profiles against the baseline cortisol levels, adjusting for age and gender in all 129 subjects. A total of 1,627 transcripts were significantly regulated by glucocorticoids with $P < 0.01$. For these transcripts, Pearson correlations between expression levels and baseline cortisol levels were calculated separately for cases and controls, further stratified by the *FKBP5* genotype carrier model (that is risk versus protective allele). The correlations between the two genotype groups were converted into *z* score differences for cases and controls. *z* score differences of <1.96 indicate significance at $P = 0.05$.

Reporter plasmid construction and transient transfection. The functional effect of differential methylation in intron 2 and intron 7 of *FKBP5* was analyzed using a CpG-free luciferase reporter construct[30] For intron 2, an 802-bp fragment containing the functional GRE was amplified from human genomic DNA with primer Intron2-F1 (containing a *Spe*I restriction site) and Intron2-R1 (containing a *Sbf*I restriction site). The reporter plasmid was digested with *Spe*I and PstI to release the original CMV enhancer fragment. The PCR amplicon was digested with *Sbf*I and *Spe*I, purified and subsequently ligated into the *Spe*I and PstI sites of the vector. The amplicon contains the single-nucleotide polymorphism rs1360780, and constructs with the A/T risk allele and the G/C protective allele were established (**Supplementary Table 5**). For intron 7, a 515-bp fragment was amplified from human genomic DNA with primer Intron7-F1 (containing a *Spe*I restriction site) and Intron7-R1 (containing a *Sbf*I restriction site). The amplicon was ligated into the vector backbone as described above. All constructs were verified by sequencing (for additional information on primers and plasmids, see **Supplementary Table 5**). *In vitro* methylation of the reporter constructs by *M.Sss*I CpG Methyltransferase (NEB) was carried out according to the manufacturer's instructions. Successful methylation was confirmed by restriction digest with *Pml*I (NEB), whereby linearization of the plasmids is blocked by CpG methylation. Methylated and unmethylated constructs were subsequently transfected into HEK-293 and HeLa cells (cultured in DMEM, supplemented with 10% fetal bovine serum, 1% sodium pyruvate and 1% antibiotic-antimycotic (vol/vol, Invitrogen)) using ExGen 500 *in vitro* Transfection Reagent (Fermentas) with Gaussia luciferase control vector for normalization of transfection efficacy. Firefly and Gaussia luciferase activity was measured in triplicates on a Tristar LB 941 luminometer with automatic injection system (Berthold Technologies). HEK 293 cells do not express the glucocorticoid receptor and are insensitive to dexamethasone stimulation. HeLa cells were transfected with the same constructs and successfully stimulated for 24 h with 50 nM dexamethasone. Data were analyzed using IBM SPSS Statistics, Version 17 as indicated.

TBP-DNA binding assay. To validate the protein DNA interaction of TBP with the genomic sequence at rs1360780, we used the EpiQuik General Protein-DNA Binding Assay Kit (P-2004, Epigentek) with 60-mer double-stranded oligonucleotide probes (**Supplementary Table 5**). The single-stranded oligonucleotides were resuspended in an equimolar concentration in 1× TE buffer and annealed by heating the mixture to 95 °C for 10 min and gradually cooling to 20 °C. The assay was performed according to the protocol of the manufacturer with 5 ng of purified recombinant TBP protein (PR-703, Jena Biosciences). We used antibody to TBP (ab818, 1 μg ml^{-1}) and horseradish peroxidase–conjugated antibody to mouse (ab6820, 0.5 μg ml^{-1}) from Abcam. Absorbance was measured at 450 nm at 20 °C on a Tecan Genios Pro microplate reader (Tecan). The assays were performed in triplicates. Background absorbance was subtracted from the measurement reading. Specificity of the interaction was tested according to the manufacturer's protocol by addition of fivefold excess of non–biotin-labeled oligonucleotide to the reaction.

Chromatin conformation capture. Chromatin conformation capture was carried out as described previously[44]. We used two human lymphoblastoid cell lines obtained from healthy individuals homozygous for the *FKBP5* SNP rs1360780 GG and AA genotypes, respectively. Cells were cultured in RMPI with stable L-glutamine (Biochrom) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Life Technologies). Crosslinking and cell lysis were performed as described[44]. Nuclei were digested using 400 U of EcoRI. Subsequent re-ligation, de-crosslinking and purification were conducted according to the protocol. DNA samples were adjusted to 20 ng μl^{-1} using the Quant-iT dsDNA BR Assay Kit and the Qubit 1.0 Fluorometer (Life Technologies). Digestion efficiency and sample purity was assessed as described previously[44]. Primers were designed with an anchor primer in the fragment containing the TSS and in potential interacting fragments in and around introns 2 and 7 of *FKBP5* using Primer3. Quantitative PCR was carried out using the Roche LightCycler 480 SYBR Green I Master on a Roche LC480 according to the instructions of the manufacturer (Roche). A 176-kb BAC clone (RP11-282I23) containing the complete *FKBP5* genomic sequence was obtained as a PCR control template from the BACPAC Resources Center of the Children's Hospital Oakland Research Institute. The BAC clone was cut with EcoRI and re-ligated by T4 DNA ligase. All primer pairs were tested on a standard curve of the BAC control library and yielded PCR efficiencies >1.7. The presence of a single PCR product was confirmed by agarose gel electrophoresis and melting curve analysis. Cycling conditions were: 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 20 s. Quantitative PCR data were normalized to *GAPDH* as a loading control and *ERCC3* to control for interaction frequencies between samples. GAPDH cycling conditions were 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 15 s, 72 °C for 20 s. *ERCC3* cycling conditions were 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 62 °C for 10 s, 72 °C for 25 s. Data analysis was carried out according to[44] and is presented as relative crosslinking frequency. The primers used for the chromatin conformation capture interaction studies are listed in **Supplementary Table 5**.

Cell culture of multipotent human hippocampal progenitor cells. Multipotent, human hippocampal progenitor cells (HPC03A/07, provided by ReNeuron) were grown in reduced modified media (RMM) as described previously[29]. To maintain proliferation, we added 10 ng ml^{-1} human bFGF (Peprotech), 20 ng ml^{-1} human EGF (Peprotech) and 100 nM 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich). To induce differentiation, HPC03A/07 cells were washed and cultured in RMM media without EGF, bFGF and 4-OHT. To assess changes in *FKBP5* methylation status in proliferating human hippocampal progenitor cells, we cultured HPC03A/07 cells in 25-cm^2 tissue culture flasks (Nunclon) for 72 h with 1 μM dexamethasone. To assess changes in *FKBP5* methylation under differentiation conditions, we treated HPC03A/07 cells for 72 h under proliferation conditions in RMM media containing EGF, bFGF and 4-OHT. After the 72-h proliferation phase, cells were washed twice for 15 min in RMM media without EGF, bFGF and 4-OHT, and subsequently cultured for another 7 d in media with 1 μM dexamethasone, but without growth factors. DNA was extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. Data were analyzed using IBM SPSS Statistics, Version 17, as indicated.

***Ex vivo* glucocorticoid receptor sensitivity.** To assess glucocorticoid receptor sensitivity, we used an *ex vivo* assay that estimates the degree of suppression of interleukin 6 secretion by the synthetic glucocorticoid, dexamethasone, in peripheral leukocytes, as described previously[45,46].

© 2013 Nature America, Inc. All rights reserved.

Endocrine measures. Serum cortisol levels were assessed in samples drawn at the same time as the whole blood samples used for DNA methylation analysis. Blood draws were performed between 8:00 and 9:00 a.m. in the Grady trauma project and at 12 p.m. in the replication sample. Cortisol levels were measured using a standard radioimmunoassay kit (Diagnostic System Laboratories).

Neuroimaging. Individuals were scanned in the Biomedical Imaging Technology Center at Emory University School of Medicine using a 3.0T Siemens Magnetom TIM Trio Scanner (Siemens Medical Solutions). We acquired high-resolution T1-weighted image scans with isotropic 1-mm resolution using a three-dimensional spoiled gradient echo acquisition protocol with sagittal volume excitation to achieve contrast and resolution sufficient for structural analysis. All images were corrected for non-uniformity[47], registered into standard stereotaxic space using the MNI template[48], and normalized for signal intensity to harmonize gray-white matter contrast across subjects. For the amygdala and hippocampal segmentation, a segmentation protocol was employed with shown reliability and validity[49].

37. Gillespie, C.F. *et al.* Trauma exposure and stress-related disorders in inner city primary care patients. *Gen. Hosp. Psychiatry* **31**, 505–514 (2009).
38. Bradley, R.G. *et al.* Influence of child abuse on adult depression: moderation by the corticotropin-releasing hormone receptor gene. *Arch. Gen. Psychiatry* **65**, 190–200 (2008).
39. Ressler, K.J. *et al.* Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature* **470**, 492–497 (2011).
40. Fink, L.A., Bernstein, D., Handelsman, L., Foote, J. & Lovejoy, M. Initial reliability and validity of the childhood trauma interview: a new multidimensional measure of childhood interpersonal trauma. *Am. J. Psychiatry* **152**, 1329–1335 (1995).
41. Howie, B.N., Donnelly, P. & Marchini, J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies 1. *PLoS Genet.* **5**, e1000529 (2009).
42. Birney, E. *et al.* Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project 1. *Nature* **447**, 799–816 (2007).
43. Huber, W., von, H.A., Sultmann, H., Poustka, A. & Vingron, M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression 7. *Bioinformatics* **18** (suppl. 1), S96–S104 (2002).
44. Hagège, H. *et al.* Quantitative analysis of chromosome conformation capture assays (3C-qPCR). *Nat. Protoc.* **2**, 1722–1733 (2007).
45. DeRijk, R.H., Petrides, J., Deuster, P., Gold, P.W. & Sternberg, E.M. Changes in corticosteroid sensitivity of peripheral blood lymphocytes after strenuous exercise in humans 2. *J. Clin. Endocrinol. Metab.* **81**, 228–235 (1996).
46. Miller, G.E., Rohleder, N., Stetler, C. & Kirschbaum, C. Clinical depression and regulation of the inflammatory response during acute stress 5. *Psychosom. Med.* **67**, 679–687 (2005).
47. Sled, J.G., Zijdenbos, A.P. & Evans, A.C. A nonparametric method for automatic correction of intensity nonuniformity in MRI data 4. *IEEE Trans. Med. Imaging* **17**, 87–97 (1998).
48. Collins, D.L., Neelin, P., Peters, T.M. & Evans, A.C. Automatic 3D intersubject registration of MR volumetric data in standardized Talairach space. *J. Comput. Assist. Tomogr.* **18**, 192–205 (1994).
49. Pruessner, J.C. *et al.* Volumetry of hippocampus and amygdala with high-resolution MRI and three-dimensional analysis software: minimizing the discrepancies between laboratories. *Cereb. Cortex* **10**, 433–442 (2000).

© 2013 Nature America, Inc. All rights reserved.

Allele–specific *FKBP5* DNA demethylation*:* a molecular mediator of gene–childhood trauma interactions

Torsten Klengel[1], Divya Mehta[1], Christoph Anacker[2], Monika Rex–Haffner[1], Jens C. Pruessner[3], Carmine M. Pariante[2], Thaddeus W.W. Pace[4], Kristina B. Mercer[8], Helen S. Mayberg[4], Bekh Bradley[5,4], Charles B. Nemeroff[6], Florian Holsboer[1], Christine M. Heim[4,7], Kerry J. Ressler[4,8,9], Theo Rein[1], and Elisabeth B. Binder[1,4]

1 Max Planck Institute of Psychiatry, Kraepelinstrasse 2–10, 80804 Munich, Germany

2 King's College London, Institute of Psychiatry, Section of Perinatal Psychiatry and Stress, Psychiatry and Immunology (SPI–lab), Department of Psychological Medicine, 125 Coldharbour Lane, SE5 9NU, London, UK

3 McGill Centre for Studies in Aging, Faculty of Medicine, McGill University, 6825 Boulevard LaSalle, Verdun, QC H4H 1R3, Canada

4 Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, 101 Woodruff Circle NE, Atlanta, GA 30322, USA

5 Atlanta VA Medical Center, 1670 Clairmont Road, Decatur, GA 300335, USA

6 Department of Psychiatry and Behavioral Sciences, University of Miami Miller School of Medicine, Clinical Research Building, 1120 Northwest 14th Street, Suite 1450, Miami, FL 33136, USA

7 Institute of Medical Psychology, Charité University Medicine Berlin, Luisenstrasse 57, 10117 Berlin, Germany

8 Yerkes National Primate Research Center, Emory University, 954 North Gatewood Road, N.E., Atlanta, GA 30329, USA

9 Howard Hughes Medical Institute, 4000 Jones Bridge Road, Chevy Chase, MD 0815–6789, USA

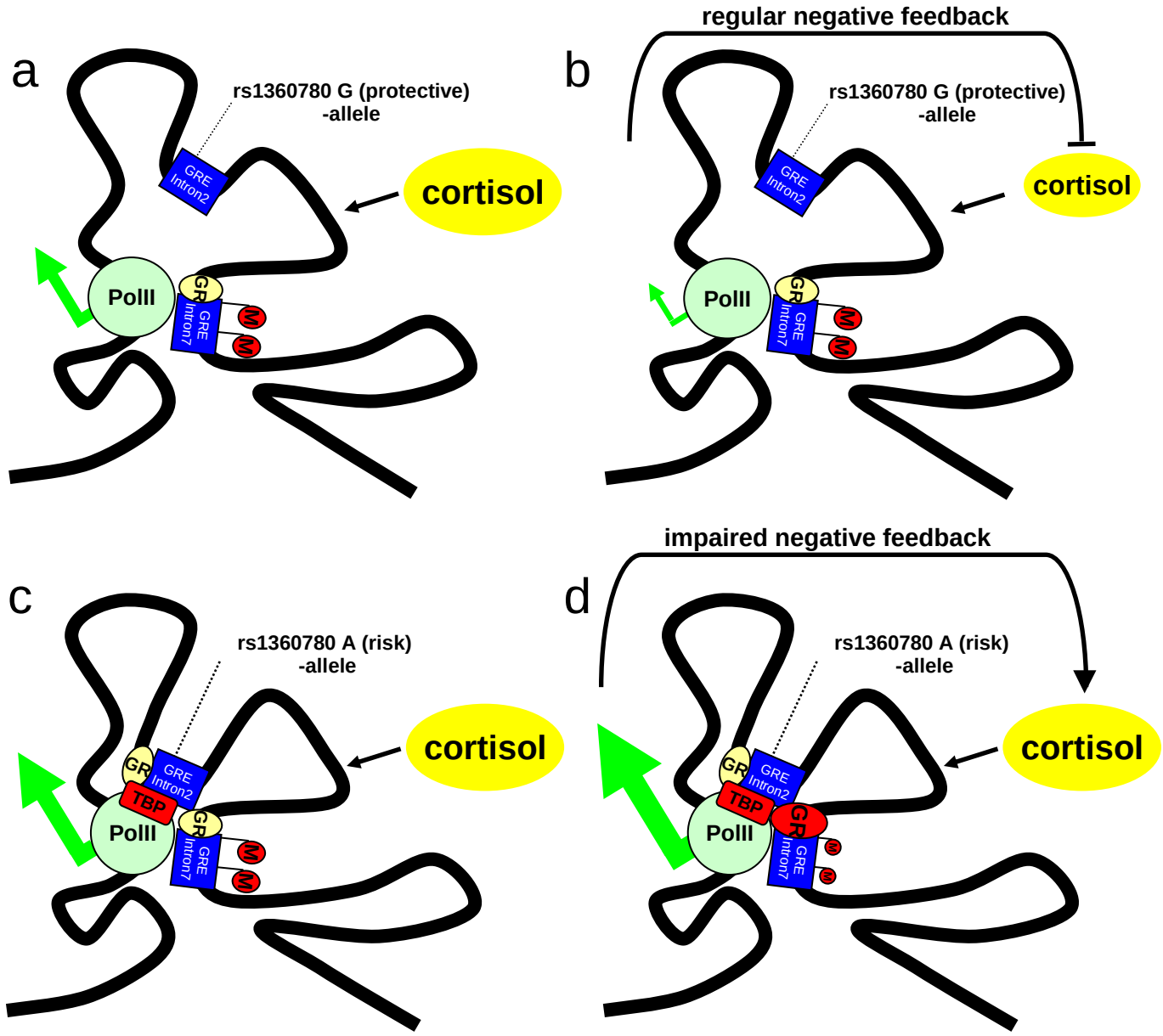
Nature Neuroscience: doi:10.1038/nn.3275

Supplementary Table 1

IDs and positions of SNPs in high linkage disequilibirium with rs1360780, rs9296158, rs9470080 and rs3800373 (r-squared 0.4 or higher).

#	Name	Position hg18	ObsHET	HWpval	MAF	Alleles	r-squared with risk haplotype SNPs
1	rs4711420	35603788	0.542	0.3096	0.474	T:G	0.4-0.8
2	rs6936515	35606241	0.516	0.7766	0.477	G:C	0.4-0.8
3	rs9368872	35607623	0.516	0.7766	0.477	G:C	0.4-0.8
4	rs9394303	35609923	0.505	1.0	0.482	G:A	0.4-0.8
5	rs2103681	35611440	0.505	1.0	0.482	C:T	0.4-0.8
6	rs7743152	35611553	0.505	1.0	0.482	A:G	0.4-0.8
7	rs7759620	35614539	0.505	1.0	0.482	G:C	0.4-0.8
8	rs7741320	35614543	0.505	1.0	0.482	T:C	0.4-0.8
9	rs7741804	35614795	0.484	0.8088	0.461	T:C	0.4-0.8
10	rs2894403	35615156	0.484	0.8088	0.461	T:C	0.4-0.8
11	rs2395631	35615292	0.495	1.0	0.466	A:G	0.4-0.8
12	rs11751447	35618099	0.484	0.8088	0.461	C:T	0.4-0.8
13	rs9368874	35622058	0.516	0.786	0.482	T:C	0.4-0.8
14	rs9394304	35622515	0.51	0.909	0.49	T:C	0.4-0.8
15	rs11307625	35625231	0.49	0.9526	0.453	A:T	0.4-0.8
16	rs9800506	35625367	0.474	0.2456	0.315	G:T	0.4-0.8
17	rs873941	35632854	0.5	0.0939	0.328	T:A	0.4-0.8
18	rs6911901	35636971	0.583	0.0226	0.453	C:G	0.4-0.8
19	rs3800373	35650453	0.526	0.5663	0.477	A:C	>= 0.8
20	rs7757037	35656213	0.464	0.8976	0.346	G:A	0.4-0.8
21	rs3798345	35670448	0.542	0.3222	0.484	C:T	>= 0.8
22	rs9296158	35675059	0.542	0.3167	0.479	A:G	>= 0.8
23	rs2395634	35675737	0.542	0.3222	0.484	G:A	>= 0.8
24	rs16878812	35677539	0.411	1.0	0.284	A:G	0.4-0.8
25	rs11966198	35683633	0.396	0.3357	0.312	A:G	0.4-0.8

26	rs3777747	35686979	0.448	0.8633	0.354	A:G	0.4-0.8
27	chr6:35693257	35693257	0.401	0.9536	0.284	A:C	0.4-0.8
28	rs9368878	35693591	0.521	0.6901	0.495	C:T	>= 0.8
29	chr6:35696209	35696209	0.432	0.8393	0.299	C:T	0.4-0.8
30	rs16879318	35698368	0.432	0.8393	0.299	A:C	0.4-0.8
31	rs34356639	35700253	0.417	1.0	0.292	T:A	0.4-0.8
32	rs7747121	35701960	0.432	0.8393	0.299	A:G	0.4-0.8
33	rs10947562	35704889	0.427	0.4661	0.276	C:T	0.4-0.8
34	rs16879378	35705680	0.432	0.8393	0.299	A:C	0.4-0.8
35	rs3798347	35709753	0.526	0.5856	0.492	A:T	>= 0.8
36	rs1591365	35712084	0.542	0.2547	0.448	A:G	>= 0.8
37	chr6:35712673	35712673	0.417	1.0	0.292	T:A	0.4-0.8
38	rs7751598	35713177	0.51	0.9126	0.495	A:G	>= 0.8
39	rs1360780	35715548	0.484	0.7587	0.482	C:T	>= 0.8
40	rs6902124	35718285	0.49	0.8753	0.479	A:C	>= 0.8
41	rs9380525	35741015	0.484	0.7677	0.477	G:C	>= 0.8
42	rs9368881	35742265	0.479	0.6649	0.474	A:G	0.4-0.8
43	rs9348980	35747030	0.458	0.6866	0.391	T:A	0.4-0.8
44	rs9470080	35754412	0.479	0.6649	0.474	T:C	>= 0.8
45	chr6:35759185	35759185	0.464	0.8179	0.341	C:G	0.4-0.8
46	rs3800372	35763222	0.495	1.0	0.466	G:A	>= 0.8
47	rs9380526	35766304	0.484	0.7677	0.477	C:T	>= 0.8
48	rs6902321	35778584	0.51	0.1561	0.354	T:C	0.4-0.8



Supplementary Figure 1. Long-distance interaction of Glucocorticoid Responsive Elements in *FKBP5* and suggested epigenetic mechanism of trauma induced demethylation.

(a) *FKBP5* mRNA transcription is induced by cortisol via a three-dimensional interaction and loop formation of predominantly distal enhancer regions harbouring GREs, that are located in the promoter region, intron 2, 5 and 7 with the core promoter site. Only interactions confirmed by 3C are depicted by blue boxes. The GR as activating transcription factor is represented in light yellow (PolII = RNA Polymerase II).

(b) When the acute stressor is over, a negative feedback mechanism via the GR leads to a normalization of cortisol levels in carriers of the G (protective) -allele of rs1360780 and in consequence less transcriptional effects on GR target genes, including *FKBP5* (*FKBP5* transcription is represented by green arrows).

(c) The A (risk) -allele of rs1360780 confers a stronger interaction of intron 2 with the promoter region and is associated with an increased binding of TATA box binding protein (TBP, represented in red), potentially by formation of an additional TATA-box like sequence in intron 2. This leads to a stronger transcriptional induction by cortisol in carriers of the risk allele vs. carriers of the protective genotype as depicted in panel (a) and indicated by the green arrow. In risk allele carriers, the initial rise in cortisol and GR activation leads to higher levels of FKBP5, which have been associated with an impaired negative feedback of the stress hormone axis and prolonged elevations of cortisol levels after a single stressor.

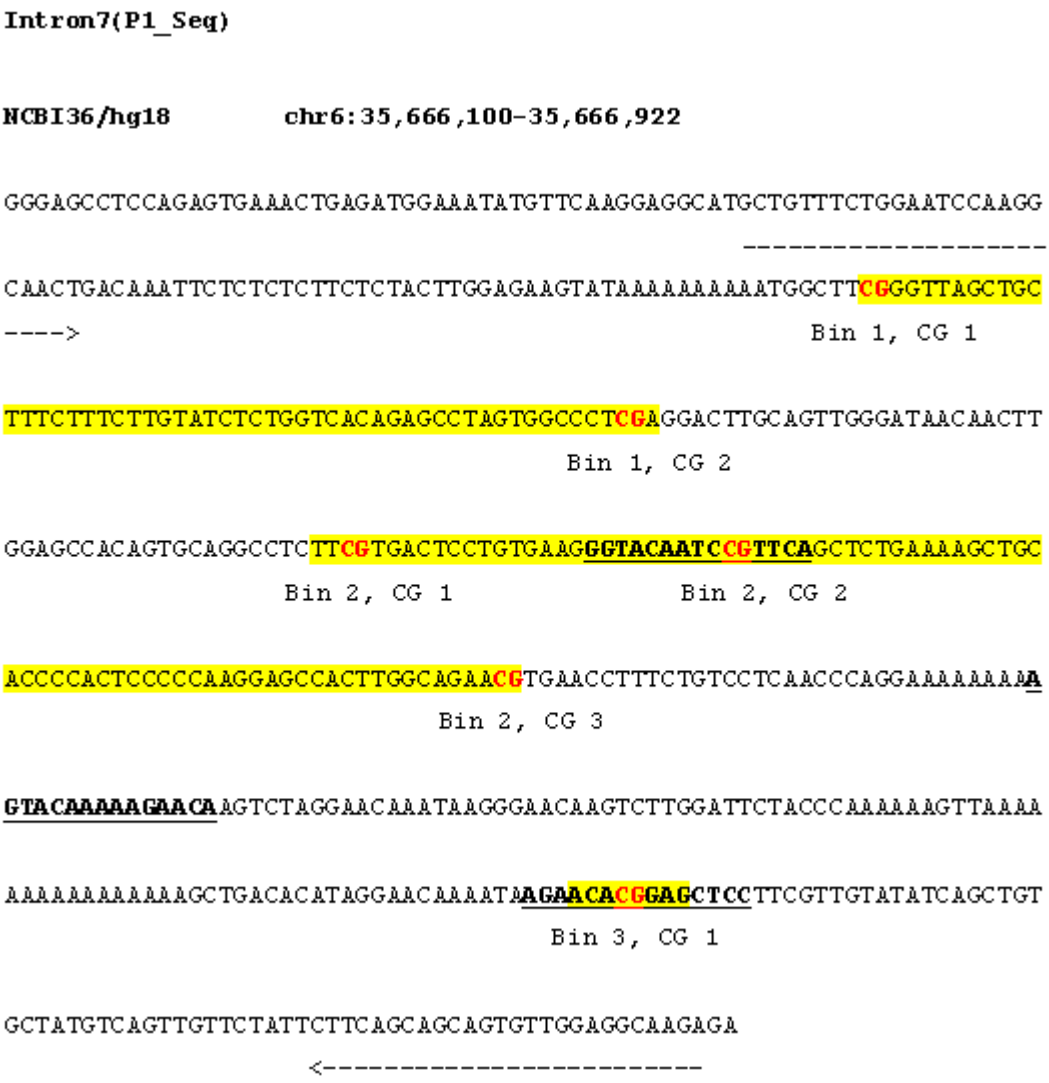
(d) This long-lasting cortisol exposure then induces DNA demethylation around functional GREs in intron 7 (depicted by red ‘M’ circles). This further enhances the genetic predisposition for a stronger GR-induced transcription of *FKBP5* (stronger GR activation in intron 7 represented by the red GR element), leading to changes in GR sensitivity.

Supplementary Table 2

Primers for bisulfite pyrosequencing and genomic location of the regions investigated

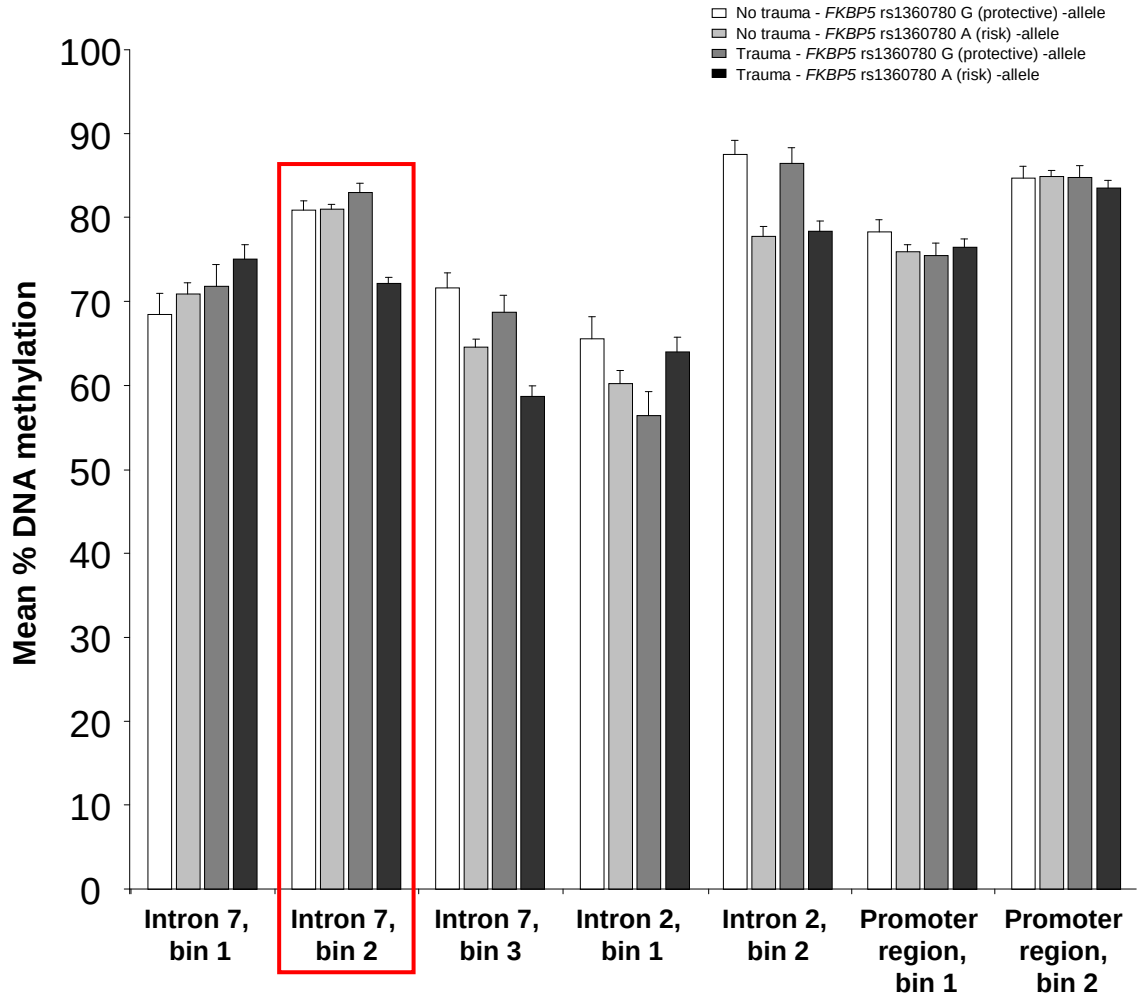
Amplicon name and genomic location (hg18)	Amplicon primer (Bisulfit-DNA), name	Amplicon primer (Bisulfit-DNA), Sequence 5'-3'	Sequencing primer, name	Sequencing primer, Sequence 5'-3'	No. Of CpG dinucleotides interrogated
P1	P1_F_MPI	GTTGTTTTTGGAATTTAAGGTAATTG			
		BIOTIN-			
chr6:35,666,288-35,666,763	P1_R_MPI	TCTCTTACCTCCAACACTACTACTAAAA			
Intron 7			P1_S1	AAGTATAAAAAAAAAATGGTTT	
			P1_S2	TGGAGTTATAGTGTAGGTTTT	
			P1_S3	GTTGATATATAGGAATAAAATAAGA	6
P2	P2_F_MPI	BIOTIN-ATTTAAAGGGGGAGGGA			
chr6:35,677,658-35,677,924	P2_R_MPI	CCTTTTTTCCCCCCTAT			
Intron 5			P2_S1	TCCCCCCTATTTTAATC	
			P2_S2	ACTCCAATTTAAATAATATTTTAC	5
P3	P3_F_MPI	GGAAAAGTTGAGAATTATTGTATTG			
chr6:35,686,677-35,687,097	P3_R_MPI	CAAAATATTCTACAAATTCCAATTAT			
Intron 5			P3_S1	GAGAATTATTGTATTGGAGG	
			P3_S2	TGTGTATTTTTGTTTAAGTTTT	
			P3_S3	TGGAGAGGGAAAGGAG	4
P4	P4_F_MPI	TGTATAGGTTTGTAGTTTTGGAGTAGT			
chr6:35,715,732-35,716,027	P4_R_MPI	BIOTIN-CCTTTCTCAAATTCAATTTATTCAC			
Intron 2			P4_S1	TTTGGAGTAGTAGGTTAAA	
			P4_S2	TTTAGAATATATTTTTGTTTTTAAG	4
P5	P5_F_MPI	GTTTTGAATTATATTGAAGGGTATTT			
location according to P10	P5_R_MPI	CAAAACTCCTTATACTCTTCTATTCTAA			
TSS			P5_S1	TGAATTATATTGAAGGGTATTTA	
			P5_S2	GGTTAGTTAATAGAAGAAAGTTATTT	15
P6	P6_F_MPI	GTTTTAGAATAGAAGAGTATAAGGAGTT			
location according to P10	P6_R_MPI	ACCTAACCAAACTATAAAAAAAATAATA			

TSS			P6_S1	AGAATAGAAGAGTATAAGGAGTTT	
			P6_S2	GATAGAGGAGATGAGGTTTTTTAA	25
P7	P7_F_MPI	GAGGAGGTAGGTGAAGG			
location according to P10	P7_R_MPI	CCCCAAATAACAAATCC			
TSS			P7_S1	GGTTATTTAGTTATTATTTTTTTTAT	
			P7_S2	AAAGAGAAGTTTGGTTTT	39
P8	P8_F_MPI	BIOTIN-GGAGAGGGATTTGTTATTT			
location according to P10	P8_R_MPI	CTACCTCAACTCCCACC			
TSS			P8_S1	CTCAACTCCCACCCC	
			P8_S2	CCTCCACCCCACTAC	
			P8_S3	CCCCCCCACCTCTCCC	43
P9	P9_F_MPI	GGGGTGGGAGTTGAGGTA			
location according to P10	P9_R_MPI	BIOTIN-ACAACCCCCAAACCCTATAA			
TSS			P9_S1	GGTGGGAGTTGAGGTA	
			P9_S2	TTTATTTTAGTGGTTATG	
			P9_S3	TTTTTAGTTAGTTTAGTAGG	49
P10	P10_F_MPI	GATAGGAGGGGTAGTGTTTTTAGAGA			
Amplicon P5 to P10 are located on chr6:35,763,522-35,764,770	P10_R_MPI	BIOTIN-CAAAACCCCCCTTCCATAAA	P10_lower_S1	GGGTAGTGTTTTTAGAGAGA	
			P10_lower_S2	AAGGGTAAGGGTTAGATA	
TSS			P10_lower_S3	GGGTTTTATTTGTATGGA	19
GRE-3	GRE3-F-MPI	GGTTGTAGGGAATTATGA			
chr6:35,798,260-35,798,490	GRE3-R-MPI	BIOTIN-TAACCAAACAAAACAAAAATA			
			GRE3_S1_MPI	GGGAATTATGAGGTTG	
			GRE3_S2_MPI	TTTTTAGTGTTTATTAGAG	6
				Sum of all CGs interrogated	**215**



Supplementary Figure 2. Bin structure of *FKBP5* intron 7.

FKBP5 intron 7 bins were selected according to their spatial and functional relationship to consensus GREs and the technological limitations of pyrosequencing. There are 3 consensus GRE bindings sites (sequences in bold and underlined) within the chosen 476 bp amplicon of intron 7 (primer position indicated as dashed arrows). Interrogated CpGs are highlighted in red/bold. Since pyrosequencing can only reliably generate short reads, we had to select three sequencing primers (see Supplementary Table 5) to cover the CpGs of interest (bold and red) and each high quality sequencing read is highlighted in yellow. The bin structure was thus determined by the relative position of the CGs to the consensus GREs as well the length limitation of the pyrosequencing reads. In intron 7 the CGs in bin 1 were upstream of all GREs, the 3 CGs of bin 2 are surrounding the first GRE and bin 3 represents the CG within the third GRE.



Supplementary Figure 3. *FKBP5* x childhood abuse interaction on DNA methylation.

For statistical analysis CpGs are combined into bins of 1-3 CpGs with spatial and likely functional proximity. The analysis showed that *FKBP5* DNA methylation in intronic regions is genotype and trauma dependent. Intron 7, bin 2 revealed significant trauma ($F_{73,1}$ = 8.2, P = 0.006), genotype ($F_{73,1}$ = 34.33, P < 0.001) and interaction between genotype and trauma effect ($F_{73,1}$ = 31.01, P_{corr} < 0.001, Bonferroni corrected for 7 CG bins and 3 tests (main effect of trauma, *FKBP5* rs1360780 genotype and their interaction) (age and gender as covariates). A significant genotype effect (age and gender as covariates as above) was observed in intron 2, bin 2 ($F_{70,1}$ = 27.88, P < 0.001, no trauma or interactive effects) and intron 7, bin 3 ($F_{73,1}$ = 26.39, P < 0.001, no trauma or interactive effects). For intron 2, bin 1 we observed no main trauma (P = 0.222) or genotype (P = 0.593) effect but the interaction revealed a nominal significant interaction (P = 0.006, not surviving Bonferroni correction for multiple testing, P = 0.126 for 21 tests). No other bins showed a significant effect of trauma, genotype or their interaction term.

Supplementary Table 3

Correlation between Intron 7, Bin 2 methylation and CTQ scores, Grady and Conte cohort. The differences on correlation between CTQ sexual abuse in the Conte cohort and the Grady cohort are most likely caused by a differential traumatization of both cohorts with less sexual abuse in the Conte cohort (see also Table 1).

Grady trauma prolect cohort

	FKBP5 risk allele carrier N=55		**FKBP5 protective allele carrier N=19**			
	Intron 7, Bin 2	**p value**	**Intron 7, Bin 2**	**p value**	**Fisher z score**	**p value (two tailed)**
CTQ total	-0.646	<0.001	0.414	0.078	-4.23	<0.001
Log CTQ total	-0.690	<0.001	0.410	0.081	-4.49	<0.001
Log CTQ physical abuse	-0.641	<0.001	0.378	0.111	-4.05	<0.001
Log CTQ sexual abuse	-0.656	<0.001	0.599	0.007	-5.17	<0.001
Log CTQ emotional abuse	-0.685	<0.001	0.321	0.181	-4.1	<0.001

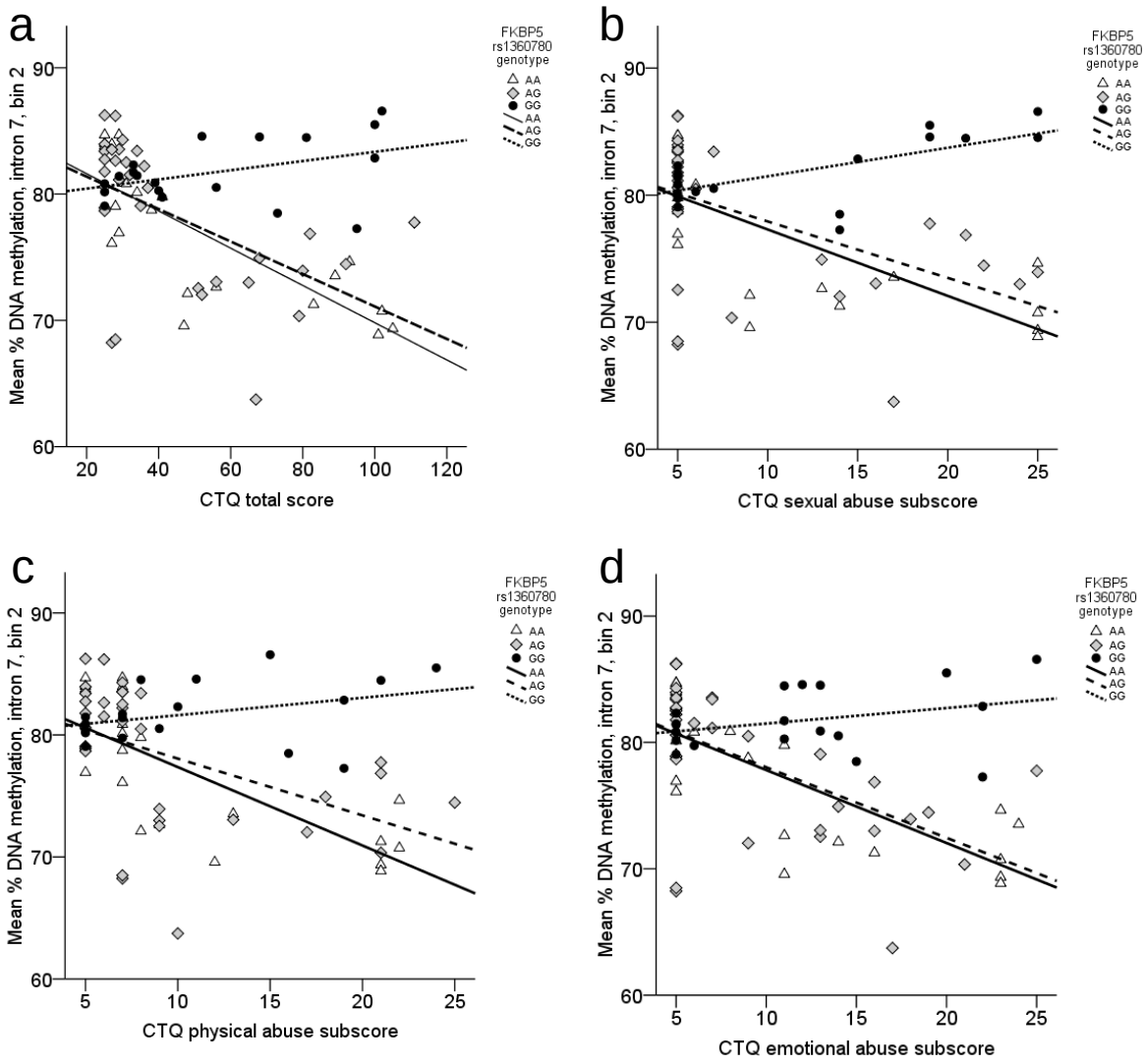
	partial correlation with age and gender df=51		**partial correlation with age and gender df=15**	
	Intron 7, Bin 2	**p value**	**Intron 7, Bin 2**	**p value**
CTQ total	-0.678	<0.001	0.423	0.091
Log CTQ total	-0.718	<0.001	0.429	0.086
Log CTQ physical abuse	-0.700	<0.001	0.423	0.091
Log CTQ sexual abuse	-0.690	<0.001	0.570	0.017

Log CTQ emotional abuse	-0.707	<0.001	0.377	0.136		

Conte Center cohort

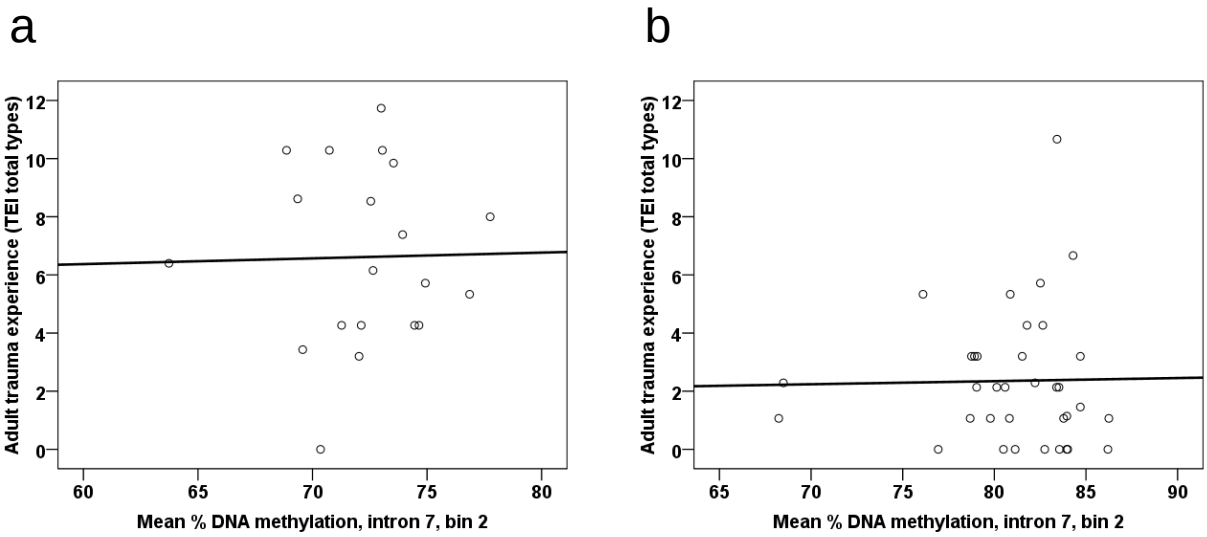
	FKBP5 risk allele carrier N=33		**FKBP5 protective allele carrier N=23**			
	Intron 7, Bin 2	**p value**	**Intron 7, Bin 2**	**p value**	**Fisher z score**	**p value (two tailed)**
CTQ total	-0.259	0.145	0.158	0.471	-1.47	0.141
Log CTQ total	-0.273	0.124	0.153	0.485	-1.5	0.133
Log CTQ physical abuse	-0.397	0.022	0.246	0.258	-2.33	0.019
Log CTQ sexual abuse	0.118	0.514	0.305	0.922	-0.68	0.496
Log CTQ emotional abuse	-0.397	0.022	0.022	0.922	-1.53	0.126

	partial correlation with age df=30		**partial correlation with age df=20**	
	Intron 7, Bin 2	**p value**	**Intron 7, Bin 2**	**p value**
CTQ total	-0.274	0.128	0.197	0.379
Log CTQ total	-0.255	0.159	0.172	0.433
Log CTQ physical abuse	-0.411	0.019	0.243	0.276
Log CTQ sexual abuse	0.121	0.508	0.324	0.141
Log CTQ emotional abuse	-0.394	0.025	0.016	0.944



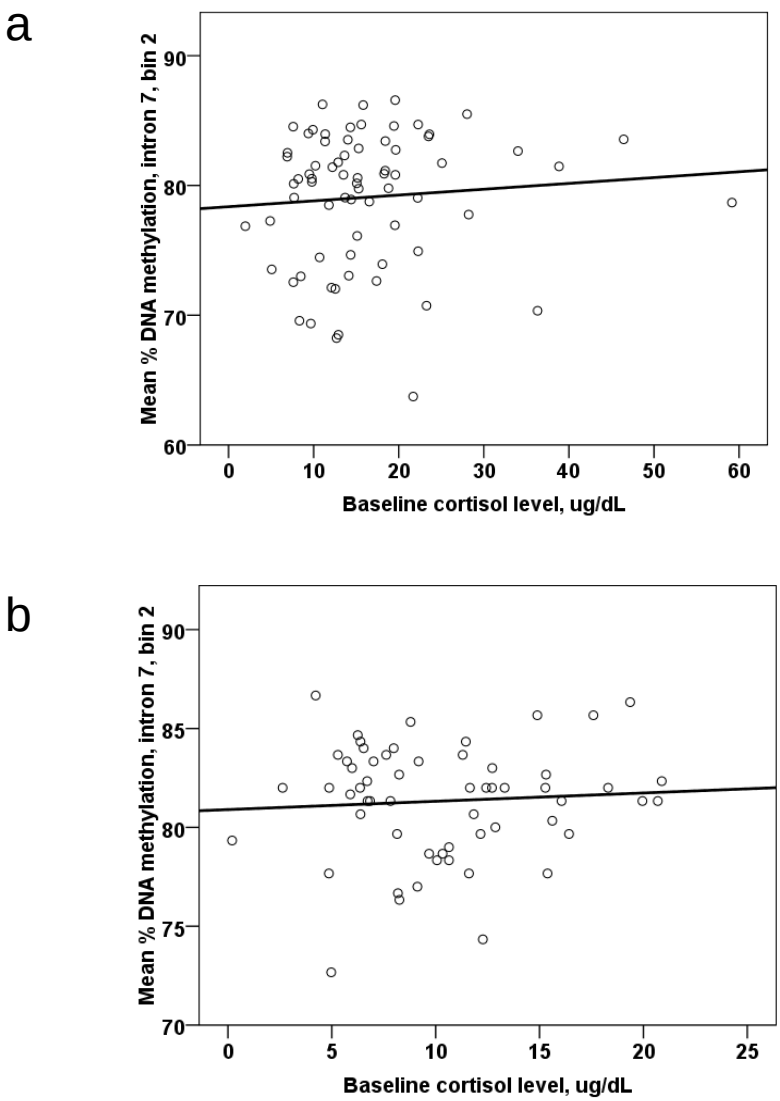
Supplementary Figure 4. A single allele confers *FKBP5* demethylation in response to childhood abuse.

The correlation of the trauma exposure (overall (a) and subsequently divided into sexual, physical and emotional abuse (b-d)) and DNA methylation in intron 7 for individuals of the Grady cohort separated for the homozygous risk (AA), heterozygous (AG) and homozygous protective (GG) genotype. The panels above show that heterozygous individuals show a response similar to homozygous risk carriers, suggesting that a single allele conferring increased *FKBP5* transcription would already lead to an enhanced cortisol response to stress and thus similar demethylation in homozygous and heterozygous risk allele carriers.



Supplementary Figure 5. *FKBP5* methylation and adult trauma.

Absence of correlation of the types of adult trauma experience with *FKBP5* intron 7 bin 2 methylation in both risk allele carriers (a) with (R = 0.202, *P* = 0.933, N = 20) and (b) without (R = 0.018, *P* = 0.917, N = 35) child abuse.



Supplementary Figure 6. *FKBP5* and current cortisol levels.

Absence of correlation of baseline cortisol levels with mean % DNA methylation in intron 7, bin 2. Baseline cortisol levels in the Grady trauma project (a) and the Conte replication cohort (b) are not correlated with mean % DNA methylation in intron 7, bin 2 (R = 0.083, *P* = 0.489, N = 72 and R = 0.069, *P* = 0.603, N = 59, respectively).

Supplementary Table 4

Cortisol x transcript correlation for Grady Trauma Project cohort, cases

Illumina HT v3 Probe NID	Gene Symbol	Correlation of transcript expression with baseline cortisol, p-value	Pearson correlation of transcript expression with baseline cortisol in cases risk genotype, r-score	Pearson correlation of transcript expression with baseline cortisol in cases protective genotype, r-score	Fisher z score between genotypes in cases
ILMN_1670723	MSL3L1	2,00E-004	-0,15	0,87	3,402
ILMN_2326591	ANXA6	8,57E-003	0,11	-0,87	3,272
ILMN_1780898	PRKCH	1,37E-003	-0,33	-0,94	3,203
ILMN_2380698	DSTN	6,65E-003	0,06	-0,87	3,148
ILMN_1713156	MSL3L1	1,54E-004	-0,07	0,86	3,088
ILMN_2415529	CDK5RAP2	1,61E-003	-0,21	0,81	3,029
ILMN_1695025	CD2	8,89E-003	-0,21	-0,90	2,894
ILMN_1787461	RUNX3	1,62E-003	-0,43	-0,94	2,888
ILMN_2387285	MSL3L1	2,95E-003	-0,16	0,80	2,859
ILMN_1736806	PAG1	1,52E-003	-0,15	0,79	2,785
ILMN_1748283	PIM2	7,94E-003	-0,25	0,75	2,773
ILMN_1736026	MMP8	1,53E-005	0,07	0,86	2,719
ILMN_1681301	AIM2	1,45E-003	-0,24	0,74	2,708
ILMN_1688279	PVRIG	3,27E-003	-0,11	-0,86	2,697
ILMN_2404539	C20orf30	4,47E-003	0,05	-0,81	2,686
ILMN_2056479	TXNL2	5,74E-003	0,17	-0,76	2,648
ILMN_1813671	SLC25A1	1,01E-003	-0,24	-0,89	2,637
ILMN_1741755	TRIM29	3,76E-005	0,31	-0,67	2,589
ILMN_1792092	ZCCHC8	3,84E-003	-0,58	0,45	2,581
ILMN_1770824	ARHGAP4	5,29E-003	-0,42	0,60	2,576
ILMN_1677920	LTF	4,92E-005	-0,06	0,79	2,572
ILMN_1708041	PLEKHF1	3,28E-003	-0,31	-0,90	2,553
ILMN_1660732	PPP2R2B	8,79E-003	-0,12	-0,84	2,519

ILMN_1699496	PHF21A	3,25E-004	0,19	0,86	2,517
ILMN_1703123	AXUD1	8,84E-005	-0,03	0,79	2,465
ILMN_1692572	KIF27	5,49E-003	0,67	-0,27	2,453
ILMN_2072178	ECHDC3	3,88E-010	0,31	-0,64	2,441
ILMN_1703316	LOC255783	2,50E-003	0,16	0,84	2,421
ILMN_1703697	LANCL1	2,54E-004	-0,15	-0,84	2,407
ILMN_2307598	SLC37A3	1,03E-003	0,02	0,79	2,399
ILMN_1708934	ADM	8,48E-003	0,09	0,82	2,396
ILMN_1852022	KIAA1881	5,16E-004	0,29	0,87	2,366
ILMN_1699991	LCK	3,45E-004	-0,19	-0,84	2,358
ILMN_1753342	SAT1	1,27E-003	0,15	0,83	2,343
ILMN_1791328	STK39	4,02E-003	-0,14	-0,83	2,337
ILMN_1789596	ETV6	7,40E-003	-0,12	0,72	2,321
ILMN_1754811	FBXO38	1,26E-003	0,18	0,83	2,303
ILMN_1733627	NEDD4L	5,29E-003	-0,27	0,63	2,297
ILMN_2316236	HOPX	2,05E-003	-0,28	-0,86	2,288
ILMN_1798886	NUDT21	2,86E-003	-0,19	-0,83	2,286
ILMN_2061405	NUP54	8,22E-003	-0,26	-0,85	2,281
ILMN_1666713	LYPLA1	4,94E-003	-0,18	0,68	2,280
ILMN_2405602	OSBPL1A	9,68E-008	0,15	0,82	2,268
ILMN_1733421	PRKCQ	4,63E-003	-0,22	-0,84	2,267
ILMN_1758418	TNFSF13B	5,41E-003	-0,15	0,69	2,265
ILMN_1781184	MYBPC3	1,72E-003	0,15	0,82	2,252
ILMN_1751958	NSUN5	1,53E-003	-0,51	0,41	2,246
ILMN_1667371	SLA	5,73E-006	0,77	0,03	2,233
ILMN_1907834		6,58E-008	0,55	-0,36	2,225
ILMN_1739914	ZNF618	2,30E-003	0,32	-0,57	2,215
ILMN_1784292	ANKMY2	1,52E-005	0,02	-0,74	2,212
ILMN_1767193	CR1	1,10E-005	0,15	0,81	2,209
ILMN_1705679	SAFB2	5,64E-003	-0,49	0,41	2,191
ILMN_1812281	ARG1	4,18E-008	0,23	0,83	2,172
ILMN_1770963	NAALADL1	3,92E-004	0,03	-0,73	2,170
ILMN_1808566	TMEM180	4,06E-005	0,25	0,84	2,154
ILMN_1789839	GTF3C1	6,28E-003	-0,14	-0,80	2,146
ILMN_1706598	ACPL2	4,36E-003	-0,09	0,69	2,144

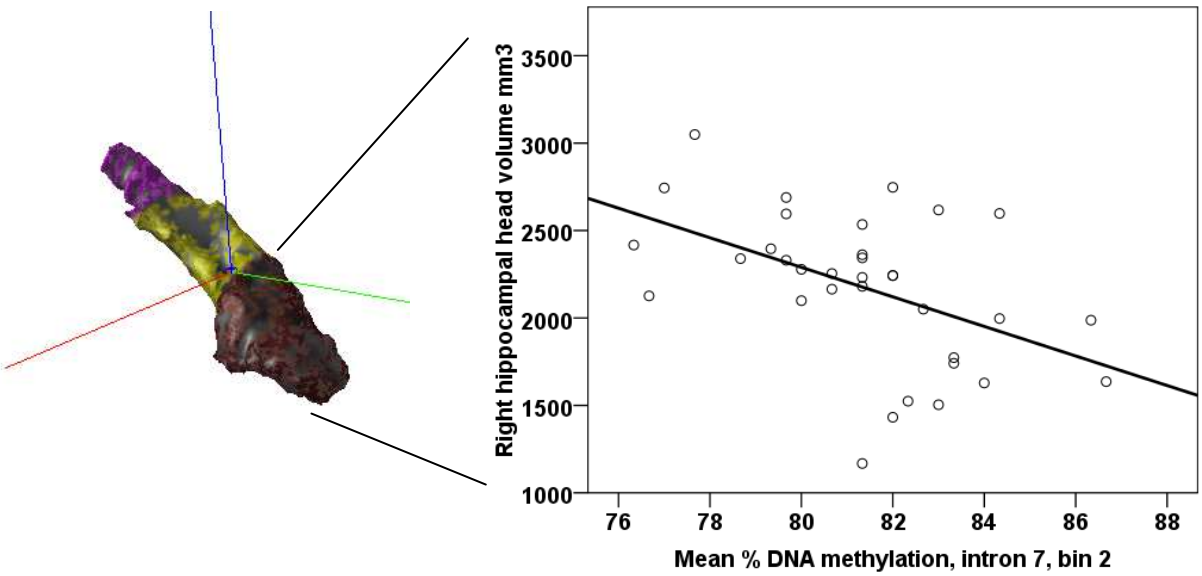
ILMN_2215355	LOC400499	2,02E-003	0,14	0,80	2,143
ILMN_1688034	COIL	6,12E-004	-0,28	-0,84	2,101
ILMN_1784287	TGFBR3	4,10E-004	-0,41	-0,88	2,096
ILMN_1737157	GRAMD1A	5,34E-004	0,21	0,81	2,085
ILMN_1669447	PYHIN1	5,63E-003	-0,46	-0,89	2,083
ILMN_1661461	LOC283547	1,82E-003	0,51	-0,35	2,076
ILMN_1775569	CRISP2	3,91E-003	0,43	-0,43	2,064
ILMN_1796146	EIF4E3	1,32E-003	-0,05	0,69	2,055
ILMN_1701837	KLHL2	8,44E-003	0,63	-0,17	2,054
ILMN_2051232	SDHA	1,05E-003	-0,25	-0,82	2,043
ILMN_2207291	IFNG	5,50E-003	0,07	-0,68	2,042
ILMN_1801307	TNFSF10	8,50E-003	-0,05	0,69	2,029
ILMN_2377109	LCK	1,60E-003	-0,04	-0,73	2,020
ILMN_1765880	C16orf57	7,16E-004	0,33	0,84	2,015
ILMN_1666902	GPR114	8,18E-004	-0,31	-0,84	2,002
ILMN_1701731	AKR1B1	2,38E-004	-0,19	-0,79	2,000
ILMN_1660976	LOC653204	2,00E-003	-0,20	0,59	1,984
ILMN_1764431	COPS6	5,23E-004	-0,15	-0,77	1,972

Cortisol x transcript correlation for Grady Trauma Project cohort, controls

Illumina HT v3 Probe NID	Gene Symbol	Correlation of transcript expression with baseline cortisol, p-value	Pearson correlation of transcript expression with baseline cortisol in controls risk genotype, r-score	Pearson correlation of transcript expression with baseline cortisol in controls protective genotype, r-score	Fisher z score between genotypes in controls
ILMN_1656910	TRIM6	3,46E-003	0,01	0,80	3,306
ILMN_1697268	EMILIN2	2,24E-003	-0,13	0,73	3,217
ILMN_1743570	CEACAM3	6,09E-003	0,19	0,84	3,124
ILMN_2404625	LAT	1,99E-004	-0,20	-0,84	3,093
ILMN_1783500	HOXD12	2,88E-005	0,34	0,87	2,973

ILMN_1654685	MCTP1	5,37E-004	0,19	0,82	2,929
ILMN_1788540	LOC732075	6,23E-003	0,42	-0,45	2,831
ILMN_1808979	CLEC4D	1,00E-003	0,26	0,83	2,800
ILMN_2231985	STCH	7,10E-003	0,38	-0,47	2,764
ILMN_2371055	EFNA1	5,33E-003	0,44	-0,38	2,649
ILMN_1871311		6,06E-003	0,36	-0,45	2,616
ILMN_2326512	CASP1	2,30E-003	0,09	0,74	2,612
ILMN_1810066	LOC650140	3,95E-003	0,38	-0,42	2,574
ILMN_1717594	DKFZp761E198	1,60E-003	0,25	0,80	2,560
ILMN_1722981	TLR5	7,90E-006	0,28	0,81	2,549
ILMN_1780698	ZFYVE19	3,03E-004	-0,45	0,33	2,513
ILMN_1758798	LOC728734	1,10E-003	-0,08	-0,72	2,513
ILMN_1688423	FCER1A	2,12E-003	-0,45	0,31	2,445
ILMN_1788002	MAPK14	5,72E-003	0,24	0,78	2,431
ILMN_1804988	MOAP1	5,99E-003	-0,24	-0,78	2,431
ILMN_1755954	CPEB3	1,47E-003	0,19	0,75	2,370
ILMN_2339705	MED8	3,10E-003	-0,03	0,63	2,342
ILMN_1678494	ZNF438	7,93E-003	0,20	0,75	2,339
ILMN_1688098	TBC1D4	1,90E-003	-0,20	-0,75	2,339
ILMN_1752526	RNF144B	1,06E-006	0,27	0,78	2,334
ILMN_1771861	LOC642250	4,01E-003	0,50	-0,21	2,315
ILMN_1859493		1,71E-003	0,42	-0,30	2,299
ILMN_1719696	PLD1	2,07E-003	0,18	0,73	2,268
ILMN_1778177	ZNF207	6,33E-003	-0,47	0,22	2,228
ILMN_1747347	C17orf60	4,98E-003	-0,09	0,56	2,196
ILMN_1707901	DHrs7B	9,34E-003	0,19	0,72	2,172
ILMN_1872182		6,24E-003	0,41	-0,27	2,163
ILMN_1881526		8,78E-004	-0,10	-0,67	2,157
ILMN_1673933	LOC440341	2,22E-003	-0,05	-0,64	2,150
ILMN_1678504	RHOT1	1,24E-004	0,20	0,72	2,140
ILMN_1763322	CCR3	8,02E-003	-0,45	0,21	2,119
ILMN_2412927	GMPPB	8,82E-003	-0,39	0,27	2,091
ILMN_2323944	FAM110A	1,91E-004	-0,38	0,28	2,088
ILMN_2140389	TMEM185A	9,21E-003	-0,16	-0,69	2,085
ILMN_1718207	SETDB1	7,75E-003	-0,44	0,21	2,081

ILMN_1678766	DYNLT1	1,06E-003	0,20	0,71	2,078
ILMN_1893129		4,19E-003	0,45	-0,19	2,056
ILMN_1724668	REPS2	3,16E-004	0,29	0,75	2,048
ILMN_1674394	C20orf3	1,98E-003	0,29	0,75	2,048
ILMN_1769282	FRMD6	9,51E-003	0,41	-0,23	2,034
ILMN_1709820	AOAH	7,76E-003	0,04	0,61	2,031
ILMN_1752333	SLC35E1	9,49E-003	-0,06	-0,62	2,019
ILMN_2397776	ASB6	3,92E-003	-0,11	-0,65	2,019
ILMN_1732615	ASMTL	6,51E-004	-0,46	0,16	2,000
ILMN_1752111	SMARCAL1	9,52E-003	-0,44	0,18	1,987
ILMN_1785266	OFD1	8,37E-004	-0,04	-0,60	1,983
ILMN_1794165	PGD	3,31E-003	0,23	0,71	1,983
ILMN_1747205	JDP2	6,84E-003	0,25	0,72	1,981
ILMN_2152028	LOC642452	5,08E-004	0,49	-0,11	1,963



Supplementary Figure 7. *FKBP5* DNA methylation correlates with hippocampal volume.

The left panel of the figure shows the 3D reconstruction of the right hippocampus with the three subregions – head (brown), body (yellow) and tail (purple) from the analyzed data set. The right panel shows that *FKBP5* intron 7, bin 2 mean methylation is negatively correlated with right hippocampal head volume (R = -0.484 *P* = 0.014, Bonferroni corrected, N = 34). For the left hippocampal head volume we also observed a negative correlation, however, it failed to reach nominal significance (R = –0.283, *P* = 0.105). These findings illustrate that early adverse experiences with subsequent enduring epigenetic changes in peripheral tissue specifically correlate with structural changes in the hippocampal head in the absence of more global correlations with brain morphology.

Supplementary Table 5

Plasmids and primer used in this study

Cloning of the reporter assay constucts

Primer	**Sequence 5' – 3'**
Intron2-F1	tctacACTAGTGCCCTTATTCTATAGCTGCAAGTC *Spe*I restriction site underlined
Intron2-R1	tctacCCTGCAGGTCACTGCTCACTCGCTGA *Sbf*I restriction site underlined
Intron7-F1	tctacACTAGTGGGAGCCTCCAGAGTGAAAC *Spe*I restriction site underlined
Intron7-R1	tctacCCTGCAGGCCAACACTGCTGCTGAAGAA *Sbf*I restriction site underlined

Plasmid	**Description**
pCpGL-CMV/EF1	CpG free reporter plasmid containing the basal EF-1α promoter, the CMV enhancer and an optimized luciferase gene. The plasmid was kindly provided by Maja Klug and Martin Rehli, University of Regensburg, Germany
pCpGL-Int2A	802 bp fragment of FKBP5 Intron 2 ligated into pCpGL-CMV(del)/EF1 containing the A allele of rs9296158
pCpGL-Int2G	802 bp fragment of FKBP5 Intron 2 ligated into pCpGL-CMV(del)/EF1 containing the G allele of rs9296158
pCpGL-Int7	515 bp fragment of FKBP5 Intron 7 ligated into ppCpGL-CMV(del)/EF1
pCpGL-EF1	Control vector, containing the EF-1α promoter and luciferase gene without CMV enhancer element. The plasmid was kindly provided by Maja Klug and Martin Rehli, University of Regensburg, Germany

Oligonucleotides for the TATA-Box binding protein assay

	Biotin-
TBP-AF	GCAGTAGCAAGTAAGAATTTTTGTTTTGT**A**TAACTTTGCTTATGTGAAAGCCTTCTGTGC
TBP-AR	Biotin-GCACAGAAGGCTTTCACATAAGCAAAGTTA**T**ACAAAACAAAAATTCTTACTTGCTACTGC
	Biotin-
TBP-GF	GCAGTAGCAAGTAAGAATTTTTGTTTTGT**G**TAACTTTGCTTATGTGAAAGCCTTCTGTGC
TBP-GR	Biotin-GCACAGAAGGCTTTCACATAAGCAAAGTTA**C**ACAAAACAAAAATTCTTACTTGCTACTGC

rs1360780 alleles are highlighted in bold, the putative TATA-box binding side is underlined

Oligonucleotides for Chromatin Conformation Capture

TSSanchor	CTACAATTTCAGTTTCAAAAATTAAGCA
Int2_prae1	TGCAGTGGTACCGTCATAGC
Int2	GGCAAGCCAATGTAACCAAC
Int2_post1	GGAGCTTGGAAAGAGCACAG
Int2_post2	TTCTGCTCACCAAGATAACTGC
Int7_prae1	AATCTCACGGGACCACTGTC
Int7	TATTCTGGCAGCACCTACCC
Int7_post1	CTGGGAAAGAAAGGGAGACC
Int7_post2	TGGCAAAATCATTCCCACTT
GAPDH-F1	TACTAGCGGTTTTACGGGCG
GAPDH-R1	TCGAACAGGAGGAGCAGAGAGCGA
ERCC3-F1	TCTTACCTGTTGGCCACTGACA
ERCC3-F2	GTCTGACCTTGCCCAGTGATAG

Paper II

Mehta D, **Klengel T**, Conneely KN, Smith AK, Rex-Haffner M, Loeschner A, Gonik M, Mercer KB, Bradley B, Müller-Myhsok B, Ressler KJ, Binder EB. Childhood maltreatment is associated with distinct genomic and epigenetic profiles in posttraumatic stress disorder. Proc. Natl. Acad. Sci. USA. 110(20), 8302-8307, 2013 doi: 10.1073/pnas.1217750110

Childhood maltreatment is associated with distinct genomic and epigenetic profiles in posttraumatic stress disorder

Divya Mehta[a,1], Torsten Klengel[a], Karen N. Conneely[b], Alicia K. Smith[c], André Altmann[a], Thaddeus W. Pace[c,d], Monika Rex-Haffner[a], Anne Loeschner[a], Mariya Gonik[a], Kristina B. Mercer[e], Bekh Bradley[c,f], Bertram Müller-Myhsok[a], Kerry J. Ressler[c,e,g], and Elisabeth B. Binder[a,c]

[a]Max Planck Institute of Psychiatry, 80804 Munich, Germany; [b]Department of Human Genetics, [c]Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, GA 30322; [d]Winship Cancer Institute, Emory University, Atlanta, GA 30322; [e]Howard Hughes Medical Institute, Chevy Chase, MD 20815-6789; [f]Veteran's Affairs Medical Center, Decatur, GA 30033; and [g]Yerkes National Primate Research Center, Atlanta, GA 30322

Edited by Bruce S. McEwen, The Rockefeller University, New York, NY, and approved March 19, 2013 (received for review October 12, 2012)

Childhood maltreatment is likely to influence fundamental biological processes and engrave long-lasting epigenetic marks, leading to adverse health outcomes in adulthood. We aimed to elucidate the impact of different early environment on disease-related genome-wide gene expression and DNA methylation in peripheral blood cells in patients with posttraumatic stress disorder (PTSD). Compared with the same trauma-exposed controls (n = 108), gene-expression profiles of PTSD patients with similar clinical symptoms and matched adult trauma exposure but different childhood adverse events (n = 32 and 29) were almost completely nonoverlapping (98%). These differences on the level of individual transcripts were paralleled by the enrichment of several distinct biological networks between the groups. Moreover, these gene-expression changes were accompanied and likely mediated by changes in DNA methylation in the same loci to a much larger proportion in the childhood abuse (69%) vs. the non-child abuse-only group (34%). This study is unique in providing genome-wide evidence of distinct biological modifications in PTSD in the presence or absence of exposure to childhood abuse. The findings that nonoverlapping biological pathways seem to be affected in the two PTSD groups and that changes in DNA methylation appear to have a much greater impact in the childhood-abuse group might reflect differences in the pathophysiology of PTSD, in dependence of exposure to childhood maltreatment. These results contribute to a better understanding of the extent of influence of differences in trauma exposure on pathophysiological processes in stress-related psychiatric disorders and may have implications for personalized medicine.

epigenome | biomarkers | psychiatry | development

Childhood maltreatment is a complex problem that exerts an enormous impact on individuals, families, and society, and is of great significance for public health. Maltreatment during childhood likely influences fundamental biological processes and engraves long-lasting epigenetic marks, leading to adverse health outcomes in adulthood (1). Exposure to adverse life events in childhood has not only been linked to an increased susceptibility for a number of psychiatric disorders, but also to cardiovascular disease, diabetes, and chronic lung disease, possibly via long-term influences on the immune system (2–9). This finding suggests that early adverse experiences not only alter neurobiological systems leading to an increased risk for psychiatric disorders, but may have a long-lasting effect on a number of organ systems. Experience of childhood abuse might influence these biological systems via epigenetic modifications conferring lifelong susceptibility to disease. In fact, in humans, distinct epigenetic and gene-expression changes have been observed in postmortem brains of suicide victims with a history of childhood abuse compared with suicide victims without a history of childhood abuse or control subjects (10).

Early life trauma is a strong risk factor for stress-related psychiatric disorders, which themselves have been shown to be associated with distinct changes in gene-expression and epigenetic profiles, both in the brain as well as in peripheral tissues (11–13). Posttraumatic stress disorder (PTSD) is one example of a common and disabling disorder that occurs after exposure to potentially life-threatening traumatic events during childhood and adulthood. Individuals who experience early life stress have been shown to develop PTSD in adulthood more often than individuals with no history of early life stress (14–16), and are also more likely to be exposed to traumatic events in adulthood (17, 18). PTSD and other stress-related disorders can thus occur both in the presence and absence of early life trauma.

It has been suggested that the impact of trauma can depend on the type and timing of the adverse events, and this in turn determines downstream consequences, such as perturbation of biological pathways (19–21). Although individuals with PTSD with or without a history of exposure to childhood maltreatment meet diagnostic criteria for the same disorder, it is not clear whether disease would be associated with similar biological modifications.

The central aim of this study was to search for evidence at the genome-wide level of such distinct disease-related biological modifications associated with the presence or absence of a history of childhood maltreatment. This goal was addressed by interrogating the influence of early life trauma on gene-expression and epigenetic signatures in immune cells occurring in patients with PTSD as an example of a stress-related psychiatric disorder.

Results

In the present study we selected 169 individuals, all of whom had experienced at least two types trauma other than childhood abuse. Of these individuals, 108 had a trauma history but did not have a life-time history of PTSD or current PTSD symptoms, and these were thus considered trauma-exposed controls. Sixty-one individuals met criteria for current PTSD, of which 32 reported a history of childhood maltreatment and 29 did not report childhood abuse. Clinical and epidemiological demographics of the participants across the three investigated groups are depicted in Table 1. To control for the observed significant group differences in age, sex, ethnicity, adult trauma severity, and substance abuse, these variables were used as covariates in the association analyses.

Effects of Childhood Maltreatment on PTSD-Associated Transcriptional Profiles. To test for gene-expression differences between individuals with PTSD and trauma-exposed controls, linear regression

Author contributions: B.B., K.J.R., and E.B.B. designed research; D.M., T.K., A.K.S., T.W.P., M.R.-H., A.L., and K.B.M. performed research; D.M., K.N.C., A.A., M.G., and B.M.-M. analyzed data; and D.M., T.K., K.N.C., A.K.S., A.A., T.W.P., M.R.-H., A.L., M.G., K.B.M., B.B., B.M.-M., K.J.R., and E.B.B. wrote the paper.

Conflict of interest statement: K.J.R. has an unrelated role as cofounder of Extinction Pharmaceuticals for development of *N*-methyl-D-aspartate-based therapeutics.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: mehta@mpipsykl.mpg.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1217750110/-/DCSupplemental.

Table 1. Distribution and comparisons of demographic and clinical variables of the samples included in the study

	Demographics			Group comparisons		
Sample	Controls (n = 108)	PTSD + child abuse (n = 32)	PTSD no child abuse (n = 29)	P value Controls ~ PTSD + child abuse	P value Controls ~ PTSD no child abuse	P value PTSD + child abuse ~ PTSD no child abuse
Age – mean (SD)	44.23 (1.2)	39.56 (1.8)	43.69 (2.0)	0.059	0.044	0.134
Sex						
Men	30 (27.8%)	5 (15.6%)	13 (44.8%)	0.244	0.113	0.023
Women	78 (72.25%)	27 (84.4%)	16 (55.2%)			
Ethnicity						
African American	101 (93.5%)	24 (75%)	25 (86.2%)	0.006	0.245	0.343
Other	7 (6.5%)	8 (25%)	4 (13.8%)			
PSS – mean (SD)	3.49 (0.32)	35.06 (1.2)	29.83 (1.7)	1.89×10^{-72}	1.14×10^{-52}	0.011
CAPS current – mean (SD)	26.19 (3.3)	112.73 (9.8)	79.71 (10.2)	1.68×10^{-17}	4.46×10^{-9}	0.054
PTSD diagnosis						
No	108 (100%)	0	0	2.59×10^{-32}	1.4×10^{-26}	1
Yes	0	32 (100%)	29 (100%)			
BDI mean (SD)	8.08 (0.7)	30.13 (2.3)	25.27 (2.1)	2.36×10^{-24}	8.47×10^{-18}	0.128
Child abuse (CTQ) – mean(SD)	34.35 (1.0)	76.23 (3.5)	37.24 (2.0)	4.69×10^{-32}	0.206	5.15×10^{-9}
No of types of child abuse (moderate to severe)						
None	75 (69.4%)	0	29 (100%)	1.19×10^{-21}	0.317	2.54×10^{-13}
One	21 (19.4%)	0	0			
Two	12 (11.2%)	32 (100%)	0			
Total types adult trauma (TEI) – mean (SD)	4.63 (0.23)	7.91 (0.6)	6.49 (0.6)	4.69×10^{-9}	0.005	0.154
Substance abuse current						
No	103 (95.4%)	28 (87.5%)	25 (86.2%)	0.111	0.077	0.881
Yes	5 (4.6%)	4 (12.5%)	4 (13.8%)			
PTSD treatment lifetime						
No	108 (100%)	24 (72%)	24 (82.8%)	8.9×10^{-5}	0.03	0.352
Yes	0	8 (28%)	4 (17.2%)			

models were built to assess the influence of the case status on expression profiles. A total of 303 transcripts were differentially expressed between PTSD individuals with child abuse vs. controls, and 244 transcripts were differentially expressed between PTSD individuals without child abuse vs. controls after corrections for multiple testing (adjusted $P < 0.05$) (Fig. 1 and Dataset S1). An overlap of only 14 transcripts was observed (2%), indicating distinct biomarker profiles in PTSD in the presence or absence of child abuse. This overlap is no more than expected by random chance (Fisher's exact P value = 0.095). Post hoc analysis identified that only 4% of the differentially expressed transcripts might be confounded by lifetime PTSD treatment and only 1.5% of the transcripts might be confounded by different proportions of immune cells types, suggesting that these factors are not major confounds in the current analysis (Dataset S1). Among the differentially expressed transcripts, we successfully validated the gene-expression changes in the corresponding groups by using quantitative PCR (qPCR) in five of five tested transcripts (Dataset S2).

Correlation of Gene-Expression Differences with DNA Methylation Changes. Childhood maltreatment has an influence on biological processes via epigenetic modifications (22). To identify if the above gene-expression changes were correlated with DNA methylation differences in loci encoding these transcripts, we investigated DNA methylation profiles of the transcripts differentially regulated in either of the PTSD case groups versus controls (n = 547) using the Illumina 450 k HumanMethylation array. Transcripts with at least one CpG within their respective gene locus on the Illumina HumanMethylation 450 k array (n = 304) were assessed for methylation differences in the same subjects tested for gene-expression differences. Although at least one significantly differentially methylated CpG site was identified in 69.3% of transcripts unique to PTSD cases with child abuse, only 33.6% of transcripts unique to PTSD cases without child abuse also showed differences on the DNA methylation level for at least one CpG after correction for multiple testing. This difference was even more accentuated in transcripts for which the locus showed significant differences in DNA methylation in five or more CpGs; here, 11.7% of the transcripts showed such matching epigenetic differences in the PTSD child abuse group, but this was only the case for 0.8% of the transcripts from the PTSD non-child abuse-only group (Fig. 2 *A* and *B*, and Dataset S3). DNA methylation results were validated for seven CpG sites in two distinct loci using an independent method, the Sequenom EpiTYPER (Dataset S4).

The relation between DNA methylation and gene expression is complex. In line with other studies (23, 24), we observe negative correlations of CpGs with gene expression close to the transcription start sites and an increasing proportion of positive correlations in proximal and distal regulatory sites (Fig. 2*B* and Dataset S5).

Compared with all tested CpGs within the differentially expressed transcripts, the CpGs that also show significantly different methylation levels are more often located close to the 3′ UTR and in the body of the gene as well as in the shelves of the CpG islands and the open sea. In addition, the vast majority of these CpGs were hypermethylated in the case group (Fig. 2*C*). In addition, even though the absolute differences in methylation levels between the groups are small (for most less than 5%), the direction of the differences in DNA methylation between the case and the control groups agrees to a large extent (73% for individual CpGs and 94% at the locus level) with the observed group differences in gene expression (see Fig. 3 and Dataset S5 for more detail).

These results indicate that changes in gene expression are more often associated with changes in DNA methylation in PTSD occurring after child abuse than PTSD occurring in the absence of child abuse, and that these changes in DNA methylation may indeed mediate the observed gene-expression changes.

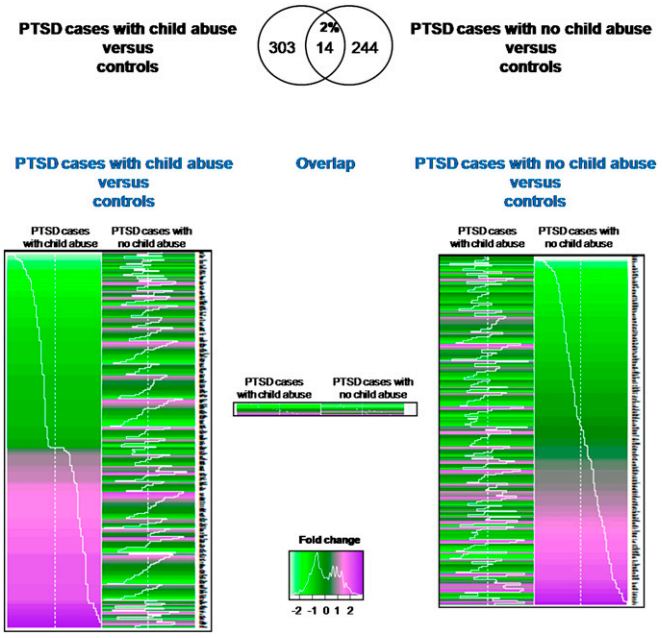


Fig. 1. Influence of child abuse on gene expression profiles in peripheral blood in PTSD. (*A*) Venn diagram representing the overlap of transcripts significantly differentially regulated between individuals with PTSD and child abuse vs. trauma-exposed controls and individuals with PTSD without child abuse vs. trauma-exposed controls. (*B*) Heatmap of differentially expressed transcripts. Gene-expression fold-changes in comparison with the controls is depicted. Up-regulation is depicted in magenta and down-regulation is depicted in bright green.

Enrichment of Biological Processes Among Differentially Expressed Transcripts. As we observed differences in gene-expression profiles and epigenetic marks in PTSD occurring in the presence or absence of childhood maltreatment, we next sought to better comprehend how the transcripts interacted with each other. We built subnetworks from the transcript relationships using a modified gene-set enrichment analysis to identify overrepresented networks in Ariadne Pathway Studio 8.0.

Although functional annotation revealed overlapping pathways between the groups, such as cell survival, cell development, cell migration, cell adhesion, T-cell activation, and immunity networks, several nonoverlapping networks were observed between the groups. The most notable differences included the enrichment of the central nervous system development and tolerance induction pathways in the PTSD group with child abuse and the apoptosis and growth rate networks in the PTSD group without child abuse (Fig. S1 and Dataset S6).

Interestingly, despite the completely divergent transcripts differentially expressed between the two groups, several overlapping networks were identified, suggesting a possible common downstream effect of disease in blood cells.

Discussion

The objective of this study was to interrogate biomarker profiles at the level of gene expression and DNA methylation in individuals with PTSD with or without childhood abuse. We observed that although robust gene-expression differences could be identified for both PTSD groups compared with trauma-exposed controls, the significant changes hardly overlapped at the transcript-specific level (2%) and only modestly (35%) at the biological pathway level. In fact, the differentially expressed transcripts in each group showed highly variable regulation and often the opposite pattern in the respective other group (Fig. 1). Moreover, the extent of epigenetic modifications (i.e., DNA methylation changes) concurrent with these gene-expression changes were up to 12-fold higher in the childhood trauma-exposed PTSD group. Our results imply that biological perturbations in individuals with PTSD who have a history of childhood maltreatment may systematically and meaningfully differ from those individuals with PTSD who do not have a history of childhood maltreatment. These findings suggest that taking into account exposure to childhood maltreatment is of great importance in the search for and application of gene-expression and DNA methylation signatures as biomarkers for PTSD and other trauma-related disorders.

Despite the fact that the differentially expressed transcripts in each PTSD group were distinct, similar cellular processes enriched across both transcript groups, suggesting that these initially different changes could be associated with perturbation of common downstream biological pathways. On the other hand, several specific cellular processes were significantly overrepresented only in one of the PTSD groups, indicating that different biological pathways maybe altered in PTSD with or without child abuse.

Long-term effects of child abuse may be mediated by epigenetic modifications, especially DNA methylation changes (10, 25, 26). The fact that we see up to 12 times as many differentially expressed transcripts also showing differences in DNA methylation in the early abuse group seems consistent with this hypothesis and may even suggest that the biological mechanisms leading to the observed differences in gene expression are distinct depending on the type of trauma exposure. Although the measured changes in DNA methylation were small (98% were less than 5%), the fact that we observe the expected directional association of DNA methylation with gene expression for 94% of the loci (Fig. 3 and Dataset S5), suggests that even such modest methylation differences could be of functional relevance. Comparison of DNA methylation profiles between brain and blood has demonstrated that interindividual DNA methylation differences were the highest in peripheral blood and, even though several DNA methylation signatures were tissue-specific, interindividual variability patterns were consistent across the tissues, suggesting that peripheral blood might reflect at least some of the DNA methylation changes in the brain (27). Furthermore, a recent study in rhesus macaques demonstrated that DNA methylation changes occurring in response to early-life adversity were persistent into adulthood and reflected in both prefrontal cortex and T cells (28). Our finding that the top enriched biological process in the transcripts from peripheral blood cells in PTSD with childhood trauma in central nervous system development may be an indication of such potential blood/brain overlap.

The analyses were performed using a common set of trauma-exposed controls, not matched by exposure to childhood maltreatment. This approach allowed identifying distinct biomarker profiles between the two case groups. The use of two distinct but trauma-matched controls groups would make it more difficult to determine if the differences originate in the case or the control groups. We acknowledge that this approach has limitations and therefore performed a post hoc analysis using matched controls with or without child abuse and did not see any differences in the percentage of overlap in differentially expressed genes (Dataset S7) between the two PTSD case group comparisons, indicating that these results are not likely confounded by the study design. Furthermore, comparisons of the transcripts defining the signature of child abuse [by (*i*) direct comparison within PTSD cases and (*ii*) comparison of individuals with and without child abuse using the whole sample] with transcripts significantly different between any of the two PTSD case groups and controls showed a minimal overlap (0.7%), suggesting that the gene-expression signature of PTSD with and without child abuse is distinct from the signature of child abuse within PTSD cases (Dataset S7).

Another potential confound of these results is the possibility that the observed gene-expression and DNA methylation profiles are only a reflection of differences in peripheral blood cell types. Comparison with transcripts correlated with the relative amount of the main immune cells subtypes (CD15–CD16, CD14, CD4, and CD8) revealed that only up to 1.5% of the transcripts might be confounded by differential cell counts. The possibility of the

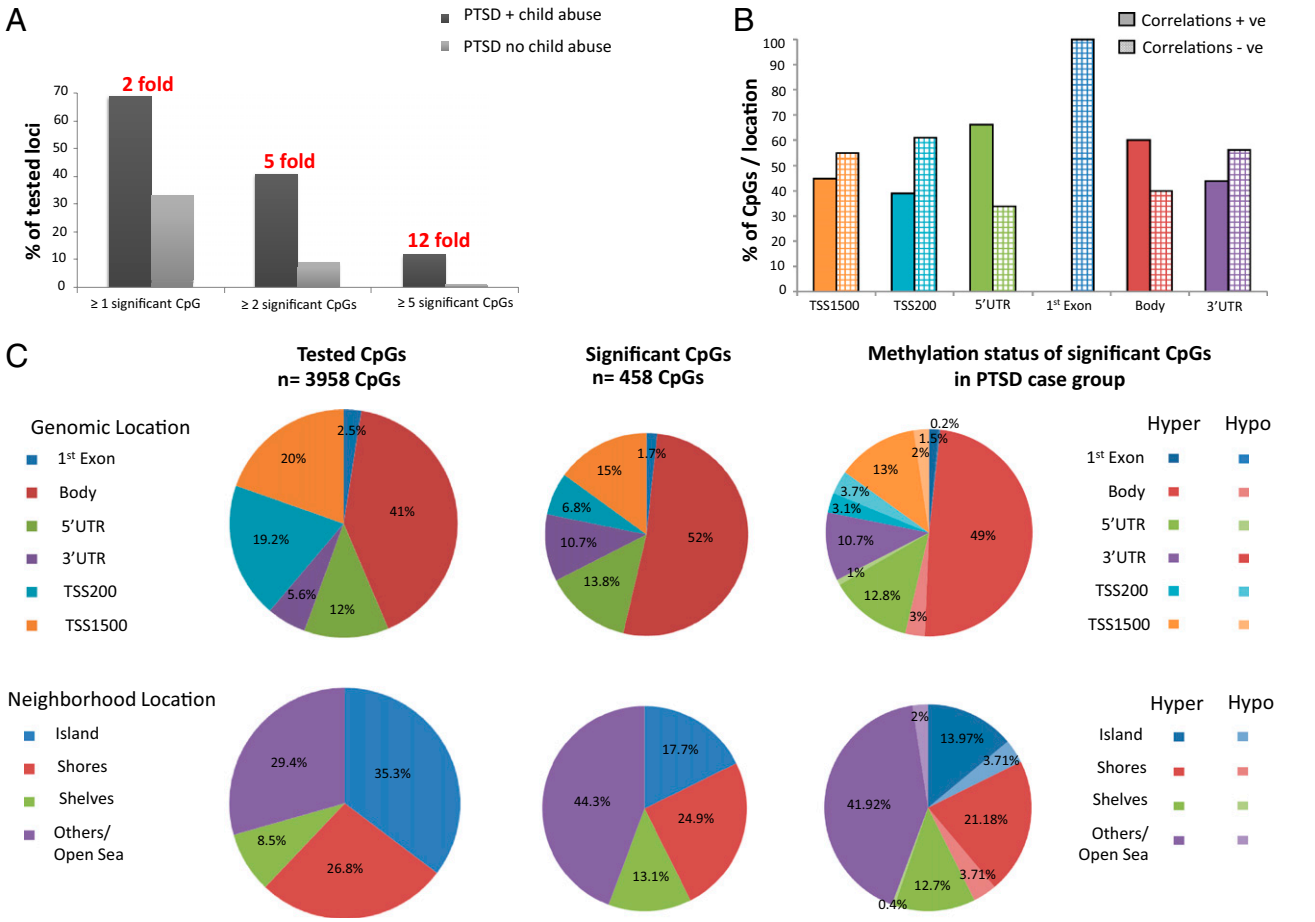


Fig. 2. Influence of child abuse on DNA methylation in peripheral blood in PTSD. (*A*) DNA methylation differences are more frequent in PTSD with child abuse. The bar graph shows the percentage of transcripts differentially regulated in the two respective group comparisons having one or more, two or more, or five or more differentially methylated CpGs in the locus. DNA methylation changes underlying the observed gene expression differences were 2- to 12-fold higher in the PTSD with child abuse group compared with the PTSD with no child abuse group. (*B*) Correlations of DNA methylation with gene expression. The bar graph shows the percent of CpGs with positive vs. negative correlations with gene expression of the closest transcript in the whole sample stratified by location with respect to the regulated gene. Inverse correlations close to the transcription start sites and an increasing proportion of positive correlations in proximal and distal regulatory sites were observed. (*C*) Distribution of the tested (left panels) and significant (middle panels) CpGs and direction of methylation across all differentially methylated CpGs in both groups. The right panels denote the relative distributions of all tested and significant CpG sites with respect to the closest gene and the CpG islands and the directions of methylation differences of the significant CpGs. Of the significant CpGs, 90% were hypermethylated and 10% were hypomethylated in the PTSD case group with respect to the controls.

impact of other specific immune-cell subtypes, not measured in our analysis can, however, not be excluded. Furthermore, because the gene-expression levels and methylation patterns were only measured at one time-point, it is difficult conclude whether the expression and methylation changes are indeed the direct consequence of exposure to child abuse per se or a reflection of other factors changed by the response to child abuse or related to the disease itself.

To the best of our knowledge, this genome-wide study assessing the influence of exposure to childhood maltreatment on gene expression and DNA methylation profiles in peripheral blood in PTSD is unique. Our findings are in line with a recent study demonstrating that epigenetic alterations in the hippocampus among suicide completers with or without childhood trauma were distinct (29), although this study did not explore related changes in gene expression. These data provide further evidence that there are substantial differences in disease-related biological perturbations in the presence or absence of exposure to maltreatment in childhood. The fact that several nonoverlapping biological pathways seem to be affected in the two PTSD groups and that changes in DNA methylation appear to have a much greater impact in the childhood maltreatment group may indicate differences in the pathophysiology of PTSD, in dependence of exposure to type of trauma. This finding could not only have implications for biomarker research for stress-related disorders but may help to elucidate pathophysiological differences in dependence of trauma exposure in immune disturbances often accompanying these disorders (3, 4, 30, 31). If these distinct changes are not limited to peripheral blood cells but extend to other organ systems, as indicated by some studies (27, 28, 32), they might also aid in the search for disease mechanisms and therapeutic intervention.

Materials and Methods

Samples. Participants in this study belonged to a larger study investigating the contribution of genetic and environmental factors in PTSD (33). All study procedures were approved by the institutional review boards of Emory University School of Medicine and Grady Memorial Hospital and all subjects gave written informed consent to the study.

The 396 participants were selected from a larger study, which has been described previously (34). Microarray gene-expression results had been reported in a previous report (34) for a subset of these patients ($n = 211$). For this study, we selected patients from a now expanded set of microarray gene-expression results (adding 185 new samples with gene-expression arrays to the previously reported ones) and we have now also added DNA methylation array data for all 396 individuals.

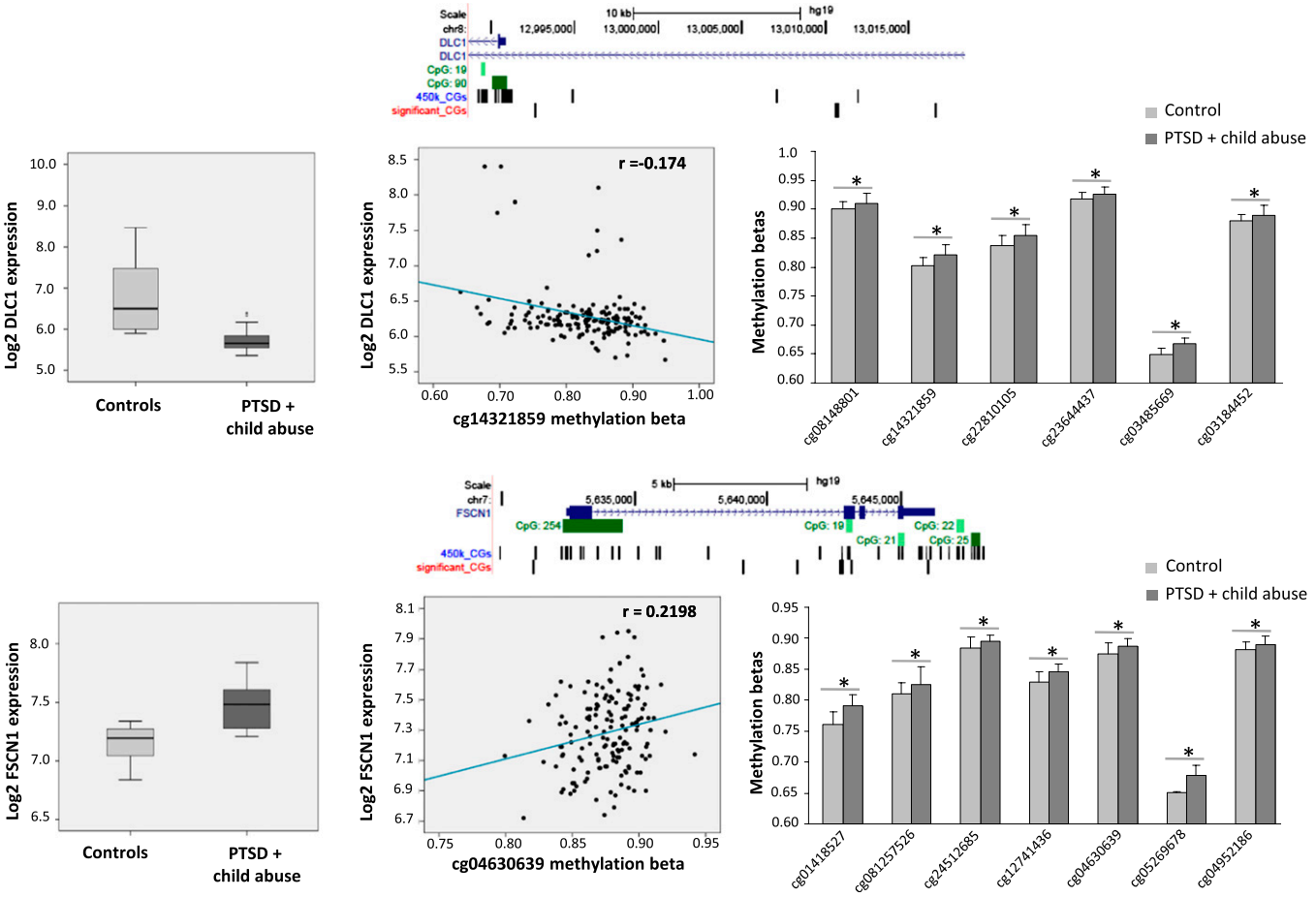


Fig. 3. Two examples of directional associations between DNA methylation and gene-expression changes. This figure shows gene-expression and DNA methylation data for two genes, *DLC1* (deleted in liver cancer 1 gene) and *FSCN1* (fascin homolog 1, actin-bundling protein). The position of the significant CpGs in relation to the gene, CpG islands and other CpGs on the 450 k methylation array are on top of the series of diagrams for each gene (with *DLC1* on top and *FSCN1* on the bottom). Below, we depict the difference in gene expression for the respective group comparison (*Left*), the correlation of methylation levels of a representative CpG in that locus with the expression level of the gene in the whole sample (*Center*), and the group differences in DNA methylation of the significant CpGs in the respective locus (*Right*). These data illustrate that the direction of the differences in DNA methylation and gene expression between the groups are as expected from the direction of correlation between gene expression and DNA methylation in the overall sample. * indicates adjusted *P* value of less than or equal to 0.05.

Psychological Assessments. ***PTSD symptomatic scale and clinician administered PTSD scale.*** The modified PTSD symptomatic scale (mPSS) was used as a measure of PTSD symptoms (35). PSS frequency items were summed to obtain a continuous measure, with values of above 20 considered as clinically significant PTSD symptoms. In addition, a categorical PTSD classification was made by using the scores of the B, C, and D clusters of the PSS. Individuals classified as having PTSD needed to have clinically significant symptoms in all three clusters, representing intrusive, avoidance/numbing, and hyperarousal (36). The clinician-administered PTSD scale (CAPS) was available for a subset of 349 of the 396 individuals (37). There was a significant strong positive correlation between PSS and current CAPS in this dataset (Pearson $r = 0.69$, $P = 3.15 \times 10^{-29}$). The CAPS was also used to assess the presence or absence of lifetime PTSD.

Beck depression inventory. The Beck depression inventory (BDI) was administered to measure current depression symptoms (38). Current depression and PTSD symptoms were highly correlated ($r = 0.653$, $P = 1.56 \times 10^{-42}$). Diagnostic tests for variance inflation factor and tolerance revealed a variance inflation factor of 1.72 and tolerance of 0.58, indicating no evidence for multicollinearity between depressive and PTSD symptoms.

Substance abuse. As described previously (34), two self-report items were used to assess for current alcohol and substance use-related problems.

Trauma-Exposure Measurements. ***Childhood trauma questionnaire.*** The childhood trauma questionnaire (CTQ) was used as a measure of child abuse. The CTQ is a consistent and stable self-report inventory assessing five types of childhood trauma: sexual, physical, and emotional abuse, and emotional and physical neglect (39). In accordance with prior research, we restricted our analyses to the three abuse subscales only (40). As described previously, participants were dichotomized into two groups for each of the three categories of abuse (presence or absence of moderate to severe physical, sexual, or emotional abuse). Finally, we created a composite variable across the three abuse types, grouping participants into (*i*) those with no exposure to any childhood abuse (scores less than cut-off for all three abuses), and (*ii*) those with moderate to severe exposure to at least two types of childhood abuse (scores above cut-off for at least two of the abuses).

Trauma events inventory. The trauma events inventory (TEI) is the primary measure of non-child abuse trauma in this study (41). Total numbers of different types of trauma among the participants was summed up for each category into a continuous variable. Although the TEI instrument also includes exposure to non-child abuse traumatic events in childhood, the mean age of exposure was 23.81 (0.70) y; hence, in the current study this refers to mostly adult trauma.

Sample Selection. Of the sample of 396 described above, 169 individuals who had experienced at least two types of moderate-to-severe childhood abuses or at least two other types of traumas during their lifetime were included in the analysis for this study. Of these, individuals with PSS ≤10 and moderate-to-severe child abuse or two other types of traumas were selected as trauma-exposed individuals with neither current nor a history of lifetime PTSD symptoms (trauma controls) ($n = 108$). From a total of 61 individuals with current PTSD symptoms (PSS > 20 and significant symptoms in all PTSD B, C, and D clusters), we selected individuals (*i*) who had experienced at least two types of moderate-to-severe childhood abuses ($n = 32$) and (*ii*) who had experienced at least two other types of traumas but no child abuse ($n = 29$).

Statistical Analysis. ***Gene-expression data.*** Raw microarray scan files from Illumina HT-12 v3.0 arrays (Illumina) were exported using the Illumina Beadstudio program and loaded into R for downstream analysis (www.R-project.org). Evaluation of the different microarray steps was done using the Illumina internal controls. Samples which were >5% SD were excluded. The data were transformed and normalized using the variance stabilizing normalization (42). A total of 15,877 probes passing the filter criteria of Illumina probe detection *P* value of <0.01 in 5% of the individuals were used for subsequent analysis. To correct for confounding as a result of batch effects, the data were normalized using an empirical Bayes method for batch correction (43). Reproducibility of the gene-expression data were assessed using six pairs of technical replicates, yielding average Pearson correlations of 0.996. General linear models were constructed by regressing the gene-expression profiles against the PTSD group status and adjusting for sex, age, ethnicity, substance abuse, and treatment. The significance of association was estimated by two-tailed *P* values using the ANOVA *F* test. Results were corrected for multiple testing by 10,000 permutations using the permutation of regressor residuals test (http://cran.r-project.org/web/packages/glmperm/index.html). Briefly, the general linear models for each transcript were built as described above and the residuals of the regressions were permuted 10,000 times for each transcript using the shuffle-*Z* method to obtain the empirical *P* values corrected for multiple testing as described previously (34).

DNA methylation data. Raw methylation Beta values from the HumanMethylation 450k BeadChip (Illumina) were determined via the Illumina Beadstudio program and loaded into R. Internal Illumina controls were used to assess the quality of staining, extension, hybridization, bisulfite conversion, and specificity. Samples with probe detection call rates <90% and those with an average intensity value of either <50% of the experiment-wide sample mean or <2,000 arbitrary units (AU) were excluded from further analysis, allowing 163 samples for subsequent analysis. Unsupervised hierarchical clustering was performed to identify extreme outliers and global trends in methylation. One sample of male DNA was included on each BeadChip as a technical control throughout the experiment and assessed for reproducibility, with average Pearson correlation coefficient of 0.993 across all replicates. Signals from methylated (M) and unmethylated (U) bead types were used to calculate a beta value as $\beta = M/(U + M)$. Hybridization and chip batch effects were accounted for using an empirical Bayes method (43). The samples were quantile normalized and peak-corrected using the IMA package functions in R (44, 45). Hybridization and chip batch effects were accounted for using an empirical Bayes method (43). Methylation differences were calculated using generalized linear models in R by regressing the β-values against the PTSD group and adjusting for age, sex, ethnicity, and substance abuse. Results were corrected for multiple testing by 10,000 permutations using the permutation of regressor residuals test (http://cran.r-project.org/web/packages/glmperm/index.html).

ACKNOWLEDGMENTS. The authors thank Maik Koedel and members of the Grady Trauma Project at Emory—particularly Allen Graham, Kimberly Kerley, Jennifer Davis, Angelo Brown, Alicia Nelson, Jennifer Winkler, Will Holland, Sarah Spann, Kimberly Dement, Dana Goodenough, and Emily Reiser—for excellent technical assistance. This work was supported by the Max-Planck Society; the Behrens-Weise Foundation (E.B.B.); National Institute of Mental Health Grants MH071537 (to K.J.R.) and MH085806 (to A.K.S.); and the Burroughs Wellcome Fund.

1. Szyf M (2011) The early life social environment and DNA methylation: DNA methylation mediating the long-term impact of social environments early in life. *Epigenetics* 6(8):971–978.
2. Danese A, et al. (2011) Biological embedding of stress through inflammation processes in childhood. *Mol Psychiatry* 16(3):244–246.
3. Danese A, et al. (2009) Adverse childhood experiences and adult risk factors for age-related disease: Depression, inflammation, and clustering of metabolic risk markers. *Arch Pediatr Adolesc Med* 163(12):1135–1143.
4. Danese A, et al. (2008) Elevated inflammation levels in depressed adults with a history of childhood maltreatment. *Arch Gen Psychiatry* 65(4):409–415.
5. Danese A, Pariante CM, Caspi A, Taylor A, Poulton R (2007) Childhood maltreatment predicts adult inflammation in a life-course study. *Proc Natl Acad Sci USA* 104(4):1319–1324.
6. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM (2001) C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* 286(3):327–334.
7. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH (1997) Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 336(14):973–979.
8. Sin DD, Man SF (2003) Why are patients with chronic obstructive pulmonary disease at increased risk of cardiovascular diseases? The potential role of systemic inflammation in chronic obstructive pulmonary disease. *Circulation* 107(11):1514–1519.
9. Widom CS, DuMont K, Czaja SJ (2007) A prospective investigation of major depressive disorder and comorbidity in abused and neglected children grown up. *Arch Gen Psychiatry* 64(1):49–56.
10. McGowan PO, et al. (2009) Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 12(3):342–348.
11. Drury SS, et al. (2012) Telomere length and early severe social deprivation: Linking early adversity and cellular aging. *Mol Psychiatry* 17(7):719–727.
12. Oberlander TF, et al. (2008) Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. *Epigenetics* 3(2):97–106.
13. Ressler KJ, et al. (2011) Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature* 470(7335):492–497.
14. Bremner JD, Southwick SM, Johnson DR, Yehuda R, Charney DS (1993) Childhood physical abuse and combat-related posttraumatic stress disorder in Vietnam veterans. *Am J Psychiatry* 150(2):235–239.
15. Breslau N, Chilcoat HD, Kessler RC, Davis GC (1999) Previous exposure to trauma and PTSD effects of subsequent trauma: Results from the Detroit Area Survey of Trauma. *Am J Psychiatry* 156(6):902–907.
16. Cougle JR, Timpano KR, Sachs-Ericsson N, Keough ME, Riccardi CJ (2010) Examining the unique relationships between anxiety disorders and childhood physical and sexual abuse in the National Comorbidity Survey-Replication. *Psychiatry Res* 177(1-2):150–155.
17. Brewin CR, Andrews B, Valentine JD (2000) Meta-analysis of risk factors for posttraumatic stress disorder in trauma-exposed adults. *J Consult Clin Psychol* 68(5):748–766.
18. Widom CS (1999) Posttraumatic stress disorder in abused and neglected children grown up. *Am J Psychiatry* 156(8):1223–1229.
19. Andersen SL, Teicher MH (2008) Stress, sensitive periods and maturational events in adolescent depression. *Trends Neurosci* 31(4):183–191.
20. Heim C, Newport DJ, Mletzko T, Miller AH, Nemeroff CB (2008) The link between childhood trauma and depression: Insights from HPA axis studies in humans. *Psychoneuroendocrinology* 33(6):693–710.
21. Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat Rev Neurosci* 10(6):434–445.
22. Klengel T, et al. (2013) Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. *Nat Neurosci* 16(1):33–41.
23. Brenet F, et al. (2011) DNA methylation of the first exon is tightly linked to transcriptional silencing. *PLoS ONE* 6(1):e14524.
24. Chuang TJ, Chen FC, Chen YZ (2012) Position-dependent correlations between DNA methylation and the evolutionary rates of mammalian coding exons. *Proc Natl Acad Sci USA* 109(39):15841–15846.
25. Davies MN, et al. (2012) Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. *Genome Biol* 13(6):R43.
26. Gunnar M, Quevedo K (2007) The neurobiology of stress and development. *Annu Rev Psychol* 58:145–173.
27. Sommershof A, et al. (2009) Substantial reduction of naïve and regulatory T cells following traumatic stress. *Brain Behav Immun* 23(8):1117–1124.
28. Provençal N, et al. (2012) The signature of maternal rearing in the methylome in rhesus macaque prefrontal cortex and T cells. *J Neurosci* 32(44):15626–15642.
29. Labonté B, et al. (2012) Genome-wide epigenetic regulation by early-life trauma. *Arch Gen Psychiatry* 69(7):722–731.
30. Smith AK, et al. (2011) Differential immune system DNA methylation and cytokine regulation in post-traumatic stress disorder. *Am J Med Genet B Neuropsychiatr Genet* 156B(6):700–708.
31. Uddin M, et al. (2010) Epigenetic and immune function profiles associated with posttraumatic stress disorder. *Proc Natl Acad Sci USA* 107(20):9470–9475.
32. Lee RS, et al. (2011) A measure of glucocorticoid load provided by DNA methylation of Fkbp5 in mice. *Psychopharmacology (Berl)* 218(1):303–312.
33. Gillespie CF, et al. (2009) Trauma exposure and stress-related disorders in inner city primary care patients. *Gen Hosp Psychiatry* 31(6):505–514.
34. Mehta D, et al. (2011) Using polymorphisms in FKBP5 to define biologically distinct subtypes of posttraumatic stress disorder: Evidence from endocrine and gene expression studies. *Arch Gen Psychiatry* 68(9):901–910.
35. Foa EB, Tolin DF (2000) Comparison of the PTSD symptom scale-interview version and the clinician-administered PTSD scale. *J Trauma Stress* 13(2):181–191.
36. Jovanovic T, et al. (2010) Impaired fear inhibition is a biomarker of PTSD but not depression. *Depress Anxiety* 27(3):244–251.
37. Blake DD, et al. (1990) A clinician rating scale for assessing current and lifetime PTSD: The CAPS-1. *Behav Therapist* 13:187–188.
38. Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J (1961) An inventory for measuring depression. *Arch Gen Psychiatry* 4:561–571.
39. Bernstein DP, et al. (2003) Development and validation of a brief screening version of the Childhood Trauma Questionnaire. *Child Abuse Negl* 27(2):169–190.
40. Bradley RG, et al. (2008) Influence of child abuse on adult depression: Moderation by the corticotropin-releasing hormone receptor gene. *Arch Gen Psychiatry* 65(2):190–200.
41. Hovens JE, Bramsen I, van der Ploeg HM, Reuling IE (2000) Test-retest reliability of the trauma and life events self-report inventory. *Psychol Rep* 87(3 Pt 1):750–752.
42. Huber W, von Heydebreck A, Sültmann H, Poustka A, Vingron M (2002) Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 18(Suppl 1):S96–S104.
43. Johnson WE, Li C, Rabinovic A (2007) Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* 8(1):118–127.
44. Dedeurwaerder S, et al. (2011) Evaluation of the Infinium Methylation 450K technology. *Epigenomics* 3(6):771–784.
45. Wang D, et al. (2012) IMA: An R package for high-throughput analysis of Illumina's 450K Infinium methylation data. *Bioinformatics* 28(5):729–730.

Supporting Information

Mehta et al. 10.1073/pnas.1217750110

SI Materials and Methods

RNA Extraction and Microarray Procedures. Whole blood was collected between 8:00 and 9:00 AM under fasting conditions in Tempus RNA tubes (Applied Biosystems). RNA was isolated using the Versagene kit (Gentra Systems), quantified using the Nanophotometer, and quality checks were performed on the Agilent Bioanalyzer. Only samples with RNA integrity numbers of ≥6 with clear 18S and 28S peaks on the Bioanalyzer were used for amplification, the average RNA integrity number was 7.87 (SD of 1.1). Next, 250 ng total RNA was reverse-transcribed and biotin-labeled using the Ambion kit (AMIL1791; Applied Biosystems), 750 ng of cRNA were hybridized to Illumina HT-12 v3.0 arrays (Illumina) and incubated overnight for 16 h at 55 °C. Arrays were washed, stained with Cy3 labeled streptavidin, dried and scanned on the Illumina BeadScan confocal laser scanner.

The 450 k Methylation Array Procedures. Genomic DNA was isolated from whole blood stored in EDTA tubes using the Gentra Puregene Kit (Qiagen). The DNA was quantified using the PicoGreen (Invitrogen) and the quality was checked on an agarose gel. A total of 1,000 ng DNA was treated with sodium bisulfite using the Zymo EZ-96 DNA Methylation Kit (Zymo Research) according to the manufacturer's protocol. The methylation assay was performed on 4 μL bisulfite-converted genomic DNA at 50 ng/μL in accordance with the Infinium HD Methylation Assay protocol. The DNA was amplified, fragmented and hybridized on the HumanMethylation 450k BeadChip (Illumina).

Separate Cohort Controls for Lymphocyte and Monocyte Cell Fractions. Data from 54 individuals [36 men and 18 women, mean (SD) age of 30 (1)] was used to interrogate potential correlations between the relative lymphocyte and monocyte fraction in whole blood and mRNA expression levels, while correcting for age and sex (2). The amount of $CD15–CD16^+$, $CD14^+$, $CD4^+$, and $CD8^+$ white blood cells was measured using FACS analysis from whole blood drawn at the same time-point as a Tempus RNA tube.

qPCR Procedures. cDNA was synthesized from 250 ng total RNA using SuperScript II Reverse Transcriptase (Invitrogen). qPCR was performed using the Universal Probe Library on the Roche LightCycler 480 (Roche Applied Science). Assays were designed using the Probe Finder Software (Roche Applied Science) and run in duplicates according to the manufacturer protocol, except for a total reaction volume of 10 μL. TBP was used as the endogenous control gene. Primer sequences are provided in Dataset S8.

DNA Methylation Validation Using Sequenom's EpiTYPER MassARRAY System. A total of 11 CpGs spanning two amplicon regions (SPON1 and TSPAN32) with a high density of CpGs were chosen for validation. Sequencing was performed at varionostic. Amplicons were designed using the Sequenom EpiDESIGNER software. All experimental procedures were performed under routine conditions as outlined in the Sequenom Methylation Analysis Application Note (www.sequenom.com/files/genetic-analysis-files/dna-methylation-pdfs/8876-007-r02_epityper-app-note) using the MassARRAY system from Sequenom. Robustness of the assay was tested using a pair of technical replicates (correlations: $r = 0.998$ for SPON1 and $r = 0.983$ for TSPAN32) for each and three controls (positive, negative methylation, and negative template control). Data analysis was performed by regressing the methylation percentage against the PTSD group using generalized linear models and adjusting for age, sex, ethnicity and substance abuse in R.

Pathway Analyses. The Ariadne Pathway Studio 8.0 (Ariadne Genomics/Elsevier) was used to deduce relationships between differentially expressed candidates using the Ariadne ResNet database based on cellular processes criteria using subnetwork enrichment analysis. Only categories with ≥five transcripts within a cellular process and relationships with ≥two references were considered were the analysis. Subnetwork enrichment analysis applies the Mann–Whitney version of gene-set enrichment analysis algorithm to each sub network to calculate P values. Results were corrected for multiple testing using the Bonferroni correction.

1. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH (1997) Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 336(14):973–979.

2. Pace TW, et al. (2011) Increased peripheral NF-kappaB pathway activity in women with childhood abuse-related posttraumatic stress disorder. *Brain Behav Immun* 25(1):6–13.

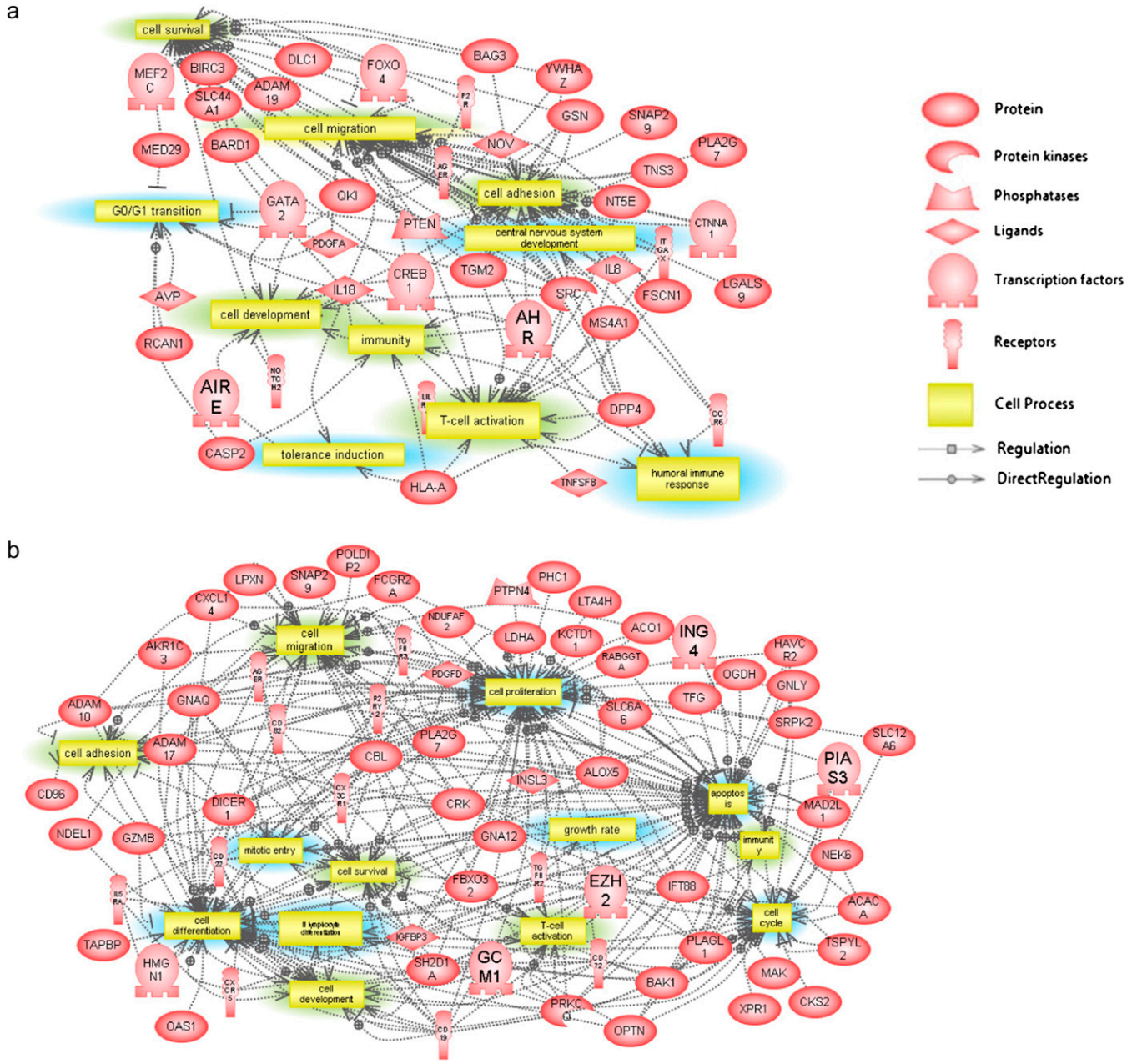


Fig. S1. Distinct biological networks enriched in child abuse vs. no child abuse groups. Significantly overrepresented cellular processes among transcripts in PTSD (*A*) with or (*B*) without child abuse using Pathway Studio after Bonferroni corrections for multiple testing. Details of the subnetwork cellular processes enrichment *P* values are shown in Dataset S3.

Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLS)
Dataset S3 (XLS)
Dataset S4 (XLS)
Dataset S5 (XLS)
Dataset S6 (XLS)
Dataset S7 (XLS)
Dataset S8 (XLS)

Discussion

Allele specific demethylation in FKBP5

Gene by environment interaction studies investigate the risk for disease in response to an environmental factor moderated by the genetic disposition of the individual. So far, GxE studies have generated high hopes to advance our understanding of the pathophysiology of complex psychiatric disorders but are at the same time contestable due to the purely statistical approach and related limitations. Knowledge on the molecular mechanisms underlying GxE thus not only corroborates the statistical findings but may also generate innovative approaches towards prevention and therapy of psychiatric disorders caused by genetic and environmental factors.

The GxE of *FKBP5* with childhood abuse on various psychiatric phenotypes is an approach to investigate the combined effects of genetic variants in FKBP5 and childhood abuse exposure on disease risk. The genetic variants implicated in the GxE of *FKBP5* and childhood abuse are in high linkage disequilibrium and distributed across the whole gene locus. Most of them are located in intronic regions and therefore possess no obvious relevant effects on protein structure and function. Hence it is difficult to determine the functional variant that mediates the biological effects. Extending previous findings from 2008, we increased the sample size and replicated the initial GxE of *FKBP5* and childhood abuse on PTSD (Figure 5) and hypothesized that the functional genetic variant may be located in or close to known regulatory elements such as glucocorticoid response elements (GREs) mediating the effect of GR-activation on the transcriptional activation of *FKBP5*. In fact, we were able to show that a SNP in intron 2 of FKBP5 named rs1360780 influences the enhancer properties of the adjacent GRE. By cloning the intron 2 enhancer region with both alleles into a luciferase reporter vector, transfecting in HeLa cells and stimulating in-vitro with the GR agonist dexamethasone, we were

able to show that the SNP alters the transcriptional response of the enhancer element in response to GR-stimulation with the T (risk) allele leading to a stronger activation compared to the C (protective) allele. In addition, the T-allele may lead to an additional TATA-box like sequence structure and in-vitro binding assays of both alleles showed that in deed TATA-binding protein (TBP) bind stronger to the sequence harboring the T-allele compared to the C-allele. The presence of a potential TBP binding site in an enhancer region, about 50kb downstream of the transcription start site (TSS) lead to the question of if and how this region might interact with the TSS.

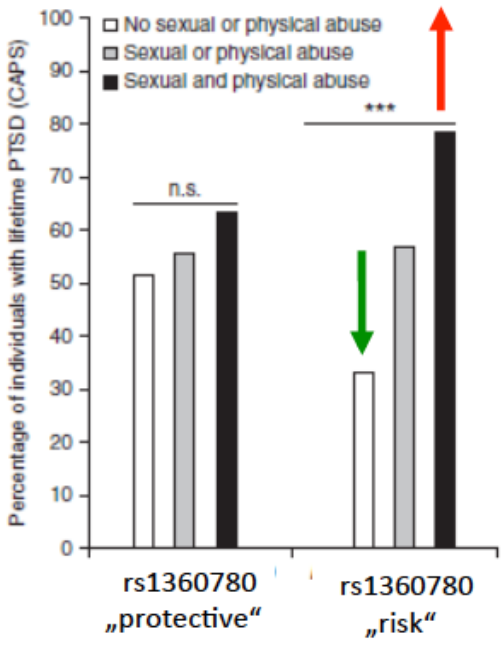


Figure 5. Replication and extension of previous GxE of *FKBP5* with childhood abuse on PTSD diagnosis in N=519. This GxE shows the interaction of *FKBP5* with childhood abuse on PTSD symptom level. Of note, the presence of the so-called risk allele lead to an increased risk for PTSD in adulthood when individuals are exposed to severe sexual and physical trauma in childhood. In contrast, when "risk" allele carrier are not exposed to severe sexual and physical abuse, the "risk" allele confers a protective effect on PTSD risk. Thus, the designation of "risk" and "protective" alleles depend on the environmental context suggesting environmentally sensitive genetic variants in FKBP5

mediating environmental effects in both directions, for positive and negative outcomes. Figure taken from Klengel et al., Nat Neurosci., 2013 [126].

Studies using chromatin conformation capture (3C) and related techniques revealed that chromatin is a highly organized non-linear structure [127]. This three-dimensional structure enables regulatory elements to form physical contact across long distances and even across chromosomes influencing gene regulation. More recently, it has been suggested that genetic variations may not only influence epigenetic pattern and transcription factor binding but also the three-dimensional structure of chromatin [128]. This form of long-range regulation has just been recognized to have a potential influence on psychiatric disorders also with the notion that most genetic variants found in recent association studies are located in intronic or intergenic regions [129].

We subsequently performed 3C and showed that the downstream enhancer regions in intron 2 and intron 7 form a three-dimensional structure with the promoter region leading to a contact of the intron 2 GRE enhancer region with the promoter region in risk allele carriers but not in individuals harboring the protective genotype (Figure 6). This loop formation and interaction of the enhancer with the promoter site thus also contributes to the stronger transcriptional activation of *FKBP5* in risk allele carriers compared to protective genotype carriers. In summary, these experiments showed that rs1360780, a common SNP in high linkage disequilibrium with other SNPs implicated in several GxE studies might be the functional SNP that contributes to the genetic predisposition for an increased transcriptional response of *FKBP5* in response to GR stimulation and stress, respectively.

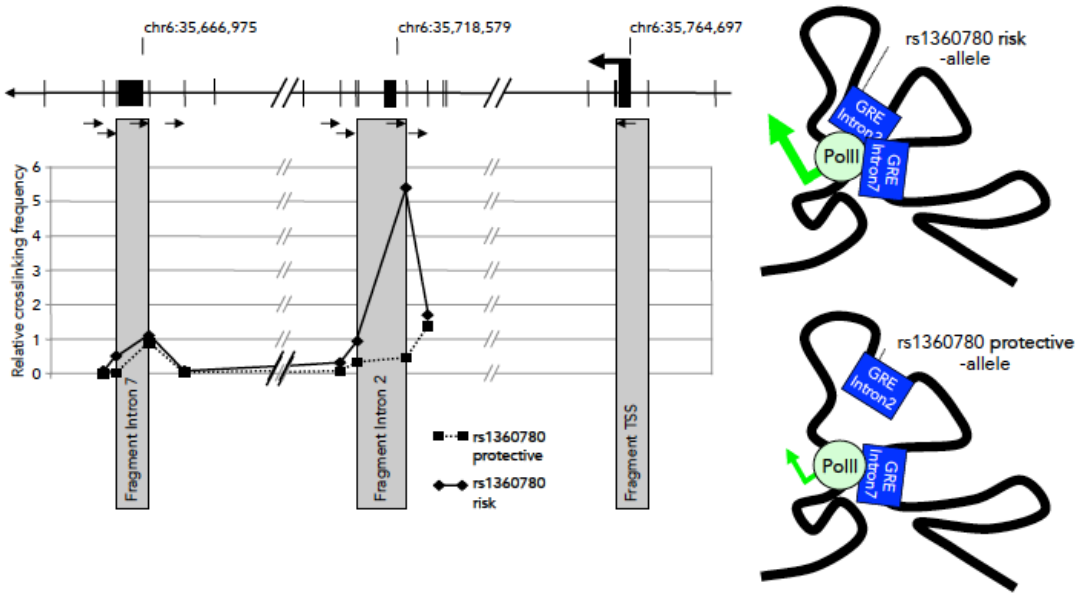


Figure 6. Chromatin conformation capture (3C) of FKBP5 shows the interaction of intronic downstream regulatory elements with the transcription start site (TSS). The left panel shows the genomic locus of FKBP5 on top with the transcription start site represented by an arrow (minus strand). Horizontal lines represent EcoRI restriction sites on the genomic DNA. Below, the small arrows represent primer used in this experiment with grey boxes representing intron 2 (middle) and intron 7 (left) as well as the fragment representing the TSS (right). The lines represent relative crosslinking and therefore interaction frequencies with the solid line depicting the FKBP5 risk allele and the dashed line depicting the protective allele. The risk allele leads to an interaction of the intron 7 as well as the intron 2 fragments with the TSS whereas the protective allele does not lead to an interaction of intron 2 with the TSS. The right panels represent the 3C results in a three-dimensional model. The physical interaction of intron 2 with the TSS in risk allele carrier may contribute to a stronger transcriptional activation of FKBP5 in response to GR activation. Figure adpted from Klengel et al., Nat Neurosci., 2013 [126].

We next asked the question if childhood abuse induces long-lasting epigenetic changes that might be associated with the risk to develop PTSD. We focused on DNA methylation as a potentially long-lasting epigenetic modification and established DNA methylation assays using bisulfite pyrosequencing and screened relevant regulatory regions in FKBP5 including the promoter region and known

functional GREs. The CpG island around the TSS and all GREs in intron 5 were found to be unmethylated. Significant DNA methylation above the technical limitation of bisulfite pyrosequencing of 5% was detected at GREs in the promoter, intron 2 and intron 7. We then investigated DNA methylation at these GREs in adult individuals recruited by the Grady Trauma Project, a cross-sectional study investigating childhood and adult trauma exposure with regard to stress-related disorders in an urban population characterized by a low socioeconomic status and high civilian trauma exposure in Atlanta, GA, USA [130]. Individuals were stratified according to severe childhood trauma exposure and *FKBP5* rs1360780 genotype. In summary, we were able to show demethylation in and around *FKBP5* intron 7 GREs in trauma exposed risk allele carriers compared to trauma-exposed carriers of the protective genotype that retained a stable methylation pattern and were similar to non-trauma exposed individuals irrespective of their genotype (Figure 7). This result was replicated in an independent cohort. Therefore, this is the first example for a genotype dependent epigenetic modification in response to childhood trauma. It highlights that the long-lasting epigenetic effects of childhood abuse are genotype dependent paralleling previous statistical GxE.

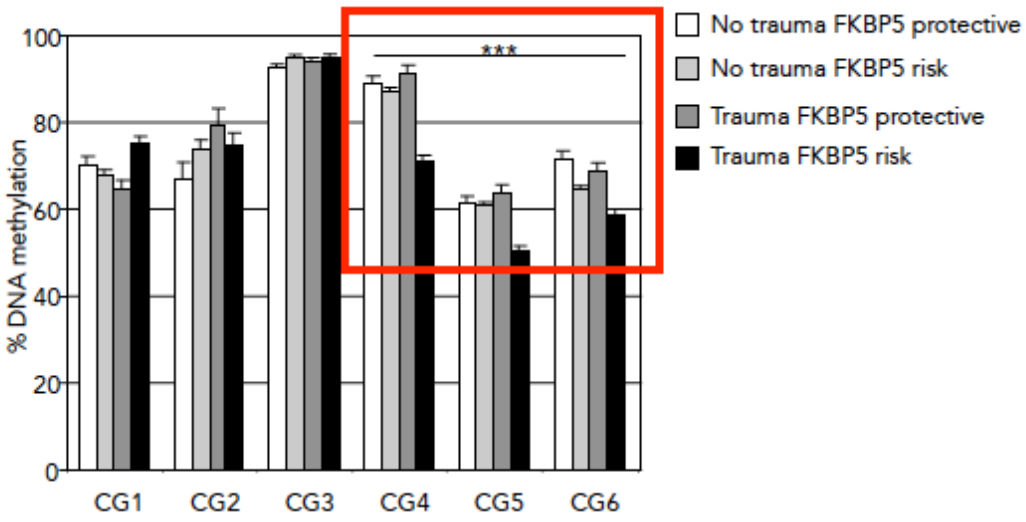


Figure 7. Genotype-dependent demethylation in intron 7 of FKBP5. Here, DNA methylation levels of 6 CpG dinucleotides in and around functional GREs in intron 7 are depicted. Risk allele carriers exposed to childhood trauma (black columns) exhibit a demethylation at CpG 4, 5 and 6 compared

to individuals carrying the protective genotype (dark grey columns). Individuals not exposed to childhood trauma do not show significant methylation differences (white and light grey columns). Figure adapted from Klengel et al., Nat Neurosci., 2013 [126].

A major limitation of these results is the investigation of DNA methylation in peripheral blood, a heterogeneous tissue that may not be informative for pathophysiologic mechanisms in the brain. Therefore we controlled for white blood cell composition in our replication cohort and found no significant influence of different white blood cell subpopulations present. Moreover, we used a human hippocampal progenitor cell line heterozygous for rs1360780 in order to test the effect of in-vitro activation of the GR on intron 7 DNA methylation in neuronal cells. In fact, parallel to the observations in our human cohorts we found that GR activation by dexamethasone led to a demethylation in exactly the same CpGs in intron 7 in this cell culture model (Figure 8).

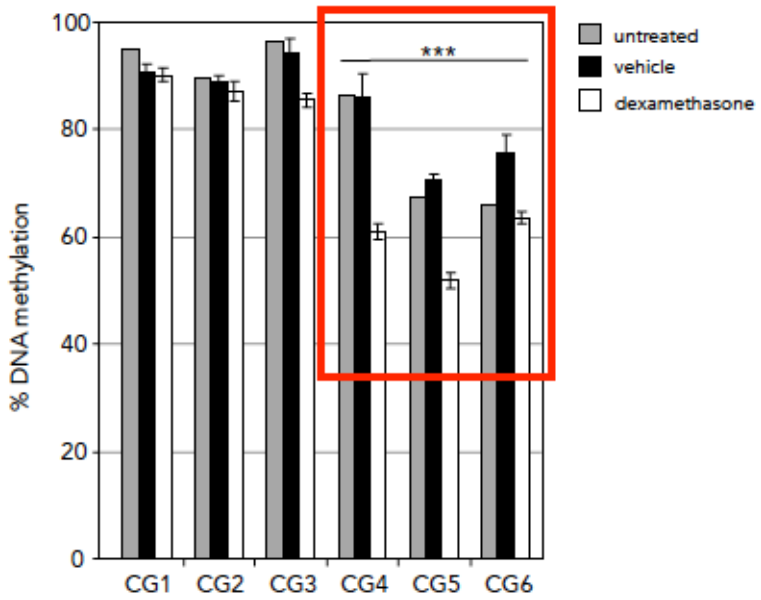


Figure 8. Demethylation of FKBP5 intron 7 in a human hippocampal progenitor cell line. GR activation by dexamethasone, a synthetic GR agonist, leads to a demethylation in intron 7 CpG 4,5 and 6 paralleling the effects of childhood trauma in FKBP5 risk allele carriers. This suggests that the activation of the GR by stress and subsequent demethylation may be present also in neuronal tissue. Figure adapted from Klengel et al., Nat Neurosci., 2013 [126].

The observed demethylation was dependent on the timing of the treatment whereby treatment in the proliferation and differentiation phase led to a demethylation stable over time but treatment of differentiated neuronal/glial cells did not result in this demethylation again emphasizing the importance of the timing of the GR-activation and stress exposure. This will be discussed at the end of this section in more detail. The functional consequence of demethylation in and around intron 7 GREs is enhanced transcriptional activation as shown by in-vitro luciferase assays using methylated and unmethylated constructs (Figure 9a and 9b). Here, the unmethylated construct was more responsive to GR activation than the methylated construct presumably through stronger binding of the GR to the unmethylated sequence. Moreover, we also observed a negative correlation of DNA methylation in intron 7 with ex-vivo GR sensitivity (Figure 9c). Lower methylation in intron 7 will therefore result in higher *FKBP5* transcription, a stronger feedback to the GR and thus a less sensitive GR with higher cortisol level over time, which is a risk factor for stress-related psychiatric disorders.

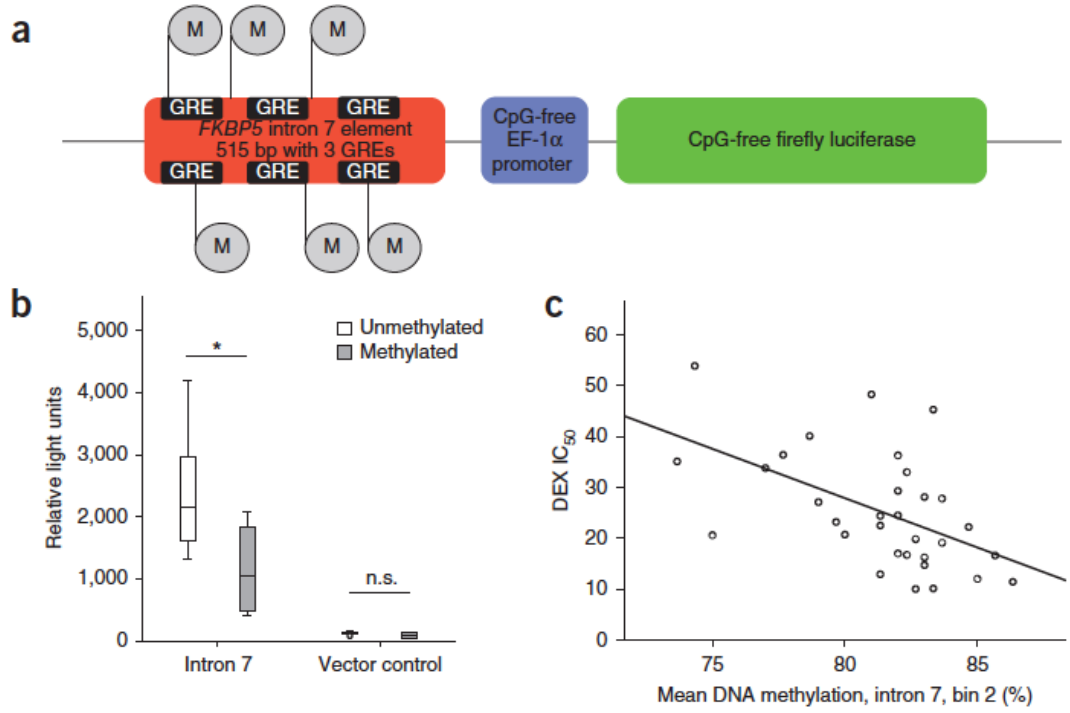


Figure 9. Demethylation of *FKBP5* intron 7 leads to enhanced GR activation and GR resistance. (a) Schematic representation of the CpG-free luciferase vector harboring an intron 7 fragment containing 3 GREs. (b) The unmethylated construct is more responsive to GR activation in HeLa cells by dexamethasone than the methylated construct. (c) DNA methylation in intron 7 is correlated with the ex-vivo GR sensitivity of human PBMCs with decreased DNA methylation leading to a less sensitive GR and thus GR resistance and elevated cortisol levels. Figure taken from Klengel et al., Nat Neurosci., 2013 [126].

Genome-wide epigenetic and transcriptional differences of childhood abuse and adult trauma

Starting from initial candidate gene studies, technical improvements have quickly enabled researchers to investigate DNA methylation changes on a broader scale up to the genome-wide level. As shown by McGowan et al., the response of the methylome to maternal care in rats includes both hyper- and hypomethylated regions across a 6.5 million nucleotide DNA stretch [131]. Similar, Provencal et al investigated the genome-wide promoter methylation profile of rhesus macaque blood and prefrontal cortex tissue in response to rearing conditions. Here, regional

increases and decreases in DNA methylation in response to rearing conditions were observed and the regions tended to cluster in gene families influencing gene expression of gene pathways despite a relatively low absolute methylation difference [132]. In particular the study by Provencal provided more evidence on the similarities of environmentally induced epigenetic modifications between central neuronal tissue and peripheral tissue such as blood; suggesting a system wide and potentially common epigenetic response to stress.

On a genome wide level, we were able to show that gene expression and DNA methylation in peripheral blood cells is distinct in PTSD patients exposed to childhood and adult trauma versus PTSD patients only exposed to adult trauma, both compared to a common trauma-exposed but PTSD negative control group [133]. Moreover, the transcripts differentially expressed were non-overlapping and these differences were paralleled by DNA methylation changes (Figure 10).

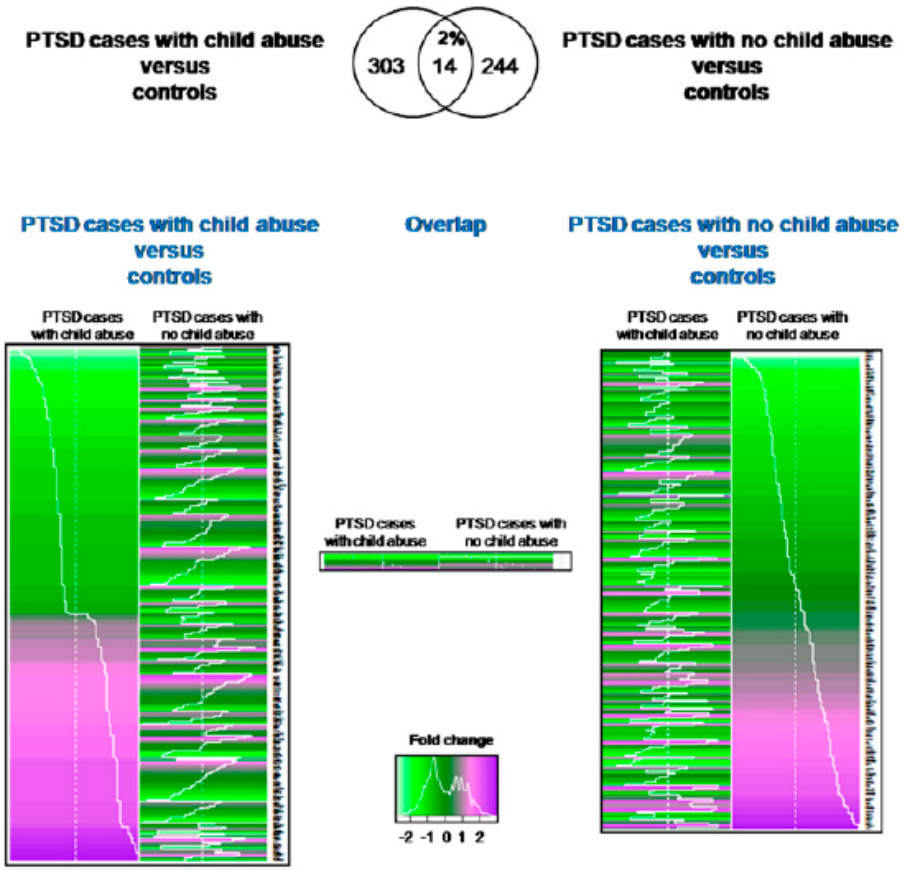


Figure 10. Heat map of gene expression profiles between PTSD cases with childhood absue and adult trauma vs. trauma exposed controls (left) and PTSD cases with adult trauma only vs. trauma exposed controls (right). Individuals suffering from PTSD with and without exposure to childhood abuse present nearly non-overlapping gene expression profiles. Both groups were exposed to similar levels of adult trauma and compared to a common non-PTSD control group. Figure taken from Mehta et al., PNAS, 2013 [133].

Interestingly, DNA methylation changes were pronounced in PTSD cases with childhood abuse compared to PTSD cases with adult trauma only suggesting not only a distinct biological mechanism but also more long-lasting biological effects by epigenetic adaptations provoked by early life stress (Figure 11).

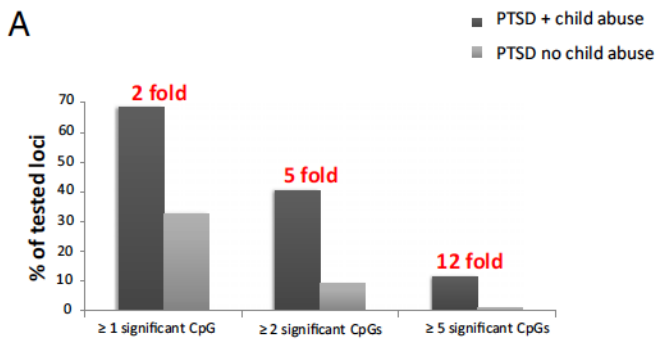


Figure 11. DNA methylation changes may contribute more in PTSD in response to childhood abuse than in PTSD in response to adult trauma. This graph shows the percentage of differentially expressed loci that harbor differentially methylated CpGs as detected by the Illumina 450k chip. Restricting the analysis to ≥1 CpG that is differentially methylated, a two fold enrichment in PTSD cases with childhood abuse over adult trauma only is detectable. This enrichment increases to twelve fold when restricting the analysis to ≥5 CpGs. Figure taken from Mehta et al., PNAS, 2013 [133].

It is still debatable whether or not epigenetic changes are the driver or the consequence of early life trauma induced gene expression changes. We addressed this question by correlating the DNA methylation changes we observed with gene expression profiles and found that for over 70% of the differentially methylated CpGs correlated with the expression of the corresponding transcript. Moreover, this correlation was strictly negative for CpGs around the transcription start site as expected but negative as well as positive for CpGs at other gene regions suggesting a role for DNA methylation at enhancer and repressor sites in response to childhood abuse fine-tuning the transcriptional response. Finally, case-control status of gene expression was paralleled by DNA methylation differences in over 90% of the transcripts investigated again suggesting a causal relationship between DNA methylation changes and gene expression of adjacent transcripts, although the majority of differentially methylated CpGs showed small absolute methylation differences below 5% suggesting that even small differences might be functional relevant. Surprisingly, CpGs that were associated with childhood abuse were more

likely to be located outside CpG dense regions such as CpG islands, arguing for an influence of childhood abuse on long-range regulatory elements.

In summary, these data suggest that distinct biological mechanisms underlie the pathophysiology of PTSD of individuals exposed to childhood trauma compared to individuals that were exposed to adult trauma only and trigger the development of PTSD. These data also highlight the importance of the timing and the type of the stressor in determining the molecular pathways that are involved in disease development. These results also support the hypothesis that broad clinical entities such as PTSD or MDD might subsume disorders that present with a common clinical phenotype but originate from separate biological mechanisms. In turn, this would also argue not only for more sophisticated diagnostic but also therapeutic approaches using patient-specific therapies potentially reducing the high variation in treatment response and resistance by current common but less specific drugs and therapies [134].

Timing of trauma is important

Both studies included in this thesis highlight the fact that the timing of the stressor is an important variable influencing epigenetic, gene regulation and disease outcomes. This is not surprising because epigenetic regulation is highly controlled with regard to cell type specificity and timing. This is also reflected by the differential expression of epigenetic writers, readers and erasers such as the DNMT enzymes that are highly expressed in proliferating and developing cells but are also detectable in post-mitotic neuronal cell types. Therefore, the impact of any given environmental factor not only depends on the cell type that is affected but also on the developmental stage at which the effect occurs. Developmental stages such as early childhood or adolescence that include high cellular turnover and complex rearrangements of the epigenetic as well as functional profile of the cell are

therefore more prone to long-term epigenetic deregulation than more stable stages later in life. On an epidemiological level, the differential sensitivity to trauma is reflected by the differential impact of environmental stressors on the development of psychiatric disorders with a stronger influence of early life trauma compared to adult trauma exposure [135].

Furthermore, animal studies such as the example of maternal care influencing GR methylation in rats argue for a time-dependent differential susceptibility. In contrast to the initial study by Weaver et al., Witzmann et al. subjected ten to twelve week old rats to a chronic stress paradigm. Although this paradigm lead to DNA methylation changes at the I_7 promoter of the rat GR, the CpG site at the NGFI-A site remained unchanged emphasizing distinct effects of timing and type of the stressor on the epigenetic profile [136]. Moreover, GR methylation in human post-mortem brain tissue of childhood abused individuals suffering from MDD and MDD controls (without exposure to childhood abuse) was similar although the expression of NGFI-A and the 1_F was altered [108,110]. On the level of the *FKBP5* GxE, this is paralleled by the strong impact of childhood abuse exposure but not adult trauma on the risk to develop PTSD [37]. Moreover, we were able to show that the peripheral DNA methylation pattern in individuals suffering from PTSD is distinct in response to childhood abuse compared to adult trauma exposure [133].

The use of peripheral tissue to investigate psychiatric disorders

As already discussed, using peripheral tissue such as blood or saliva is a necessity especially in large human studies where neuronal tissue is not readily available. Whether or not this tissue actually contains relevant information on pathophysiological mechanisms in the brain or represents mere surrogate markers is controversial; however, studies including our work suggest similarities between blood and neuronal tissue [126,132,137]. In particular this may be relevant for signaling cascades that are present in many tissues such as the GR activation by cortisol. In

addition, it remains elusive when the observed methylation changes get established or even if they represent existing risk factors since longitudinal studies investigating these marks over the lifetime are not available at the moment. As discussed already, DNA methylation is a binary readout with either a methylated or an unmethylated cytosine present at a certain genomic position. Though, intermediate methylation marks such as hydroxymethyl-, carboxyl- or formylcytosine may contribute to a more complex picture of DNA methylation. The partial methylation changes that we usually observe thus reflect either a specific subpopulation of cells that is most susceptible to certain environmental conditions or that the methylation pattern is not stably maintained across cell divisions. This applies for current gene expression studies as well, where the readout is not binary but clearly a composite measurement of a plethora of different cells in the given tissue. Single cell gene expression and epigenetic techniques will provide more insights into the cell-to-cell variability of transcription and regulation by epigenetic factors.

Genetic and epigenetic interplay

Future studies on the pathophysiology of psychiatric disorders need to address the complex interaction of several levels of genetic and environmental regulation. Although current genetic studies point towards a marginal contribution of main genetic effects to disease risk, the genome wide contribution of genetic variants needs to be further studied by increasing the power to reliably detect genetic effects. In addition to common polymorphisms, rare variants, but also structural variants such as insertions/deletions and larger copy number variations (CNVs) may contribute to the genetic predisposition. Although candidate GxE studies showed that genetic variants can modulate the impact of the environment, genome-wide GxE studies will provide a more comprehensive and certainly also more complex picture of GxE which is a statistical challenge given the amount of variables contributing to these interactions. In this regard, it will be important to characterize

the environmental factors contributing but also preventing psychiatric disorder in more detail. Here, epidemiological studies on detrimental and also protective environmental factors need to inform future GxE studies. Proximate to genetic and environmental measurements, knowledge on how these factors influence epigenetic conditions will be pivotal to understand the long-lasting influence of early life stressors and childhood abuse on psychiatric disorders. Here, we need to understand how the environment engages cellular signaling cascades and which cell types actually reflect the environmental exposure through its epigenetic profile, which is a complex network of DNA methylation, post-translational histone modification and non-coding RNAs. It remains an open question if epigenetic profiles such as DNA methylation reflect a predisposition or a consequence of environmental exposure. Longitudinal studies and animal models might help to understand the dynamics of this relationship. In addition, we have shown that genetic variation influences epigenetic profiles and their response to the environment on a candidate gene levels but similar effects on a genome wide level remain to be probed. Finally, to understand the consequence of genotype-specific, environmentally induced epigenetic changes, functional studies investigating the impact of those modifications on chromatic organization, gene expression and protein function are needed.

Global versus specific changes in response to childhood abuse

The studies included here make on one side a case for very specific epigenetic changes at one gene locus and on the other side argue for global changes in response to childhood trauma. It is unlikely that stress in the form of traumatic experiences will result in the modification of singular genes that in turn are responsible for the spectrum of observed molecular and behavioral changes. In fact, the response to stress is system wide including the immune system, the HPA axis, and the metabolic system and brain tissue. Thus, the impact of trauma is likely

genome wide, influencing multiple signaling cascades that result in specific phenotypes. Nonetheless, the effect of childhood abuse can induce very specific changes at even single CpG dinucleotides in *FKBP5* that in turn influence transcription factor binding and result in modification of the HPA axis. Therefore, global as well as local epigenetic changes may play a role in the pathogenesis of stress-related disorders. However, the DNA methylation changes we and others have observed are not random. We have shown that DNA methylation at specific enhancer elements in *FKBP5* influence the transcriptional activation of the gene. On a global level, childhood abuse induced pronounced alterations in DNA methylation of distal regulatory elements. It is important to mention however, that current approaches might not be able to detect DNA methylation in regions that are implicated in stress-related psychiatric disorders. For example, the *FKBP5* GRE regions investigated in the first study are not covered by the widely used Illumina 450k methylation array nor would they be covered by promoter tiling arrays or techniques such as reduced representative bisulfite sequencing (RRBS). In addition, although we gained substantial knowledge by using the Illumina 450k array, it covers only a fraction of the 28 million CpG sites in the genome and disregards non-CpG methylation and other forms of DNA methylation such as hydroxymethylation.

Aside from the question of where to look for DNA methylation changes or other epigenetic modifications in the genome, it also remains an open question of which cell type will be most informative on the level of disease marker but also to gain pathophysiological insight. Clearly, it would be interesting to investigate single neurons from different brain regions to look into the tissue and cell type that most likely mediates the effects of stress but this will remain difficult in larger human studies. On the other side, stress has been shown to affect multiple cell types on an organism wide level. Thus, the investigation of cell types not directly related to the brain but affected by common stress pathways may be beneficial. The investigation of heterogeneous tissue such as blood, saliva or even neuronal tissue leads to a composite readout over multiple different cell types that may cover true signals and

introduce confounders by changing cell type composition. This is especially relevant for psychiatric studies that so far have yielded rather small DNA methylation differences compared to other fields such as cancer. Future studies therefore may benefit from cell sorting and single cell techniques.

Implications on study design in human epigenetic studies

Most studies investigating DNA methylation in psychiatry to date are cross sectional. The advantage is that many individuals can be included in a short time frame and stratified by a case-control design. Nevertheless, this design does not enable us to follow epigenetic profiles over time and answer questions regarding the timing of the stressor and sequential molecular and behavioral alterations. Thus, longitudinal studies in populations at risk for psychiatric disorders are needed to get more insight in the dynamics of epigenetic changes in response to stress. Although stress is a major risk factor for PTSD and MDD and convincing evidence for epigenetic alterations in response to stress exist, positive environments can also influence disease development and thus need to be included. More recently, studies in rodents showed that aversive environmental conditions might induce effects in subsequent generations and suggest that apart from a behavioral transmission, epigenetic marks might be inherited through the gametes [138]. At the moment it is unclear if this form of inheritance is relevant for human psychiatric disorders is but future studies may need to consider controlling for environmental exposure of the ancestral generation. In sum, future studies need to take a complex relationship of environmental exposure, genetic predisposition, dynamic epigenetic alterations and functional readout into account.

Can environmental epigenetics inform psychiatric therapy?

The reversibility of epigenetic marks offers the possibility to modulate environmentally induced or disease associated epigenetic marks. Although first examples of epigenetic drugs influencing behavior in rodents were reported, it remains speculative at the moment if unidirectional drugs such as DNMT inhibitors would reverse the bidirectional changes in DNA methylation seen in early life stress models. In addition, not only is the direction of epigenetic marks in response to the environment highly variable but also the genomic location, thereby challenging the idea of an epigenetic drug restoring the complex epigenetic pattern in a given neuronal cell, while at the same time not damaging other cell types. Promising research tools that might be able to deliver induced epigenetic modification in a temporal and spatial manner are the protein-guided Transcription Activator-Like Effectors (TALEs) and the-RNA guided Clustered Regularly Interspaced Short Palindromic Repeats CRISPR/Cas9 systems [139]. However, the direct manipulation of epigenetic marks will be challenging because of potential off target effects and epigenetic modifiers might be more useful in manipulating the epigenome towards an enhanced plasticity to enable the reversal of pathological marks by conventional techniques such as psychotherapy or medications [140].

Apart from therapeutically targeting epigenetic marks, a profound knowledge on disease associated epigenetic imprints will be helpful in predicting and tracking therapeutic approaches [141-143].

References

1 Kessler, R. C. *et al.* Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. *Arch Gen Psychiatry* **51**, 8-19 (1994).

2 Kessler, R. C., Sonnega, A., Bromet, E., Hughes, M. & Nelson, C. B. Posttraumatic stress disorder in the National Comorbidity Survey. *Arch Gen Psychiatry* **52**, 1048-1060 (1995).

3 Ferrari, A. J. *et al.* Burden of depressive disorders by country, sex, age, and year: findings from the global burden of disease study 2010. *PLoS medicine* **10**, e1001547, doi:10.1371/journal.pmed.1001547 (2013).

4 Sullivan, P. F., Neale, M. C. & Kendler, K. S. Genetic epidemiology of major depression: review and meta-analysis 19. *Am J Psychiatry* **157**, 1552-1562 (2000).

5 Sartor, C. E. *et al.* Common heritable contributions to low-risk trauma, high-risk trauma, posttraumatic stress disorder, and major depression. *Arch Gen Psychiatry* **69**, 293-299, doi:10.1001/archgenpsychiatry.2011.1385 (2012).

6 Kendler, K. S., Gatz, M., Gardner, C. O. & Pedersen, N. L. A Swedish national twin study of lifetime major depression. *Am. J. Psychiat.* **163**, 109-114, doi:10.1176/appi.ajp.163.1.109 (2006).

7 Biological insights from 108 schizophrenia-associated genetic loci. *Nature* **511**, 421-427, doi:10.1038/nature13595 (2014).

8 Consortium, M. D. D. W. G. o. t. P. G. A mega-analysis of genome-wide association studies for major depressive disorder. *Mol Psychiatry* **18**, 497-511. (2012).

9 Muglia, P. *et al.* Genome-wide association study of recurrent major depressive disorder in two European case-control cohorts. *Mol Psychiatry* **15**, 589-601 (2010).

10 Shi, J. *et al.* Genome-wide association study of recurrent early-onset major depressive disorder. *Mol Psychiatry* **16**, 193-201 (2011).

11 Sullivan, P. F. *et al.* Genome-wide association for major depressive disorder: a possible role for the presynaptic protein piccolo. *Mol Psychiatry* **14**, 359-375 (2009).

12 Guffanti, G. *et al.* Genome-wide association study implicates a novel RNA gene, the lincRNA AC068718.1, as a risk factor for post-traumatic stress disorder in women. *Psychoneuroendocrinology* **38**, 3029-3038, doi:10.1016/j.psyneuen.2013.08.014 (2013).

13 Logue, M. W. *et al.* A genome-wide association study of post-traumatic stress disorder identifies the retinoid-related orphan receptor alpha (RORA) gene as a significant risk locus. *Mol Psychiatry* **18**, 937-942, doi:10.1038/mp.2012.113 (2013).

14 Xie, P. *et al.* Genome-wide association study identifies new susceptibility loci for posttraumatic stress disorder. *Biol Psychiatry* **74**, 656-663, doi:10.1016/j.biopsych.2013.04.013 (2013).

15 Stein, M. B., Jang, K. L., Taylor, S., Vernon, P. A. & Livesley, W. J. Genetic and environmental influences on trauma exposure and posttraumatic stress disorder symptoms: A twin study. *Am J Psychiat.* **159**, 1675-1681, doi:10.1176/appi.ajp.159.10.1675 (2002).

16 Rutter, M., Moffitt, T. E. & Caspi, A. Gene-environment interplay and psychopathology: multiple varieties but real effects. *Journal of Child Psychology and Psychiatry* **47**, 226-261, doi:10.1111/j.1469-7610.2005.01557.x (2006).

17 Lupien, S. J., McEwen, B. S., Gunnar, M. R. & Heim, C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature reviews. Neuroscience* **10**, 434-445, doi:10.1038/nrn2639 (2009).

18 McLaughlin, K. A. *et al.* Childhood adversities and first onset of psychiatric disorders in a national sample of US adolescents. *Arch Gen Psychiatry* **69**, 1151-1160, doi:10.1001/archgenpsychiatry.2011.2277 (2012).

19 Kessler, R. C., Davis, C. G. & Kendler, K. S. Childhood adversity and adult psychiatric disorder in the US National Comorbidity Survey. *Psychol Med.* **27**, 1101-1119 (1997).

20 Kendler, K. S., Prescott, C. A., Myers, J. & Neale, M. C. The structure of genetic and environmental risk factors for common psychiatric and substance use disorders in men and women. *Arch Gen Psychiatry* **60**, 929-937, doi:10.1001/archpsyc.60.9.929 (2003).

21 Kendler, K. S., Prescott, C. A., Myers, J. & Neale, M. C. The structure of genetic and environmental risk factors for common psychiatric and substance use disorders in men and women. *Arch Gen Psychiatry* **60**, 929-937 (2003).

22 Kendler, K. S. *et al.* Childhood sexual abuse and adult psychiatric and substance use disorders in women: an epidemiological and cotwin control analysis. *Arch Gen Psychiatry* **57**, 953-959 (2000).

23 Kessler, R. C. *et al.* Childhood adversities and adult psychopathology in the WHO World Mental Health Surveys. *The British journal of psychiatry : the journal of mental science* **197**, 378-385, doi:10.1192/bjp.bp.110.080499 (2010).

24 Sanchez, M. M., Ladd, C. O. & Plotsky, P. M. Early adverse experience as a developmental risk factor for later psychopathology: Evidence from rodent and primate models. *Dev Psychopathol.* **13**, 419-449, doi:10.1017/s0954579401003029 (2001).

25 Scharf, S. H. & Schmidt, M. V. Animal models of stress vulnerability and resilience in translational research. *Current psychiatry reports* **14**, 159-165, doi:10.1007/s11920-012-0256-0 (2012).

26 Manuck, S. B. & McCaffery, J. M. in *Annual Review of Psychology, Vol 65* Vol. 65 *Annual Review of Psychology* (ed S. T. Fiske) 41-70 (2014).

27 Belsky, J. *et al.* Vulnerability genes or plasticity genes? *Mol Psychiatry* **14**, 746-754 (2009).

28 Daskalakis, N. P., Bagot, R. C., Parker, K. J., Vinkers, C. H. & de Kloet, E. R. The three-hit concept of vulnerability and resilience: toward understanding adaptation to early-life adversity outcome. *Psychoneuroendocrinology* **38**, 1858-1873, doi:10.1016/j.psyneuen.2013.06.008 (2013).

29 Caspi, A. *et al.* Role of genotype in the cycle of violence in maltreated children. *Science* **297**, 851-854, doi:10.1126/science.1072290 (2002).

30 Caspi, A. *et al.* Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* **301**, 386-389 (2003).

31 Duncan, L. E. & Keller, M. C. A critical review of the first 10 years of candidate gene-by-environment interaction research in psychiatry. *Am J Psychiatry* **168**, 1041-1049, doi:10.1176/appi.ajp.2011.11020191 (2011).

32 Munafo, M. R., Zammit, S. & Flint, J. Practitioner Review: A critical perspective on gene-environment interaction models - what impact should they have on clinical perceptions and practice? *Journal of child psychology and psychiatry, and allied disciplines* **55**, 1092-1101, doi:10.1111/jcpp.12261 (2014).

33 Munafo, M. R., Durrant, C., Lewis, G. & Flint, J. Gene X environment interactions at the serotonin transporter locus. *Biol Psychiatry* **65**, 211-219 (2009).

34 Keller, M. C. Gene x environment interaction studies have not properly controlled for potential confounders: the problem and the (simple) solution. *Biol Psychiatry* **75**, 18-24, doi:10.1016/j.biopsych.2013.09.006 (2014).

35 Koenen, K. C. *et al.* Modification of the association between serotonin transporter genotype and risk of posttraumatic stress disorder in adults by county-level social environment. *American journal of epidemiology* **169**, 704-711, doi:10.1093/aje/kwn397 (2009).

36 Kilpatrick, D. G. *et al.* The serotonin transporter genotype and social support and moderation of posttraumatic stress disorder and depression in hurricane-exposed adults. *Am J Psychiatry* **164**, 1693-1699 (2007).

37 Binder, E. B. *et al.* Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. *Jama* **299**, 1291-1305 (2008).

38 Xie, P. *et al.* Interaction of FKBP5 with childhood adversity on risk for post-traumatic stress disorder. *Neuropsychopharmacology* **35**, 1684-1692 (2010).

39 Roy, A., Gorodetsky, E., Yuan, Q., Goldman, D. & Enoch, M. A. Interaction of FKBP5, a stress-related gene, with childhood trauma increases the risk for attempting suicide. *Neuropsychopharmacology* **35**, 1674-1683 (2010).

40 Bevilacqua, L. *et al.* Interaction between FKBP5 and childhood trauma and risk of aggressive behavior. *Arch Gen Psychiatry* **69**, 62-70 (2012).

41 Appel, K. *et al.* Moderation of Adult Depression by a Polymorphism in the FKBP5 Gene and Childhood Physical Abuse in the General Population. *Neuropsychopharmacology* **36**, 1982-91. (2011).

42 Zimmermann, P. *et al.* Interaction of FKBP5 gene variants and adverse life events in predicting depression onset: results from a 10-year prospective community study. *Am J Psychiatry* **168**, 1107-1116 (2011).

43 Holsboer, F. The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology* **23**, 477-501 (2000).

44 Zannas, A. S. & Binder, E. B. Gene-environment interactions at the FKBP5 locus: sensitive periods, mechanisms and pleiotropism. *Genes Brain Behav.* **13**, 25-37, doi:10.1111/gbb.12104 (2014).

45 Nakao, K., Myers, J. E. & Faber, L. E. Development of a monoclonal antibody to the rabbit 8.5S uterine progestin receptor. *Canadian journal of biochemistry and cell biology = Revue canadienne de biochimie et biologie cellulaire* **63**, 33-40, doi:10.1139/o85-005 (1985).

46 Kang, C. B., Hong, Y., Dhe-Paganon, S. & Yoon, H. S. FKBP family proteins: immunophilins with versatile biological functions. *Neuro-Signals* **16**, 318-325, doi:10.1159/000123041 (2008).

47 Pirkl, F. & Buchner, J. Functional analysis of the Hsp90-associated human peptidyl prolyl cis/trans isomerases FKBP51, FKBP52 and Cyp40. *Journal of molecular biology* **308**, 795-806, doi:10.1006/jmbi.2001.4595 (2001).

48 Chrousos, G. P. *et al.* The new world primates as animal models of glucocorticoid resistance. *Advances in experimental medicine and biology* **196**, 129-144 (1986).

49 Reynolds, P. D., Pittler, S. J. & Scammell, J. G. Cloning and expression of the glucocorticoid receptor from the squirrel monkey (Saimiri boliviensis boliviensis), a glucocorticoid-resistant primate. *The Journal of clinical endocrinology and metabolism* **82**, 465-472, doi:10.1210/jcem.82.2.3755 (1997).

50 Reynolds, P. D. *et al.* Glucocorticoid-resistant B-lymphoblast cell line derived from the Bolivian squirrel monkey (Saimiri boliviensis boliviensis). *Laboratory animal science* **48**, 364-370 (1998).

51 Reynolds, P. D., Ruan, Y., Smith, D. F. & Scammell, J. G. Glucocorticoid resistance in the squirrel monkey is associated with overexpression of the immunophilin FKBP51. *The Journal of clinical endocrinology and metabolism* **84**, 663-669, doi:10.1210/jcem.84.2.5429 (1999).

52 Denny, W. B., Valentine, D. L., Reynolds, P. D., Smith, D. F. & Scammell, J. G. Squirrel monkey immunophilin FKBP51 is a potent inhibitor of glucocorticoid receptor binding. *Endocrinology* **141**, 4107-4113, doi:10.1210/endo.141.11.7785 (2000).

53 Wochnik, G. M. *et al.* FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. *J Biol Chem.* **280**, 4609-4616 (2005).

54 Riggs, D. L. *et al.* The Hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling in vivo. *The EMBO Journal* **22**, 1158-1167, doi:10.1093/emboj/cdg108 (2003).

55 Riggs, D. L. *et al.* Noncatalytic role of the FKBP52 peptidyl-prolyl isomerase domain in the regulation of steroid hormone signaling. *Mol Cell Biol* **27**, 8658-8669, doi:10.1128/mcb.00985-07 (2007).

56 Schulke, J. P. *et al.* Differential impact of tetratricopeptide repeat proteins on the steroid hormone receptors. *PLoS One* **5**, e11717, doi:10.1371/journal.pone.0011717 (2010).

57 Binder, E. B. The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. *Psychoneuroendocrinology* **34 Suppl 1**, S186-S195 (2009).

58 Paakinaho, V., Makkonen, H., Jaaskelainen, T. & Palvimo, J. J. Glucocorticoid receptor activates poised FKBP51 locus through long-distance interactions. *Mol Endocrinol.* **24**, 511-525 (2010).

59 Jaaskelainen, T., Makkonen, H. & Palvimo, J. J. Steroid up-regulation of FKBP51 and its role in hormone signaling. *Curr Opin Pharmacol.* **11**, 326-331 (2011).

60 Scharf, S. H., Liebl, C., Binder, E. B., Schmidt, M. V. & Muller, M. B. Expression and Regulation of the Fkbp5 Gene in the Adult Mouse Brain. *Plos One* **6**, 10, doi:10.1371/journal.pone.0016883 (2011).

61 Menke, A. *et al.* Dexamethasone Stimulated Gene Expression in Peripheral Blood is a Sensitive Marker for Glucocorticoid Receptor Resistance in Depressed Patients. *Neuropsychopharmacology* **37**, 1455-1464, doi:10.1038/npp.2011.331 (2012).

62 Blair, L. J. *et al.* Accelerated neurodegeneration through chaperone-mediated oligomerization of tau. *Journal of Clinical Investigation* **123**, 4158-4169, doi:10.1172/jci69003 (2013).

63 Attwood, B. K. *et al.* Neuropsin cleaves EphB2 in the amygdala to control anxiety. *Nature* **473**, 372-375 (2011).

64 Sinclair, D., Fillman, S. G., Webster, M. J. & Weickert, C. S. Dysregulation of glucocorticoid receptor co-factors FKBP5, BAG1 and PTGES3 in prefrontal cortex in psychotic illness. *Scientific reports* **3**, 3539, doi:10.1038/srep03539 (2013).

65 Lee, R. S. *et al.* A measure of glucocorticoid load provided by DNA methylation of Fkbp5 in mice. *Psychopharmacology (Berl)* **18**, 303-12 (2011).

66 Binder, E. B. *et al.* Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. *Nat Genet.* **36**, 1319-1325 (2004).

67 Ising, M. *et al.* Polymorphisms in the FKBP5 gene region modulate recovery from psychosocial stress in healthy controls. *Eur J Neurosci.* **28**, 389-398 (2008).

68 Luijk, M. P. *et al.* FKBP5 and resistant attachment predict cortisol reactivity in infants: gene-environment interaction. *Psychoneuroendocrinology* **35**, 1454-1461 (2010).

69 Fani, N. *et al.* FKBP5 and attention bias for threat: associations with hippocampal function and shape. *JAMA Psychiatry* **70**, 392-400, doi:10.1001/2013.jamapsychiatry.210 (2013).

70 Menke, A. *et al.* Genetic variation in FKBP5 associated with the extent of stress hormone dysregulation in major depression. *Genes Brain Behav.* **12**, 289-296, doi:10.1111/gbb.12026 (2013).

71 Mehta, D. *et al.* Using Polymorphisms in FKBP5 to Define Biologically Distinct Subtypes of Posttraumatic Stress Disorder Evidence From Endocrine and Gene Expression Studies. *Arch Gen Psychiatry* **68**, 901-910, doi:10.1001/archgenpsychiatry.2011.50 (2011).

72 Jaenisch, R. & Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genet.* **33**, 245-254, doi:10.1038/ng1089 (2003).

73 Bonasio, R., Tu, S. & Reinberg, D. Molecular signals of epigenetic states. *Science* **330**, 612-616, doi:10.1126/science.1191078 (2010).

74 Egger, G., Liang, G., Aparicio, A. & Jones, P. A. Epigenetics in human disease and prospects for epigenetic therapy. *Nature* **429**, 457-463, doi:10.1038/nature02625 (2004).

75 Holliday, R. Epigenetics: a historical overview. *Epigenetics* **1**, 76-80 (2006).

76 Jenuwein, T. & Allis, C. D. Translating the histone code. *Science* **293**, 1074-1080, doi:10.1126/science.1063127 (2001).

77 Bird, A. Perceptions of epigenetics. *Nature* **447**, 396-398, doi:10.1038/nature05913 (2007).

78 Peschansky, V. J. & Wahlestedt, C. Non-coding RNAs as direct and indirect modulators of epigenetic regulation. *Epigenetics* **9**, 3-12, doi:10.4161/epi.27473 (2014).

79 Szulwach, K. E. *et al.* 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. *Nat Neurosci* **14**, 1607-1616, doi:10.1038/nn.2959 (2011).

80 Lienert, F. *et al.* Identification of genetic elements that autonomously determine DNA methylation states 4 200. *Nat.Genet.* **43**, 1091-1097 (2011).

81 Mercer, T. R. & Mattick, J. S. Structure and function of long noncoding RNAs in epigenetic regulation. *Nature structural & molecular biology* **20**, 300-307, doi:10.1038/nsmb.2480 (2013).

82 Morris, K. V. & Mattick, J. S. The rise of regulatory RNA. *Nature reviews. Genetics* **15**, 423-437, doi:10.1038/nrg3722 (2014).

83 Murr, R. in *Epigenetics and Cancer, Pt A* Vol. 70 *Advances in Genetics* (eds Z. Herceg & T. Ushijima) 101-141 (Elsevier Academic Press Inc, 2010).

84 Levenson, J. M. & Sweatt, J. D. Epigenetic mechanisms in memory formation. *Nat. Rev. Neurosci.* **6**, 108-118, doi:10.1038/nrn1604 (2005).

85 Maddox, S. A., Schafe, G. E. & Ressler, K. J. Exploring epigenetic regulation of fear memory and biomarkers associated with post-traumatic stress disorder. *Front Psychiatry* **4**, 62, doi:10.3389/fpsyt.2013.00062 (2013).

86 Mill, J. & Petronis, A. Molecular studies of major depressive disorder: the epigenetic perspective. *Mol. Psychiatr.* **12**, 799-814, doi:10.1038/sj.mp.4001992 (2007).

87 Zovkic, I. B. & Sweatt, J. D. Epigenetic mechanisms in learned fear: implications for PTSD. *Neuropsychopharmacology* **38**, 77-93, doi:10.1038/npp.2012.79 (2013).

88 Tsankova, N., Renthal, W., Kumar, A. & Nestler, E. J. Epigenetic regulation in psychiatric disorders. *Nat. Rev. Neurosci.* **8**, 355-367, doi:10.1038/nrn2132 (2007).

89 Bahari-Javan, S., Sananbenesi, F. & Fischer, A. Histone-acetylation: a link between Alzheimer's disease and post-traumatic stress disorder? *Frontiers in neuroscience* **8**, 160, doi:10.3389/fnins.2014.00160 (2014).

90 Sananbenesi, F. & Fischer, A. The epigenetic bottleneck of neurodegenerative and psychiatric diseases. *Biological Chemistry* **390**, 1145-1153, doi:10.1515/bc.2009.131 (2009).

91 Weaver, I. C. *et al.* Epigenetic programming by maternal behavior. *Nat Neurosci.* **7**, 847-854 (2004).

92 Murgatroyd, C. *et al.* Dynamic DNA methylation programs persistent adverse effects of early-life stress. *Nat.Neurosci.* **12**, 1559-1566 (2009).

93 Elliott, E., Ezra-Nevo, G., Regev, L., Neufeld-Cohen, A. & Chen, A. Resilience to social stress coincides with functional DNA methylation of the Crf gene in adult mice. *Nat Neurosci.* **13**, 1351-1353 (2010).

94 Lee, R. S. *et al.* Chronic corticosterone exposure increases expression and decreases deoxyribonucleic acid methylation of Fkbp5 in mice. *Endocrinology* **151**, 4332-4343 (2010).

95 Kress, C., Thomassin, H. & Grange, T. Active cytosine demethylation triggered by a nuclear receptor involves DNA strand breaks. *Proc Natl Acad Sci USA* **103**, 11112-11117 (2006).

96 Thomassin, H., Flavin, M., Espinas, M. L. & Grange, T. Glucocorticoid-induced DNA demethylation and gene memory during development. *Embo J.* **20**, 1974-1983 (2001).

97 Feldmann, A. *et al.* Transcription Factor Occupancy Can Mediate Active Turnover of DNA Methylation at Regulatory Regions. *PLoS Genet.* **9**, doi:10.1371/journal.pgen.1003994 (2013).

98 Bhutani, N., Burns, D. M. & Blau, H. M. DNA demethylation dynamics. *Cell* **146**, 866-872 (2011).

99 Gordon, L. *et al.* Expression discordance of monozygotic twins at birth: effect of intrauterine environment and a possible mechanism for fetal programming. *Epigenetics* **6**, 579-592 (2011).

100 Fraga, M. F. *et al.* Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A* **102**, 10604-10609, doi:10.1073/pnas.0500398102 (2005).

101 Wong, C. C. *et al.* Methylomic analysis of monozygotic twins discordant for autism spectrum disorder and related behavioural traits. *Mol Psychiatry* **19**, 495-503, doi:10.1038/mp.2013.41 (2014).

102 Ouellet-Morin, I. *et al.* Increased serotonin transporter gene (SERT) DNA methylation is associated with bullying victimization and blunted cortisol response to stress in childhood: a longitudinal study of discordant monozygotic twins. *Psychol Med* **43**, 1813-1823, doi:10.1017/s0033291712002784 (2013).

103 Dempster, E. L. *et al.* Genome-wide Methylomic Analysis of Monozygotic Twins Discordant for Adolescent Depression. *Biol Psychiatry*, doi:10.1016/j.biopsych.2014.04.013 (2014).

104 Byrne, E. M. *et al.* Monozygotic twins affected with major depressive disorder have greater variance in methylation than their unaffected co-twin. *Transl Psychiatry* **3**, e269, doi:10.1038/tp.2013.45 (2013).

105 Tobi, E. W. *et al.* DNA methylation signatures link prenatal famine exposure to growth and metabolism. *Nature communications* **5**, 5592, doi:10.1038/ncomms6592 (2014).

106 Heijmans, B. T. *et al.* Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* **105**, 17046-17049, doi:10.1073/pnas.0806560105 (2008).

107 Bygren, L. O. *et al.* Change in paternal grandmothers' early food supply influenced cardiovascular mortality of the female grandchildren. *BMC genetics* **15**, 12, doi:10.1186/1471-2156-15-12 (2014).

108 McGowan, P. O. *et al.* Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci.* **12**, 342-348 (2009).

109 Suderman, M. *et al.* Conserved epigenetic sensitivity to early life experience in the rat and human hippocampus. *Proc Natl Acad Sci U S A* **109**, 17266-17272, doi:10.1073/pnas.1121260109 (2012).

110 Alt, S. R. *et al.* Differential expression of glucocorticoid receptor transcripts in major depressive disorder is not epigenetically programmed. *Psychoneuroendocrinology* **35**, 544-556, doi:10.1016/j.psyneuen.2009.09.001 (2010).

111 Uddin, M. *et al.* Epigenetic and immune function profiles associated with posttraumatic stress disorder. *Proc Natl Acad Sci U S A* **107**, 9470-9475, doi:10.1073/pnas.0910794107 (2010).

112 Smith, A. K. *et al.* Differential immune system DNA methylation and cytokine regulation in post-traumatic stress disorder. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics* **156b**, 700-708, doi:10.1002/ajmg.b.31212 (2011).

113 Uddin, M. *et al.* Epigenetic and inflammatory marker profiles associated with depression in a community-based epidemiologic sample. *Psychol Med* **41**, 997-1007, doi:10.1017/s0033291710001674 (2011).

114 Fuchikami, M. *et al.* DNA methylation profiles of the brain-derived neurotrophic factor (BDNF) gene as a potent diagnostic biomarker in major depression. *PLoS One* **6**, e23881, doi:10.1371/journal.pone.0023881 (2011).

115 Rusiecki, J. A. *et al.* DNA methylation in repetitive elements and post-traumatic stress disorder: a case-control study of US military service members. *Epigenomics* **4**, 29-40, doi:10.2217/epi.11.116 (2012).

116 Sabunciyan, S. *et al.* Genome-wide DNA methylation scan in major depressive disorder. *PLoS One* **7**, e34451, doi:10.1371/journal.pone.0034451 (2012).

117 Alasaari, J. S. *et al.* Environmental stress affects DNA methylation of a CpG rich promoter region of serotonin transporter gene in a nurse cohort. *PLoS One* **7**, e45813, doi:10.1371/journal.pone.0045813 (2012).

118 Labonte, B. *et al.* Genome-wide Epigenetic Regulation by Early-Life Trauma. *Arch. Gen. Psychiatry* **69**, 722-731 (2012).

119 Yang, B. Z. *et al.* Child abuse and epigenetic mechanisms of disease risk. *American journal of preventive medicine* **44**, 101-107, doi:10.1016/j.amepre.2012.10.012 (2013).

120 Melas, P. A. *et al.* Genetic and epigenetic associations of MAOA and NR3C1 with depression and childhood adversities. *Int J Neuropsychopharmacol* **16**, 1513-1528, doi:10.1017/s1461145713000102 (2013).

121 Kang, H. J. *et al.* Association of SLC6A4 methylation with early adversity, characteristics and outcomes in depression. *Prog Neuropsychopharmacol Biol Psychiatry* **44**, 23-28, doi:10.1016/j.pnpbp.2013.01.006 (2013).

122 Zhao, J., Goldberg, J., Bremner, J. D. & Vaccarino, V. Association between promoter methylation of serotonin transporter gene and depressive symptoms: a monozygotic twin study. *Psychosomatic medicine* **75**, 523-529, doi:10.1097/PSY.0b013e3182924cf4 (2013).

123 Klengel, T., Pape, J., Binder, E. B. & Mehta, D. The role of DNA methylation in stress-related psychiatric disorders. *Neuropharmacology* **80**, 115-132, doi:10.1016/j.neuropharm.2014.01.013 (2014).

124 Kerkel, K. *et al.* Genomic surveys by methylation-sensitive SNP analysis identify sequence-dependent allele-specific DNA methylation. *Nature Genet.* **40**, 904-908, doi:10.1038/ng.174 (2008).

125 Gertz, J. *et al.* Analysis of DNA methylation in a three-generation family reveals widespread genetic influence on epigenetic regulation. *PLoS Genet* **7**, e1002228, doi:10.1371/journal.pgen.1002228 (2011).

126 Klengel, T. *et al.* Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. *Nature Neuroscience* **16**, 33-41, doi:10.1038/nn.3275 (2013).

127 Splinter, E. & de Laat, W. The complex transcription regulatory landscape of our genome: control in three dimensions. *The EMBO journal* **30**, 4345-4355, doi:10.1038/emboj.2011.344 (2011).

128 Meyer, K. B. *et al.* A functional variant at a prostate cancer predisposition locus at 8q24 is associated with PVT1 expression. *PLoS Genet* **7**, e1002165, doi:10.1371/journal.pgen.1002165 (2011).

129 Mitchell, A. C. *et al.* The Genome in Three Dimensions: A New Frontier in Human Brain Research. *Biol Psychiatry*, doi:10.1016/j.biopsych.2013.07.015 (2013).

130 Gillespie, C. F. *et al.* Trauma exposure and stress-related disorders in inner city primary care patients. *Gen Hosp Psychiatry* **31**, 505-514 (2009).

131 McGowan, P. O. *et al.* Broad Epigenetic Signature of Maternal Care in the Brain of Adult Rats. *Plos One* **6**, doi:10.1371/journal.pone.0014739 (2011).

132 Provencal, N. *et al.* The signature of maternal rearing in the methylome in rhesus macaque prefrontal cortex and T cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**, 15626-15642, doi:10.1523/jneurosci.1470-12.2012 (2012).

133 Mehta, D. *et al.* Childhood maltreatment is associated with distinct genomic and epigenetic profiles in posttraumatic stress disorder. *Proc Natl Acad Sci U S A* **110**, 8302-8307, doi:10.1073/pnas.1217750110 (2013).

134 Holsboer, F. How can we realize the promise of personalized antidepressant medicines? *Nat. Rev. Neurosci.* **9**, 638-U614, doi:10.1038/nrn2453 (2008).

135 Heim, C., Shugart, M., Craighead, W. E. & Nemeroff, C. B. Neurobiological and psychiatric consequences of child abuse and neglect. *Dev.Psychobiol.* **52**, 671-690 (2010).

136 Witzmann, S. R., Turner, J. D., Meriaux, S. B., Meijer, O. C. & Muller, C. P. Epigenetic regulation of the glucocorticoid receptor promoter 1(7) in adult rats. *Epigenetics* **7**, 1290-1301, doi:10.4161/epi.22363 (2012).

137 Smith, A. K. *et al.* DNA extracted from saliva for methylation studies of psychiatric traits: Evidence tissue specificity and relatedness to brain. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*, doi:10.1002/ajmg.b.32278 (2014).

138 Gapp, K. *et al.* Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nat Neurosci* **17**, 667-669, doi:10.1038/nn.3695 (2014).

139 Maeder, M. L. *et al.* Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. *Nat Biotechnol* **31**, 1137-1142, doi:10.1038/nbt.2726 (2013).

140 Sweatt, J. D. Experience-Dependent Epigenetic Modifications in the Central Nervous System. *Biol. Psychiatry* **65**, 191-197, doi:10.1016/j.biopsych.2008.09.002 (2009).

141 Guintivano, J., Arad, M., Gould, T. D., Payne, J. L. & Kaminsky, Z. A. Antenatal prediction of postpartum depression with blood DNA methylation biomarkers. *Mol Psychiatry* **19**, 560-567, doi:10.1038/mp.2013.62 (2014).

142 Roberts, S. *et al.* Serotonin tranporter methylation and response to cognitive behaviour therapy in children with anxiety disorders. *Transl Psychiatry* **4**, e467, doi:10.1038/tp.2014.109 (2014).

143 Yehuda, R. *et al.* Epigenetic Biomarkers as Predictors and Correlates of Symptom Improvement Following Psychotherapy in Combat Veterans with PTSD. *Front Psychiatry* **4**, 118, doi:10.3389/fpsyt.2013.00118 (2013).

Acknowledgements

Over the past years I was fortunate enough to work with Elisabeth B. Binder who has been a great mentor and role model in many aspects of scientific life. I would like to express my gratitude for her continuous guidance and mentorship.

I would like to thank Carsten T. Wotjak for his support and guidance as official thesis advisor and Wolfgang Enard for his willingness to examine this thesis. I appreciate the time and effort that Bettina Bölter, Elisabeth Weiß, Heinrich Leonhardt and Jürgen Soll spend on evaluating this thesis.

I am deeply grateful to all current and former members of the Binder lab and the Department for Translational Research in Psychiatry at the Max Planck Institute of Psychiatry. I would like to thank all current and former collaborators for their invaluable support.

Curriculum Vitae

Name: Torsten Klengel

Year of Birth: 1980

Nationality: German

Education

03/2014 – present	Research Fellow at the Department of Psychiatry and Behavioral Sciences and Yerkes National Primate Research Center, Emory School of Medicine, Advisor: Kerry J. Ressler MD, PhD
03/2012 – 03/2014	PhD student in Neurobiology at the Translational Research Department of the Max Planck Institute of Psychiatry and the Ludwig Maximilians University Munich, Director and Mentor: Elisabeth B. Binder MD, PhD, Co-Advisor: Carsten T. Wotjak PhD
08/2007 – 03/2012	Resident in Clinical Psychiatry at the Clinical Department of the Max Planck Institute of Psychiatry, Director: Florian Holsboer MD, PhD
03/2008	MD thesis, 'Molecular characterization of the carboanhydrase *NCE103* in the course of CO_2 induced polymorphism in *Candida albicans*', Julius Maximilians University Würzburg, Advisor: Matthias Frosch MD and Fritz A Mühlschlegel MD
04/2000 – 05/2007	MD, Julius Maximilians University Würzburg