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Novel Epigenetic and Genetic Biomarker Candidates in Post-Traumatic Stress Disorder

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Abbreviations

5-HTTLPR	Serotonin-transporter-linked polymorphic region
ACTH	Adrenocorticotropic hormone
ADCYAP1R1	Pituitary adenylate cyclase-activating polypeptide type I receptor (gene)
BDNF	Brain derived neurotrophic factor
CAPS	Clinician-administered PTSD scale
CpG	Cytosine-phosphate-guanine
CRF	Corticotropin releasing factor
CRF1	Corticotropin releasing factor receptor 1
CRHR1	Corticotropin releasing factor receptor 1 (gene)
CSF	Cerebrospinal fluid
CTQ	Childhood trauma questionnaire
DICER1	Dicer gene
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase
DSM-5	The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
EEG	Electroencephalography
FDA	Food and Drug Administration
FKBP5	FK506-binding protein 51 kDa (gene)
GABA	gamma-Aminobutyric acid
GAD	Generalized anxiety disorder
GR	Glucocorticoid receptor
GxE	Gene by environment interaction
HPA	Hypothalamic-pituitary-adrenal

ICD-10	International Statistical Classification of Diseases, 10th revision
ICV	Intracerebroventricular
IFN-γ	Interferon-gamma
IL-12	Interleukin 12
MDD	Major depressive disorder
miR	microRNA
NR3C1	Nuclear receptor subfamily 3 group C member 1
PAC1	Pituitary adenylate cyclase-activating polypeptide type I receptor
PBMCs	Peripheral blood mononuclear cells
PET	Positron emission tomography
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
RNA	Ribonucleic acid
SKA2	Spindle and kinetochore-associated complex subunit 2
SLC6A4	Serotonin transporter (gene)
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor
SNRI	Serotonin-norepinephrine reuptake inhibitor

List of Publications

Publication I

Pape JC, Carrillo-Roa T, Rothbaum BO, Nemeroff CB, Czamara D, Zannas AS, Iosifescu D, Mathew SJ, Neylan TC, Mayberg HS, Dunlop BW, Binder EB. (2018). DNA methylation levels are associated with CRF1 receptor antagonist treatment outcome in women with post-traumatic stress disorder. ***Clinical Epigenetics***, *10*(1). doi: 10.1186/s13148-018-0569-x

Publication II

Dunlop BW, Binder EB, Iosifescu D, Mathew SJ, Neylan TC, **Pape JC**, Carrillo-Roa T, Green C, Kinkead B, Grigoriadis D, Rothbaum BO, Nemeroff CB, Mayberg HS. (2017). Corticotropin-Releasing Factor Receptor 1 Antagonism Is Ineffective for Women With Posttraumatic Stress Disorder. ***Biological Psychiatry***, *82*(12), 866–874. doi: 10.1016/j.biopsych.2017.06.024

Publication III

Volk N, **Pape JC**, Engel M, Zannas AS, Cattane N, Cattaneo A, Binder EB, Chen A. (2016). Amygdalar MicroRNA-15a is Essential for Coping with Chronic Stress. ***Cell Reports***, *17*(7), 1882–1891. doi: 10.1016/j.celrep.2016.10.038

Summary

Post-traumatic stress disorder (PTSD) represents one of the major psychiatric disorders, yet treatment options, including pharmacological interventions, are still limited. As a regulator of the HPA axis and having been implicated in the pathogenesis of the disorder, the corticotropin-releasing factor (CRF) system represents a promising drug target in PTSD. However, results from large clinical trials using CRF receptor type 1 (CRF1) antagonists to treat other stress-related disorders like major depressive disorder (MDD) or generalized anxiety disorder (GAD) have been disappointing so far. To further improve pharmacological treatment, therapeutic concepts like precision medicine are of great value. This approach incorporates the idea of targeting the "right" patient with the "right" treatment. Here, clinically applicable biomarkers represent an essential tool. Diagnostic and prognostic markers will enable a biology-based diagnosis and allow us to apply preventive therapies for high-risk patients. Treatment biomarkers are specifically helpful in predicting individual treatment response but also tracking the effectiveness of a therapeutic intervention. This thesis aims to evaluate the efficacy of a CRF1 receptor antagonist in a cohort of PTSD diagnosed women with particular focus on biological subgroups showing differential treatment response and further to identify potential treatment biomarkers, specifically on the epigenetic level. Results from this randomized clinical trial revealed that the applied CRF1 receptor antagonist was not superior over placebo overall. However, a distinct subgroup of patients, hypothesized to have higher CRF system activity, showed significantly better treatment outcome. By examining DNA methylation levels of PTSD-relevant genes in peripheral blood before and after CRF1 receptor antagonist administration, this thesis identified potential epigenetic treatment biomarkers in PTSD for CRF1 antagonist therapy. Our previously described subgroup of responsive patients demonstrated

significantly different changes of *CRHR1* methylation levels over treatment time compared to the other patients suggesting *CRHR1* methylation as a possible treatment-tracking marker. Further, *NR3C1* methylation levels at baseline significantly interacted with early life stress to predict treatment outcome and may therefore serve as an epigenetic stratification biomarker, subgrouping patients prior to a therapeutic intervention. Interestingly, *NR3C1* methylation has previously been shown to predict PTSD treatment response after psychotherapy proposing a treatment biomarker independent of the type of therapy. In addition, the presented work investigated the role of miR-15a in the human stress response, a microRNA that has been shown to be crucially involved in stress reactivity in mice. Showing differential regulation in peripheral blood after dexamethasone treatment in healthy subjects as well as in adult patients with a history of early trauma, miR-15a represents another potential epigenetic biomarker in stress related disorders such as PTSD.

The presented data strengthen the concept of precision medicine in stress related psychiatric disorders by revealing biological subgroups with differential response to CRF1 receptor antagonist treatment. This thesis further identifies promising epigenetic biomarker candidates and highlights their future potential to improve preventive strategies, diagnosis and treatment in PTSD.

Zusammenfassung

Die Posttraumatische Belastungsstörung (PTBS) stellt eine der wichtigsten psychiatrischen Erkrankungen dar. Dennoch sind die Behandlungsmöglichkeiten einschließlich pharmakologischer Interventionen weiterhin deutlich begrenzt. Als Regulator der HPA-Achse und zudem an der Pathogenese der PTBS beteiligt, stellt das Corticotropin-Releasing-Factor (CRF) -System einen vielversprechenden Ansatzpunkt in der pharmakologischen Therapie der Erkrankung dar. Die Ergebnisse großer klinischer Studien zur Behandlung anderer stressbedingter Erkrankungen wie Major Depression (MDD) oder generalisierter Angststörung (GAD) mit CRF-Rezeptor-Typ-1-Antagonisten (CRF1) waren bisher jedoch enttäuschend. Im Rahmen der pharmakologischen Therapieoptimierung der PTBS spielen deshalb therapeutische Konzepte wie die Präzisionsmedizin eine bedeutende Rolle. Dieser Ansatz beinhaltet grundsätzlich die Idee, die „richtige“ Behandlung für den „richtigen“ Patienten zu identifizieren. In diesem Zusammenhang stellen klinisch anwendbare Biomarker ein wichtiges Instrument dar. Diagnostische und prognostische Marker gestatten eine biologiebasierte Diagnose und ermöglichen so den Einsatz von vorbeugenden Therapien für Hochrisikopatienten. Behandlungsbezogene Biomarker sind besonders hilfreich sowohl bei der Vorhersage des individuellen Ansprechens der Behandlung als auch beim Monitoring der Wirksamkeit einer therapeutischen Intervention. Die vorliegende Arbeit zielt darauf ab, die Wirksamkeit eines CRF1-Rezeptorantagonisten in einer Kohorte von PTBS-diagnostizierten Frauen zu evaluieren und legt dabei einen besonderen Schwerpunkt auf biologische Untergruppen mit unterschiedlichem Ansprechen auf die Behandlung. Ein weiterer Fokus liegt in der Identifikation potenzieller behandlungsbezogener Biomarker, insbesondere auf epigenetischer Ebene. Die Ergebnisse dieser randomisierten klinischen Studie zeigten insgesamt keine Überlegenheit des applizierten CRF1-

Rezeptorantagonisten gegenüber der Behandlung mit Placebo. Allerdings konnte bei einer spezifischen Untergruppe von Patienten, von denen angenommen wird, dass sie eine höhere Aktivität des CRF-Systems aufweisen, ein signifikant besseres Behandlungsergebnis beobachtet werden. Durch Untersuchung der DNA-Methylierungslevel von PTBS-relevanten Genen im peripheren Blut der Patientinnen vor und nach der Verabreichung des CRF1-Rezeptorantagonisten konnten potenzielle epigenetische Behandlungsmarker für die CRF1-Antagonistentherapie bei PTBS identifiziert werden. Die zuvor beschriebene Subgruppe mit einem erhöhten Ansprechen auf die Behandlung zeigte signifikant unterschiedliche Veränderungen der CRHR1-Methylierungslevel im Vergleich zu den übrigen Patienten, was darauf hindeutet, dass die CRHR1-Methylierung einen möglichen Marker darstellt, um den Behandlungsverlauf nachzuverfolgen. Darüber hinaus zeigte sich die Interaktion zwischen NR3C1-Methylierungslevel zu Behandlungsbeginn und Kindheitstrauma als signifikanter Prädiktor für das Behandlungsergebnis, und könnte somit als epigenetischer Stratifikationsmarker dienen, um Patienten vor einer therapeutischen Intervention in relevante Untergruppen zu unterteilen. Interessanterweise konnte die Methylierung von NR3C1 bereits in früheren Studien bei PTBS-Patienten als Prädiktor für das Ansprechen auf eine psychotherapeutische Behandlung identifiziert werden und stellt somit einen möglichen Behandlungsmarker dar, unabhängig von der angewandten Therapie. Die vorgestellte Arbeit untersucht ferner die Rolle von miR-15a im Rahmen der humanen Stressantwort, einer microRNA, deren entscheidender Stellenwert bei der Stressreaktivität von Mäusen bereits gezeigt werden konnte. Sowohl nach Dexamethason-Behandlung von gesunden Probanden als auch bei erwachsenen Patienten mit Kindheitstraumata in der Vorgeschichte weist miR-15a eine veränderte

Regulation im peripheren Blut auf und repräsentiert damit einen weiteren potentiellen epigenetischen Biomarker stressbedingter Erkrankungen wie PTBS.

Die hier präsentierten Daten stärken das Konzept der Präzisionsmedizin im Rahmen stressbedingter psychiatrischer Erkrankungen, indem biologische Untergruppen mit unterschiedlichem Ansprechen auf die Behandlung mit CRF1-Rezeptorantagonisten aufgedeckt werden. Darüber hinaus identifiziert die vorliegende Arbeit vielversprechende Kandidaten für epigenetische Biomarker und zeigt ihr zukünftiges Potenzial zur Verbesserung von Präventionsstrategien, Diagnose und Behandlung bei PTBS auf.

Introduction

PTSD and the need of specific pharmacological treatment

Post-traumatic stress disorder represents a common and debilitating mental disorder. This psychiatric condition typically occurs after experiencing a traumatic life event and is accompanied by characteristic symptoms such as repeated and unwanted re-experiencing of the event, emotional numbness and avoidance as well as increased arousal. By definition, these symptoms last for a minimum of one month and create distress or functional impairment (DSM-5). The overall lifetime prevalence is reported between 7-12% and women are affected twice as often as men (Breslau et al., 1998; Breslau 2001; Kessler et al., 1995). Although a history of exposure to a traumatic event is a diagnostic criteria for PTSD, it is not sufficient itself for an individual to develop the disorder. The fact that around 40-90% of the general population is exposed to a traumatic event in their life, but only a small number of individuals eventually develop the disorder, indicates a possible genetic predisposition to PTSD (Galea et al., 2005). A number of twin studies have provided evidence that the estimated genetic contribution to PTSD risk in both men and women ranges between 30 and 40%. Nonetheless the investigation of genetic main-effects in the field of PTSD has shown only very limited results (Afifi et al., 2010). As previously mentioned, experience of a traumatic event represents an essential criterion for the diagnosis of PTSD and is crucially involved in the pathogenesis of the disorder. The often long-lasting effects caused by these environmental factors are most likely mediated by epigenetic changes and other molecular and cellular mechanisms. Therefore, research in PTSD has centered on the complex interplay between genetics, environmental factors and epigenetic mechanisms (Pape and Binder, 2016).

Novel epigenetic and genetic biomarker candidates in PTSD

Due to this unique psychopathology, treatment of PTSD remains challenging. Available therapeutic approaches are psychotherapy and psychopharmacology or the combination of the two. With only two medications, the selective serotonin reuptake inhibitors (SSRIs) Sertraline and Paroxetin, approved by the Food and Drug Administration (FDA), psychotherapeutic strategies, especially exposure-based interventions, currently represent the most commonly used therapeutic approach (Powers et al. 2010). Even though recommendations regarding treatment for PTSD are inconsistent, most clinical guidelines indicate pharmacological interventions as second line treatment (Hoskins et al., 2015; Lee et al., 2016). Here, SSRIs and SNRIs (Serotonin–norepinephrine reuptake inhibitors) seem to have the broadest effect on PTSD symptoms. With remission rates of only 20-30% there is however still much room for improvement (Krystall et al., 2017).
Interestingly none of the drugs used in the treatment of PTSD has been specifically developed for this purpose. SSRIs for example have been used due to the overlapping symptoms and high comorbidity with other mood disorders like depression. Even medication like beta-blockers, originally designed to treat cardiologic disorders, represents a common off-label use in PTSD treatment. For this reason, current pharmacological interventions in PTSD are solely capable of specific symptom-based therapy instead of directly targeting the pathophysiology underlying the disease. Future research should therefore focus on more specific pharmacological treatment options developed on the basis of the pathophysiology of PTSD (Yehuda 2015).

The CRF-system in the context of PTSD

Corticotropin-releasing factor is a small 41 amino acid peptide, discovered by Vale and colleagues in 1981, that is widely expressed throughout the central nervous system showing its highest concentration in the paraventricular nucleus of the hypothalamus (Vale et al., 1981). CRF binds to two known receptors, CRF receptor type 1 (CRF1) and CRF receptor type 2 (CRF2) (Lewis et al. 2001), but its affinity to CRF1 is tenfold higher (Perrin and Vale, 1999). As the primary mediator of the hypothalamus–pituitary–adrenal (HPA) axis, CRF and its type 1 receptor (CRF1) play a crucial role in the adaptation of the organism to stress (Claes, 2004). HPA axis activation is initiated by the release of CRF from the nerve terminals of the paraventricular nucleus (PVN) following exposure to a stressor. After its secretion from the PVN neurons, terminating in the median eminence, CRF binds to CRF1 receptors in pituitary corticotrophs, which leads to the synthesis and release of adrenocorticotrophin hormone (ACTH) from the anterior lobe of the pituitary gland. ACTH in turn triggers the subsequent stimulation of glucocorticoid synthesis and secretion from the adrenal gland into the systemic circulation (Herman et al., 2016; Smith 2006). In humans, cortisol then acts on two nuclear hormone receptors, the mineralocorticoid and glucocorticoid receptor (GR) in the CNS as well as in multiple peripheral tissues, triggering metabolic, and neuromodulatory changes essential for stress adaption (Carvalho et al., 2017) (Figure 1).

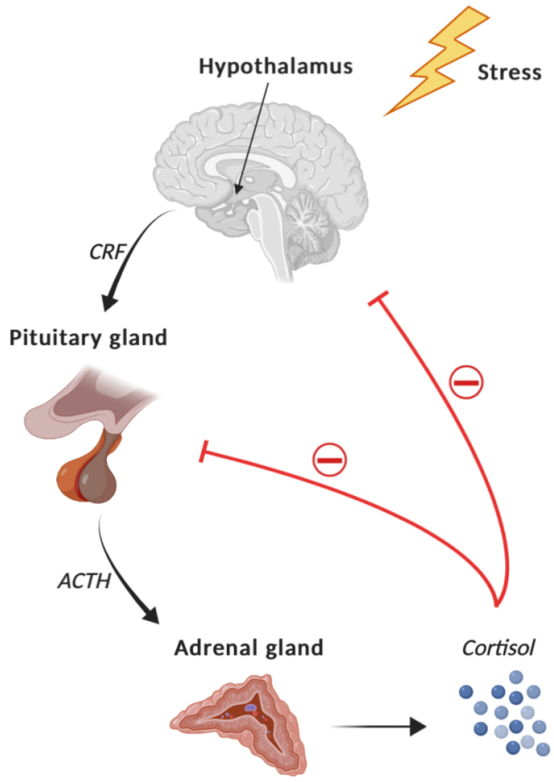

Figure 1: Schematic diagram of the hypothalamic-pituitary-adrenal (HPA) axis.

Since dysregulation of this axis is one of the most consistent findings in PTSD, corticotropin-releasing factor and its type 1 receptor have been closely linked to the pathophysiology of the disorder (Mehta and Binder, 2012). This has been investigated by numerous studies in animals as well as in humans. Intracerebroventricular (ICV) or site-specific injection of CRF followed by different behavioral paradigms is a well-studied and validated method in animal models to test for behavioral changes in a CRF hyper-activated system. One of the first studies in this field examined changes in behavior, which has been shown to be related to the degree of responsiveness to novelty. CRF administered rats showed an increase in grooming as well as a decrease in rearing and food intake, which represents fear related behaviors (Britton et al., 1982). Other studies showed an increase in locomotor activity (Matsuzaki et al. 1998; Sutton et al., 1982),

decreased active social interaction (Dunn and File 1987), potentiation of freezing behavior (Sherman and Kelin 1988) as wells as acoustic startle response in rats (Swerdlow et al.1986) after ICV-injection. Another interesting study by Brown and colleagues demonstrated the impact of injected CRF on the sympathetic nervous system by increase of blood pressure and heart rate (Brown et al., 1982). Together these studies suggest an anxiogenic action for CRF including features particularly relevant to PTSD.

Instead of exogenous administration, later studies investigating genetically modified animals overexpressing CRF, further support the crucial role of CRF/CRF1 activity in stress-induced psychiatric disorders. The observed findings were in line with earlier studies performing ICV-injection by showing induced anxiety-like behavior as well as chronic stress-like autonomic and physiological alterations (Dirks et al., 2002) after persistent central CRF hypersecretion. For example, CRF transgenic animals spent significantly less time in the open arms of the elevated plus maze (Stenzel-Poore et al., 1994) and female mice showed impairment in social interaction being sexually less receptive compared to control mice (Heinrichs et al 1997). Using viral vector technology, a recent study investigated the consequences of chronically increased CRF in the amygdala of primates. Showing similar results to rodent studies, primates overexpressing CRF demonstrated significantly increased anxious temperament compared to their cage-mate controls (Kalin et al., 2016).

Confirmatory results are obtained when decreasing levels of CRF. Skutella and colleagues suppressed translation of CRF by intracerebroventricular infusion of antisense oligodeoxynucleotides. As hypothesized by the authors, lower CRF levels in mice led to reduced anxiety-related behavior demonstrated by significantly higher amounts of time spent in the open arms of the elevated plus maze after social defeat then controls (Skutella et al., 1994). Being an important mediator of the CRF-induced response to stress, several

studies have investigated the corticotropin-releasing factor receptor 1 in the same context using CRF1-deficient mice. Overall these studies showed comparable results indicating that a lack of CRF1 results in decreased anxiety-like behavior (Contarino et al., 1999; Smith et al., 1998; Timpl et al., 1998). The above-mentioned studies clearly show that a general hyperactivity of the CRF/CRF-1 system in animals leads to increased anxiety like behavior and vice versa. However, work from the Deussing's lab suggests that specific brain regions and even neurotransmitter-specific neuronal circuits may show opposite effects (Dedic et al., 2018; Refojo et al., 2011) (Discussed in detail on pp. 73-74). Even though there are considerably less studies in humans specifically examining the role of CRF/CRF1 activity in PTSD, the observed results point in the same direction as the described animal studies, showing a strong link between CRF/CRF-1 system activity and the disorder. An important finding reported by several authors shows specific changes in cerebrospinal fluid (CSF) concentrations of corticotropin releasing hormone in PTSD patients. Hypothesizing a hypersecretion of neuronal CRF in PTSD the authors found significantly higher basal CSF CRF levels in patients with chronic combat-related PTSD than in comparison subjects, further linking the CRF system to the disorder (Baker et al., 1999; Bremner et al., 1997). This effect has been shown to be even stronger in PTSD patients with comorbid psychosis, which is hypothesized to be a more severe form of the disorder. Patients with secondary psychotic symptoms displayed significantly higher CSF concentrations of CRF than healthy controls but also significantly higher CRF levels than PTSD patients without comorbid psychosis (Sautter et al., 2003). Examining whether Plasma CRF levels might serve as a possible predictor of hypothalamic CRF levels de Kloet and colleagues obtained and tested plasma CRF from war veterans diagnosed for PTSD, traumatized veterans without PTSD and healthy controls. Interestingly PTSD patients showed higher CRF plasma concentration than controls but also higher than patients

without PTSD but a history of trauma experience. This suggests that increased CRF concentrations are specifically associated with the development of PTSD but not with trauma exposure alone (de Kloet et al., 2007).

CRHR1 polymorphisms in PTSD and other stress related psychiatric disorders

Genetic heritability represents an important contributor to the risk for PTSD and has therefore long been subject of intense research. Identifying the genetic underpinnings of the disorder would give us a better understanding of the inter-individual differences in susceptibility to the disease and might help subgrouping patients for individual treatment options.

Due to the central role of the CRF system in stress related disorders such as PTSD, variation of genes involved in this system are of special interest. Studies examining genetic variants in the CRF receptor 1 gene (*CRHR1*) and stress related disorders have found that specific *CRHR1*-polymorphisms have impact on an individual's response to environmental stressors, in this case trauma exposure. In two independent, ethnically different populations Bradley and colleagues reported that several *CRHR1* polymorphisms significantly moderated effects of early trauma on depressive symptoms in adulthood, showing the strongest interaction effect for rs110402 GG genotype by child abuse on MDD risk (Bradley et al., 2008). Further, a potentially protective effect of a haplotype formed of three single nucleotide polymorphisms rs7209436, rs110402 and rs242924 was reported and replicated in a later study (Polanczyk et al., 2009). The authors therefore suggested that these genetic variants possibly predict both risk and resilience for MDD in subjects that experienced childhood trauma (Bradley et al., 2008).

Although these reports clearly implicate genetic variants of *CRHR1* in traumatic-stress related phenotypes, only a few studies so far have specifically investigated these variants in the context of PTSD. For example, Amstadter and colleagues tested nine *CRHR1* polymorphisms in relation to PTSD severity in a small prospective study of children who had experienced medical trauma. Several of the variants were associated with both, acute symptom level and trajectory of symptoms over time, with SNP rs12944712 being most significant (Amstadter et al., 2011). The first study in this context, working with adult patients, evaluated *CRHR1* polymorphisms in association with PTSD symptoms in victims of the 2004 Florida hurricanes. Results indicate a significant relation between two *CRHR1* variants and symptom severity. More specifically, showing that the major alleles of SNP rs12938031 and rs4792887 increased the risk for post-hurricane PTSD symptoms (White et al. 2013). Another study designed to assess a cumulative risk score for PTSD from multiple polymorphisms located in different genetic loci, found that *CRHR1* SNP rs110402 was associated with PTSD symptom level. Studying outpatients with chronic, nonmalignant pain, a condition often associated with PTSD, the authors found that G allele carriers of rs110402 (*CRHR1*) were more common among PTSD cases than non-PTSD cases (Boscarino et al., 2012).

Specifically interesting in the context of the current work are reports of associations between genetic variants in the CRF receptor 1 gene (*CRHR1*) and neuroendocrine alterations including the CRF system in psychological and pharmacological challenge tests (Chichetti *et al*, 2011; Heim *et al,* 2009; Mahon *et al,* 2013; Sumner *et al,* 2014; Tyrka *et al,* 2009). Using the combined dexamethasone suppression/CRF stimulation test, Heim and colleagues as well as Tyrka and colleagues demonstrated a significant association between G allele carriers of *CRHR1* SNP rs110402 that also experienced childhood trauma

and an elevated endocrine response to this test (Dunlop and Wong, 2018; Heim et al, 2009; Tyrka et al, 2009).

These studies highlight the close link between the CRF system and stress related disorders such as PTSD. Moreover, the data suggest that certain genetic variants may moderate an individual's stress response, possibly by affecting the functional properties of the CRF system, and therefore potentially serve as specific endophenotypes for e.g. personalized treatment selection. Directly antagonizing this system represents a promising pharmaco-therapeutic approach of which individuals with enhanced CRF system activity (likely carriers of the rs110402 G allele that also experienced childhood trauma) might benefit most.

CRF1 antagonists in stress related disorders

Over the last decades the hypothesis to pharmacologically target the CRF system in order to treat stress related psychiatric diseases emerged from considerable evidence involving the CRF system in the pathogenesis of these disorders. More specifically a number of points, discussed in detail above and summarized in the following, indicate increased CRF/CRF1 signaling, suggesting antagonism of the CRF1 receptor and by that reducing endogenous CRF1 receptor neurotransmission, as a promising treatment strategy. First, a hyper-activated CRF system in animals, induced by either exogenous administration or by CRF overexpression, leads to anxiety-related behavior. Secondly, suppressing the system utilizing e.g. antisense oligodeoxynucleotides or CRF1 receptor deficient mice decreased stress-induced anxiety-like behavior compared to control animals. Third, in humans, concentrations of CRF, the ligand of the CRF1 receptor, are elevated in PTSD

patients (Baker et al., 1999; Bremner et al., 1997; Britton et al., 1982; Dunn and File 1987; Skutella et al., 1994; Smith et al., 1998; Stenzel-Poore et al., 1994).

Consequently, these findings led to multiple preclinical studies investigating the effects of CRF1 receptor antagonists in animal models on both CRF- and also stress-induced behavioral alterations. Overall, CRF1 receptor antagonists have been shown to reverse stress-related behaviors provoked by intracerebroventricular infusion or overexpression of CRF. For example, early work by Swerdlow and colleagues demonstrated that the potentiation of acoustic startle amplitude in rats induced by exogenous administration of CRF was reversed after infusion of a CRF1 receptor antagonist (Swerdlow et al., 1989). Another study showing that transgenic mice with chronic overproduction of CRF throughout their life span spend significantly less time in the open arms of an elevated plus maze than controls, further depicted that this anxiogenic effect was reversible by intracerebroventricular infusion of a CRF1 receptor antagonist (Stenzel-Poore et al., 1994). In addition, CRF1 receptor antagonists hold the potential to significantly reduce locomotor activity in animals produced by exogenous administration of CRF as reported by several groups (Menzaghi et al., 1994; Spina et al., 2000).

Considering CRF1 receptor antagonists as potential treatment options of stress related disorders, their capability to reverse behavioral alterations induced by a certain external stressor might be even more relevant. Here, exploration-based models of anxiety – like the elevated plus maze or the open field test – represent commonly used test paradigms. Several authors reported the antagonizing compounds to have a reversal effect on increased anxiety-related behavior evoked by exposure to different environmental stressors. An interesting study by Heinrichs and colleagues showed that after exposure to either social, swim or restraint stress animals displayed less exploration of the open arms of the maze and that these behavioral effects were blocked after CRF1 receptor antagonist

administration. The authors further demonstrated that the behavioral reactivity to the different kinds of stressors as well as the efficacy of the antagonist to reverse these effects were comparable, suggesting that the type or intensity of a stressor might not be particularly relevant for the outcome of antagonizing the CRF system (Heinrichs et al., 1994). Similar anti-stress effects were observed in non-human primates. Using an intruder paradigm, the authors reported that administration of the antagonist not only inhibited behaviors associated with anxiety but even increased exploratory and sexual behaviors usually decreased during stress. Moreover, the administered compound significantly suppressed elevated CRF concentration in the cerebrospinal fluid of the monkeys (Habib et al., 2000). Numerous other findings using different animal models further demonstrate the highlighted anxiolytic effects. CRF1 receptor antagonists reverse stress-induced behaviors like decreased social interaction, increased freezing behavior, potentiation of acoustic startle, aggression and sleep impairments (Farrokhi et al., 2004; Griebel et al., 2002, Kobayashi et al., 2011; Philbert et al., 2015; Robison et al., 2004; Swerdlow et al. 1989).

Interestingly the observed effects only occur under stressed but not non-stressed conditions, suggesting CRF 1 receptor antagonists to require a highly activated CRF system to perform their actions (Keck et al., 2001; Zorilla et al., 2010). Keck and colleagues tested a CRF1 receptor antagonist in a rat strain selectively bred for strong anxiety behavior measured on the elevated plus maze. A significant anxiolytic effect of the compound was only observed in animals showing high innate anxiety but not in controls (Keck et al., 2001).

Taken together, these preclinical data provide convincing evidence to consider CRF1 receptor antagonists as potential new treatment options for stress related psychiatric disorders in humans. The first clinical trials evaluated the efficacy of CRF1 receptor

antagonists in patients with major depressive disorder (MDD). In 2000, Zobel and colleagues administered a CRF1 receptor antagonist to 20 MDD patients for 30 days. The subjects were split into two groups of which one received a dose-escalation from 5 to 40 mg and the other 40 to 80 mg over treatment time. As a first finding the authors reported that the antagonist was safe and well tolerated by the patients. They further observed that the compound significantly reduced symptom severity, rated by a clinician and the patient himself, and also relapse of these symptoms after drug discontinuation (Zobel et al., 2000). Moreover, in a subsequent study investigating a subgroup (n=10) of the same sample, the CRF1 receptor antagonist showed to have beneficial effects on sleep architecture in depressed patients measured by EEG (Held et al., 2004).

Although this study was originally designed to evaluate safety and tolerability instead of drug efficacy, the sample size was small and the clinical development of this specific drug was discontinued due to hepatotoxicity, it yielded initial promising results of CRF_1 receptor antagonists as potential therapeutic agents (Ising and Holsboer, 2007). Therefore, multiple other, larger clinical trials in MDD patients followed using different newly developed compounds. However, all of these trials failed to demonstrate efficacy in the treatment of MDD or have been discontinued due to intolerable side effects. (Binnemann et al. 2008; Koob and Zorrilla, 2012) The therapeutic value of CRF1 receptor antagonism was further tested in generalized anxiety disorder. A cohort of 260 patients with GAD was treated with eather a CRF1 receptor antagonist, placebo or escitalopram. Treatment outcome (pre to post treatment score on the Hamilton Anxiety Scale) showed no significant differences between the CFR1 receptor group and placebo. Moreover, subjects treated with escitaplopram showed better outcome than patients treated with the antagonist.

Given the fact that CRF1 receptor antagonists failed to treat stress related psychiatric disorders like MDD or GAD in clinical studies on the one hand and on the other hand are particularly effective in animal models immediately after exposure to a strong external stressor, experts hypothesize, that these agents may show their greatest therapeutic value in disorders which specifically include traumatic stressors in their etiology, e.g. posttraumatic stress disorder (Kehne and Cain 2010).

Biomarkers in PTSD treatment

A major challenge in treating psychiatric diseases like post-traumatic stress disorder is to find the right therapy for each individual patient. In order to enable precision medicine biomarkers that predict or monitor treatment outcome are of great value. A biomarker in general refers to "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). Treatment response biomarkers in particular can be used to either track an individual's response to a therapeutic intervention or serve as predictive markers to stratify patients into different subgroups of potential responders and non-responders to a targeted therapy. Ideally, these objective parameters derived from either different body fluids like blood, saliva or cerebrospinal fluids or from imaging methods will help to develop more specific phenotype characterization and further customize individual therapies.

Due to the limited success of current pharmacological treatment options this might be a way to improve PTSD treatment outcome. However, the field of biomarkers in PTSD, especially markers of therapy response is still in its infancy. Potential biomarkers predicting or monitoring treatment outcome include brain activity and morphology

(Bryant et al., 2008; Dickie et al., 2013; Felmingham et al., 2007; Levy-Gigi et al., 2013), neurochemical (Rapcencu et al., 2017; Rauch et al., 2015; Yehuda et al., 2014), neurophysiological (Griffin et al., 2012; Pitman et al., 2002; Raskind et al., 2016; Wangelin and Tuerk, 2015) as well as genetic and epigenetic markers (discussed in detail below).

Genetic biomarkers

Research on genetic biomarkers to predict and monitor treatment outcome in stress related psychiatric diseases has mainly focused on major depressive disorder (reviewed in Kato and Serretti, 2010). So far only a few studies investigated the influence of genetic variants on treatment response in PTSD. Here, most authors focused on psychotherapeutical interventions.

Due to its strong association with PTSD (for review see Koenen et al., 2009), Bryant and colleagues examined whether the *5-HTTLPR* polymorphism of the serotonin transporter (*SLC6A4*) gene predicted treatment outcome following eight weeks of exposure-based cognitive behavior therapy and after six months follow up. At six month follow up but not after eight weeks carriers of the *5-HTTLPR* short (S) allele, which is associated with lower transcriptional activity compared to the long (L) allele, showed stronger PTSD severity, suggesting *5-HTTLPR*-genotype as a predictor of long term treatment response (Bryant et al., 2010).

Among other glucocorticoid-related biomarkers Yehuda and colleagues assessed a BCLI polymorphism of the glucocorticoid receptor gene, that has previously been associated with HPA-axis sensitivity in PTSD (Bachmann et al., 2005), before and after psychotherapy. In a sample of 37 combat veterans with a diagnosis of PTSD, subjects were either treated with prolonged exposure therapy or received minimal attention

intervention over twelve consecutive weeks. Responder status was determined by the absence of a PTSD diagnosis using the Clinician Administered PTSD Scale (CAPS). The authors reported that G allele carrier status of the BCLI polymorphism significantly predicted recovery from PTSD symptoms (Yehuda et al., 2014).

Another study in this line of research by Felmingham and colleagues examined whether a genetic variant in the brain-derived neurotrophic factor (BDNF) - gene would serve as a predictor for treatment response after eight weeks of exposure-based cognitive behavior therapy. The authors assessed genotype status of 55 patients diagnosed with PTSD according to a polymorphism in the BDNF gene, which results in an amino-acid substitution (valine-to-methionine) at codon 66. This functional variant (Met-allele) is associated with reduced activity-dependent secretion of BDNF, decrease in hippocampal volume and human cognitive dysfunction (Bath and Lee, 2006; Egan et al., 2003). Findings showed that PTSD patients carrying the Met-allele (Met/Met and Val/Met) showed significantly poorer response to psychotherapy then Val allele carriers, indicating this *BDNF* variant as a potential predictor of treatment outcome (Felmingham et al., 2013).

Given that SSRIs are currently considered the Gold Standard in pharmacological PTSD therapy and represent the only medication approved by the FDA, most studies investigating predictive biomarkers for pharmacotherapy focus on SSRI treatment. Findings include e.g. BDNF serum levels and changes in functional brain activity (Berger et al., 2010; Zhu et al., 2015), however studies investigating genetic biomarkers of pharmacological response are rare.

Mushtaq and colleagues assessed the serotonin transporter (5HTTLPR) genotype as a possible predictor of sertraline treatment response in PTSD. Patients were genotyped and analyzed according to their carrier status of the long (L) or the short (S) allele (SS vs SL vs LL) of *5HTTLPR*. All subjects were treated with a dose of 100 mg/day Sertralin over

twelve consecutive weeks and responder status was determined by symptom improvement of over 30% assessed by the CAPS. Patients homozygous for the long allele (LL) showed significantly better treatment response compared to the other 5HTTLPR genotypes. Additionally, the short allele was associated with a higher drop out rate due to adverse side effects (Mushtaq et al., 2012).

In line with the work of Mushtaq and colleagues several other studies investigating biomarkers of treatment response in different psychiatric disorders suggest that genetic variants in genes of the molecular target of the given drug might be particularly relevant as treatment outcome predictors. For example, in MDD patients a functional *5-HTTLPR* polymorphism has been shown to predict treatment outcome specifically following SSRI treatment but not after treatment with tricyclic antidepressants (Huezo-Diaz et al., 2009; Kenna et al., 2012; Lester et al., 2013; Pollock et al., 2000).

With an estimated contribution to disease risk of about 30-40% genetics represent an important factor in accounting for the risk of developing PTSD. However, exposure to a traumatic or stressful event is by definition mandatory for the diagnosis of the disorder (Pape and Binder, 2016). Trauma exposure further plays a crucial role in the susceptibility to PTSD. Here early life trauma seems to be particularly relevant and represents an important disease risk factor as it has been shown in numerous studies (Brewin et al., 2000; Cougle et al., 2010; Lang et al., 2008). Several authors suggest that not only the genetic background determines an individuals risk to develop PTSD but that environmental factors like early life stress might recalibrate the individual stress response system to subsequent traumatic events and by that influence susceptibility to the disease later in life (Mehta and Binder, 2012; Yehuda et al., 2010). Therefore, research in PTSD has focused on gene by environment (G x E) interaction studies, particularly investigating the joint contribution of the genetic predisposition and the environmental

trigger. Several of these studies suggest that different interactions between multiple genetic and environmental factors will affect different biological pathways resulting in distinct pathophysiological subtypes of PTSD (reviewed by Mehta and Binder, 2012). Since individuals with specific PTSD subtypes will most likely respond differently to certain treatments, accounting for G x E interactions in treatment response studies might be particularly relevant.

Epigenetic biomarkers

As described above, an interplay of genetic factors and traumatic events is thought to form the complex phenotype of PTSD. However, the exact molecular mechanisms that mediate the long-lasting effects of these interactions have not been entirely elucidated. Recently, increasing evidence suggests epigenetic changes as prime candidates for stress-induced long-term effects on DNA function like alteration of gene transcription and protein translation. These epigenetic processes such as DNA methylation, histone modification or non-coding RNAs are not limited to early developmental stages but can also occur in later life. Furthermore, they can be long lasting but also dynamic, as indicated by longitudinal studies (Klengel et al., 2014; Rusiecki et al., 2013; Wiechmann et al., 2019). Due to these unique characteristics epigenetic mechanisms represent a significant contributor to the pathophysiology of PTSD as it has been shown in numerous studies to date. Therefore, epigenetic changes observed in peripheral tissues such as blood and saliva of PTSD patients may serve as biomarkers of the disorder. These peripheral modifications may either reflect PTSD-specific epigenetic changes in the brain or simply be disease-associated changes independent of the disorder's pathophysiology (Zannas et al. 2015). Therefore, these modifications may also serve as diagnostic and prognostic biomarkers

as well as predicting and monitoring treatment outcome. Several studies highlight the possible use of epigenetic marks in peripheral tissues as potential biomarkers in PTSD. However, most of these studies focus on diagnostic or prognostic markers of disease status instead of biomarkers of treatment response, predicting and monitoring therapy outcome. In this context epigenetic modification of genes involved in the regulation of the HPA axis has been extensively investigated and most studies focus on DNA methylation, which represents the best characterized epigenetic mechanism, (Klengel et al., 2013)

DNA-methylation studies

We have previously reviewed these studies (Addendum: Pape and Binder, 2016, pp.514-515). For example, a number of authors have observed a strong association between PTSD symptoms and methylation levels of the glucocorticoid-receptor encoding gene *NR3C1* (Labonte et al., 2014; Vukojevic et al., 2014; Yehuda et al. 2015). Work by Labonte and colleagues showed significantly lower *NR3C1* promoter methylation in peripheral T lymphocytes of 30 subjects with a diagnosis of lifetime PTSD compared to healthy controls (Labonte et al., 2014). Investigating a cohort of 122 combat veterans Yehuda and colleagues reported lower blood *NR3C1* promoter methylation in individuals with a diagnosis of PTSD compared to those without. Furthermore, promoter methylation levels correlated inversely with PTSD symptom severity (Yehuda et al. 2015). Also in line with the previous results another study demonstrated that increased *NR3C1* promoter methylation in peripheral blood was associated with less intrusive memory of the traumatic event and reduced PTSD risk in male survivors of the Rwandan genocide, and further showed that it may be related to differences in recognition memory-related brain activity (Vukojevic et al., 2014).

SKA2, the spindle and kinetochore associated protein 2, which has previously been implicated in the pathophysiology of PTSD at the epigenetic level represents another promising biomarker candidate. By activating the glucocorticoid receptor, SKA2 seems to be involved in moderating the negative feedback mechanism of the HPA axis (Rice et al., 2008). Methylation levels of the SKA2 gene have been shown to predict suicidal behavior. More specifically Guintivano and colleagues examined *SKA2* methylation in human postmortem brain tissue. The authors observed significantly elevated methylation levels of a CpG (cg13989295) located in the SKA2 gene in suicide completers compared to controls. Interestingly, this increase in methylation was associated with significantly decreased *SKA2* expression levels (Guintivano et al. 2014). Due to increased suicide rates among PTSD patients, *SKA2* methylation levels were also investigated in the context of PTSD. *SKA2* methylation at the previously described CpG (cg13989295) and early trauma scores were identified as a significant predictor of PTSD status (Kaminsky et al., 2015). Two additional studies specifically examined associations between PTSD and *SKA2* methylation at cg13989295 (Boks et al., 2016; Sadeh et al., 2016). Boks and colleagues were able to confirm the previous findings by Kaminsky and further strengthend the role of *SKA2* as a potential biomarker in PTSD. Analizing DNA methylation levels in 93 Dutch war veterans, the authors observed a significant interaction of childhood trauma and pre deployment *SKA2* methylation predicting the development of PTSD after deployment (Boks et al., 2016). A study by Sadeh and colleagues investigated a military cohort of 200 trauma-exposed veterans. Results showed a positive correlation between PTSD symptom severity and *SKA2* (cg13989295) DNA methylation levels (Sadeh et al., 2016).

In a cohort of heavily traumatized subjects with or without a diagnosis of PTSD Ressler and colleagues have identified another interesting epigenetic biomarker candidate. DNA methylation levels of *ADCYAP1R1*, the gene encoding the pituitary adenylate cyclase-

activating polypeptide type I receptor (PAC1), showed a positive correlation with PTSD symptom severity (Ressler et al., 2011).

Studying DNA methylation of different subsets of PTSD-relevant genes or on a genome wide level and by that possibly identifying PTSD-specific methylation profiles across multiple genomic loci, represents another promising approach in the field of PTSD biomarker research and to date numerous such studies have been completed (Hammamieh et al., 2017; Kuan et al., 2017; Mehta et al., 2013; Rusiecki et al., 2013; Smith et al., 2011; Uddin et al., 2010, 2011). Taken together, these results suggest distinct PTSD and trauma associated genome wide differences in DNA methylation levels with possible system wide effects on the organism (Pape and Binder, 2016).

As highlighted above, the vast majority of studies investigating epigenetic biomarkers in PTSD concentrate on diagnostic or prognostic markers of disease status. So far only a single study has focused on epigenetic biomarkers of treatment response in PTSD (Yehuda et al., 2013). In a small cohort of PTSD diagnosed combat veterans treated with prolonged exposure psychotherapy over twelve consecutive weeks the authors observed that pre-treatment *NR3C1* methylation levels significantly predicted treatment outcome. Further, results showed that a decrease in *FKBP5* methylation associated with better treatment outcome (Yehuda et al., 2013).

Yehudas work represents the first study examining epigenetic treatment outcome markers in PTSD following psychotherapy. The fact that epigenetic biomarkers of response to pharmacological treatment have not been studied in PTSD makes this present study particularly relevant.

MicroRNA studies

In addition to DNA methylation, microRNAs represent another promising epigenetic biomarker candidate in stress-related psychiatric disorders. However, in PTSD specific biomarker research involving microRNAs is even less established than DNA methylation. In this context most studies were conducted in animals and mainly focus on the impact of different stressful stimuli on microRNA expression levels. Several studies in rodents have proven a potential role for microRNAs as biomarkers for stress (PTSD) related symptoms. For example, Balakathiresan and colleagues investigated microRNA expression levels in serum and amygdala of rats stressed in a daily two hours session of immobilization accompanied by tail shocks over three days. Results showed 82 (78 upregulated) differentially expressed microRNAs in serum and 60 (all upregulated) in the amygdala 14 days after stress exposure. A comparison between serum and amygdala identified a panel of 9 commonly upregugalted microRNAs which might be particularly relevant as biomarkers for fear response (Balakathiresan et al., 2014). Another study showed that exposure to acute stress results in increase of miR-34c in the amygdala of mice. The authors further reported that virus-mediated overexpression of miR-34c significantly reduces anxiety-like behavior induced by acute stress (Haramati et al., 2011). Also in mice, Volk and colleagues demonstrated an upregulation of miR-19b in the amygdala after chronic social defeat. Further findings revealed miR-19b as a possible modulator of behavioral responses to stress. Bilateral injection of the microRNA into the amygdala resulted in lower freezing time in the cue fear conditioning test, whereas miR-19b knockdown led to opposite behavioral effects (Volk et al., 2014). Several other rodent studies also show a shift in microRNA levels in response to different external stressors (Cho et al., 2014; Issler et al., 2014; Mannironi et al., 2013; Snijders et al., 2017).

Novel epigenetic and genetic biomarker candidates in PTSD

As mentioned above, most studies investigating the involvement of microRNAs in the underlying molecular mechanisms of PTSD and their potential role as epigenetic biomarkers have been completed in animals. In humans this line of research is still in its infancy with a very limited number of studies.

In an early study, for example, Zhou and colleagues examined the involvement of microRNAs in PTSD associated immunological dysfunction. Findings included a significant downregulation of miR-181c and miR-125a in peripheral blood mononuclear cells (PBMC) of PTSD patients compared to controls. Interestingly miR-125a specifically targets IFN-γ mRNA and by that decreases IFN-γ production (Zhou et al., 2014). Bam and colleagues further underlined the role of microRNAs as a potential regulator of elevated pro-inflammatory cytokine levels in PBMCs of PTSD patients. Using the same cohort, the authors observed a down regulation of miR-193a in PTSD patients compared to controls as well as increased levels of one of its targets, IL-12. Transfection of THP-1 cell with pre-mir-193a resulted in a downregulation of the transcript level of IL-12 (Bam et al., 2016).

Differential levels of DICER1, a crucial enzyme in the biogenesis of microRNAs, have also been linked to the pathogenesis of PTSD. Blood DICER1 expression was significantly decreased in PTSD patients compared to controls in a cohort of 184 mainly African American subjects. This finding was replicated in two independent cohorts and interestingly correlated with a downregulation of overall microRNA levels in cases vs. controls (Wingo et al., 2015). A recent study by Martin and colleagues further associated differentially expressed microRNAs with PTSD. In a cohort of 24 combat veterans with or without a diagnosis of PTSD the authors reported eight differentially expressed microRNAs, of which 4 were upregulated and 4 downregulated. Interestingly, pathway analyses revealed that these specific microRNAs relate to Wnt signaling as well as axonal guiding (Martin et al., 2017; Snijders et al., 2017).

Taken together, these studies emphasize the role of microRNAs in the underlying molecular mechanisms of PTSD. Furthermore, showing differential peripheral blood expression profiles between patients and controls, microRNAs represent a promising Biomarker candidate in the diagnosis of PTSD.

Aims of the thesis

The first goal of this thesis was to evaluate whether the efficacy of a CRF1 receptor antagonist in post-traumatic stress disorder was depedent on distinct biological subgroups of patients. For this, a cohort of women between 18 and 65 years of age with a diagnosis of current PTSD of at least three months was treated with the CRF1 receptor antagonist or placebo and CRHR1 genotypes as well as different psychological and environmental measures were assessed. Then differences in treatment response, specifically in subsets of patients with probable differential CRF system activity, were investigated.

The second aim was to examine whether epigenetic modifications in *CRHR1* would be associated with PTSD symptom change after CRF1 receptor antagonist treatment in the same cohort (with specific focus on a previously identified biological subgroup) and further, whether these alterations may serve as potential epigenetic biomarkers for treatment response. To test this, pre- and post-treatment peripheral blood DNA-methylation was measured in our cohort of PTSD diagnosed women.

In addition, the presented work aimed to unravel whether methylation levels of two specific PTSD-relevant genes, that had previously been associated with and shown to predict treatment outcome following psychotherapy, may also show potential as blood-biomarkers following pharmacological treatment.

Finally, this thesis followed up on animal findings showing an important role for miR-15 in stress reactivity, analysing human peripheral blood samples in order to evaluate another possible biomarker in PTSD. Therefore miR-15a levels were examined in human samples in two different stress scenarios: 1. in healthy individuals following dexamethasone treatment, and 2. in adult subjects that had experienced early life stress.

Paper I

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DNA methylation levels are associated with CRF_1 receptor antagonist treatment outcome in women with post-traumatic stress disorder

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Abstract

Background: We have previously evaluated the efficacy of the CRF_1 receptor antagonist GSK561679 in female PTSD patients. While GSK561679 was not superior to placebo overall, it was associated with a significantly stronger symptom reduction in a subset of patients with probable CRF system hyperactivity, i.e., patients with child abuse and *CRHR1* SNP rs110402 GG carriers. Here, we test whether blood-based DNA methylation levels within *CRHR1* and other PTSD-relevant genes would be associated with treatment outcome, either overall or in the high CRF activity subgroup.

Results: Therefore, we measured *CRHR1* genotypes as well as baseline and post-treatment DNA methylation from the peripheral blood in the same cohort of PTSD-diagnosed women treated with GSK561679 ($N = 43$) or placebo ($N = 45$). In the same patients, we assessed DNA methylation at the PTSD-relevant genes *NR3C1* and *FKBP5*, shown to predict or associate with PTSD treatment outcome after psychotherapy. We observed significant differences in *CRHR1* methylation after GSK561679 treatment in the subgroup of patients with high CRF activity. Furthermore, *NR3C1* baseline methylation significantly interacted with child abuse to predict PTSD symptom change following GSK561679 treatment.

Conclusions: Our results support a possible role of *CRHR1* methylation levels as an epigenetic marker to track response to CRF_1 antagonist treatment in biologically relevant subgroups. Moreover, pre-treatment *NR3C1* methylation levels may serve as a potential marker to predict PTSD treatment outcome, independent of the type of therapy. However, to establish clinical relevance of these markers, our findings require replication and validation in larger studies.

Trial registration: NCT01018992. Registered 6 November 2009.

Keywords: CRF_1 receptor antagonist, DNA methylation, Epigenetics, PTSD, CRHR1, NR3C1, FKBP5

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Background

Post-traumatic stress disorder (PTSD) is a common psychiatric disorder with a prevalence of about 5% in the general population and an overall lifetime prevalence of 7–12%. Key symptoms of the disorder include intrusive memories, avoidance, and numbing as well as hyperarousal. Typically, these symptoms are long lasting and occur after exposure to traumatic life events. Women are twice as likely to develop the disease than men. PTSD therapies include both evidence-based psychotherapies and pharmacology, but only few patients attain remission. Currently, only two medications, paroxetine and sertraline, are approved by the US Food and Drug Administration (FDA). These SSRIs are capable of significantly reducing PTSD symptoms, but with only 20–30% remission rates to these agents, there is a need for additional pharmacologic treatment options [1].

Among pathophysiologic mechanisms that have been investigated for PTSD, disruptions of regulation of the hypothalamic-pituitary-adrenal (HPA) axis are among the most frequently cited hypotheses [2]. A key regulator of the HPA axis is the corticotropin-releasing factor (CRF) and its type 1 receptor (CRF_1 receptor), and many studies have reported alterations in this system in PTSD [3]. Therefore, it represents a promising novel drug target for this disorder. In response to stress, CRF is secreted by nerve terminals of the paraventricular nucleus of the hypothalamus and binds to the CRF_1 receptor in the adenohypophysis to release adrenocorticotropic hormone (ACTH). This process acts as the initial step of HPA axis activation and leads to the release of a number of hormones from the adrenal cortex including cortisol. Numerous studies in laboratory animals as well as in humans indicate that abnormalities of these HPA axis regulators play a crucial role in stress-related disorders such as PTSD [4].

In humans, for example, a number of independent studies report increased cerebrospinal fluid concentrations of corticotropin-releasing factor in PTSD patients [5–7], suggesting hyperactivity of the hypothalamus and extra-hypothalamus CRF system. Moreover, previous investigations have found that genetic variants in the CRF receptor 1 gene (*CRHR1*) are associated with differences in CRF signaling and may also impact individual responses to environmental stressors [3]. The most studied are variants within a haplotype tagged by the intronic SNP rs110402 that also comprises rs242924 and rs7209436. Interactions with exposure to child abuse and this haplotype were shown to alter risk for major depression, with individuals homozygous for the G-allele of rs110402 and exposed to child abuse being at higher risk in several but not all studies (see [8] for review). This haplotype has also been associated with differences in the neural activation profile with emotional stimulus processing [9], as well as neuroendocrine responses in psychological and pharmacological challenge tests [10–14], in which individuals who experienced childhood abuse and carry the G-allele display stronger HPA axis disturbances.

These preclinical and clinical results, taken together, support the role of CRF/CRF_1 receptor as a potential drug target in PTSD. However, antagonism of the CRF_1 receptor may only benefit those patients with initial increases in CRF signaling, which according to the above cited endocrine studies are likely to be those with exposure to child abuse and carrying the G-allele of rs110402.

We recently published a study evaluating the efficacy of a novel CRF_1 receptor antagonist (GSK561679) in a cohort of female PTSD patients in a double-blind, placebo-controlled trial. Although the drug was not superior to placebo overall, it was associated with a significantly stronger symptom reduction in a subset of patients with probable CRF_1 receptor hyperactivity, i.e., patients with childhood abuse and carriers of the GG genotype of the *CRHR1* SNP rs110402 [15, 16]. These patients may represent a biologically distinct subtype of PTSD and show distinct biomarker profiles. Markers that predict or monitor treatment outcome would represent an important tool to offer targeted treatment for individual patients. Despite great progress in identifying the underpinnings of the pathophysiology of PTSD and some very promising results in the biomarker field [17, 18], there is still no clinically applicable marker in PTSD, neither for diagnosis nor, perhaps even more significantly, to guide treatment selection. This is likely due to the complex pathophysiology of the disease that may include an interplay of genetics, environment, and epigenetic changes. It is therefore likely that not a single but rather a combination of different biological and clinical markers will need to be identified [18].

In addition to gene variants that predispose to PTSD development, epigenetic changes have been implicated in the pathophysiology of PTSD (for review, see [19]). These modifications may also serve as diagnostic marks as well as predicting and monitoring treatment outcome. Several studies highlight the possible use of epigenetic marks in peripheral tissues such as the blood and saliva as diagnostic markers in PTSD [18, 20, 21]. So far, epigenetic marks of only two genes, also within the HPA axis, *NR3C1*—encoding the glucocorticoid receptor (GR) and *FKBP5*—a co-chaperone of the GR, have been shown to associate with treatment response. More specifically, *NR3C1* baseline promoter methylation in peripheral blood predicted treatment outcome in PTSD, and in the same study, promoter methylation of *FKBP5* decreased in association with symptom improvement [22]. These findings were observed after 12 weeks of psychotherapy and have not yet been investigated in the context of pharmacological treatment.

Extending our previous study showing potential effects of a novel CRF_1 receptor antagonist (GSK561679) in a specific subset of women with PTSD (GG homozygous for rs110402 and with a history of childhood abuse) [16], we here use the same cohort to test whether blood-based epigenetic changes of PTSD relevant genes could serve as potential markers for treatment selection and outcome monitoring in biologically defined subgroups of patients. Given that the drug targets the CRF_1 receptor, we focused our analysis on the methylation of the *CRHR1* gene using the previous subgrouping of patients based on genetic and environmental risk factors. In addition, we explored whether methylation levels of two other genes within the stress hormone system (*NR3C1* and *FKBP5*), previously shown to predict and correlate with PTSD symptom improvement after psychotherapy [22], would also be associated with pharmacological treatment response in our study, again with specific focus on patients with probable CRF system hyperactivity (rs110402 GG-carriers and exposure to child abuse).

Results

Subgroup differences in *CRHR1* baseline methylation and change in *CRHR1* methylation from baseline to post-treatment

First, we tested a model with the main effects and interaction effect of child abuse and rs110402 carrier status on mean *CRHR1* baseline methylation. Seventy-nine subjects were included in this analysis due to missing genotype data in three samples. Neither the main effects nor the interaction effect showed significance ($n = 79$; $p > 0.05$). Next, we tested a model including main effects of treatment as well as interaction effects of treatment by child abuse, treatment by rs110402, child abuse by rs110402, and the three-way interaction of treatment by child abuse by rs110402 on changes in mean methylation levels of *CRHR1* from baseline to post-treatment. Due to missing methylation data in two baseline samples and one post-treatment sample, 57 subjects with baseline and post-treatment methylation data remained for this analysis. There was a significant interaction effect of child abuse by rs110402 carrier status ($n = 57$; F (1, 41) = 9.05; $p = 0.004$; $ß = -0.449$; Cohen's $f = 0.47$; $R^2 = 0.38$; adj. $R^2 = 0.153$; post-hoc power = 0.94) on change in methylation. Further, the three-way interaction of treatment by child abuse by rs110402 showed a significant effect on *CRHR1* methylation levels from pre- to post-treatment ($n = 57$; F (1, 41) = 4.86; $p = 0.033$; $ß = -0.297$; Cohen's $f = 0.344$; $R^2 = 0.38$; adj. $R^2 = 0.153$; post-hoc power = 0.72) (Fig. 1a, b).

Genotype by childhood abuse interaction on methylation change stratified by treatment

To further explore the significant three-way interaction on *CRHR1* methylation, we investigated the interaction

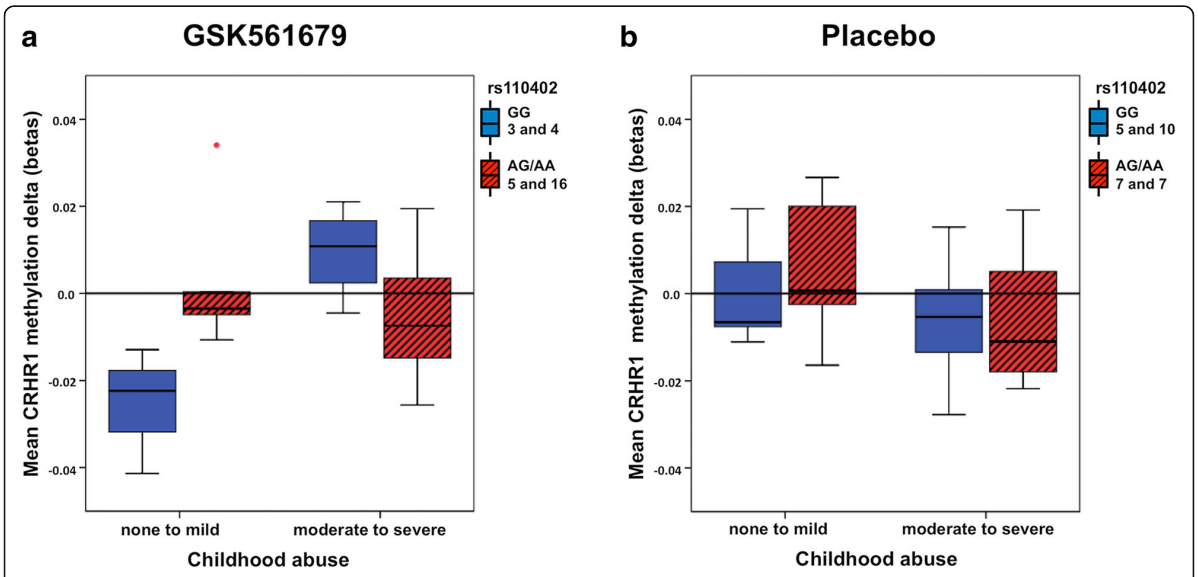

Fig. 1 The boxplots describe the mean change of CRHR1 methylation (top tertile of the most variable CpGs from pre- to post-treatment) in abused and non-abused patients treated with GSK561679 or placebo. GG carriers are shown in blue (plain boxes) and AA/AG in red (striped boxes). Positive values correspond to an increase, whereas negative values correspond to a decrease in methylation from baseline to endpoint. Dots indicate outliers. Three-way interaction of treatment × rs110402 A carrier status × child abuse was significantly associated with mean methylation change ($n = 57$; $p = 0.033$) (**a**, **b**). After treatment stratification, there was a significant interaction effect of rs110402 A carrier status and child abuse on mean methylation change in subjects treated with GSK561679 ($n = 28$; $p = 0.00005$) (**a**) but not with placebo ($n = 29$; $p > 0.05$) (**b**)

of rs110402 carrier status by child abuse on the change in methylation levels stratified by treatment. The interaction showed a significant effect on pre- to post-treatment *CRHR1* methylation change only in patients treated with the CRF_1 receptor antagonist ($n = 28$; F (1, 16) = 29.81; $p = 0.00005$; withstands Bonferroni correction for multiple testing; $ß = -0.913$; Cohen's $f = 1.366$; $R^2 = 0.73$; adj. $R^2 = 0.55$; post-hoc power = 0.99) (Fig. 1a).

Interestingly, the subset of patients with child abuse and who are also carriers of the GG genotype of rs110402 showed an increase in *CRHR1* methylation with GSK561679 treatment. This subgroup was previously described to benefit most from the drug ([16] and Additional file 1: Figure S1). The other three subsets of patients (no abuse and rs110402 GG; no abuse and rs110402 AG/AA; abuse and rs110402 AG/AA) showed no change or decreased methylation after GSK561679 treatment. There was no significant effect in the placebo group ($n = 29$; $p > 0.05$) (Fig. 1b).

Baseline methylation by treatment interaction effects on PTSD symptom change

We next tested whether baseline methylation predicted %-change of PTSD symptoms from pre- to post-treatment. Seventy-nine (CAPS)/78 (PSS) subjects were included in the analysis due to missing genotype data in three samples and missing phenotype data (PSS %-change) in one sample. Neither *NR3C1* ($n = 79/78$; $p > 0.05$) nor *FKBP5* ($n = 79/78$; $p > 0.05$) showed a significant interaction effect of treatment by baseline methylation on symptom change.

Three-way interaction effects on PTSD symptom change with treatment, baseline methylation, and SNP/child abuse

Next, we included either rs110402 or child abuse in our analysis and tested for two three-way interaction effects (rs110402 × treatment × mean baseline methylation or child abuse × treatment × mean baseline methylation) on symptom reduction measured by change in Clinician-Administered PTSD Scale (CAPS) and PTSD Symptom Scale-Self-Report (PSS-SR) scores. Treatment by baseline methylation by rs110402 carrier status was not significantly associated with differences in PTSD symptom change for neither of the genes (NR3C1: $n = 79/78$, $p > 0.05$; FKBP5: $n = 79/78$, $p > 0.05$).

The three-way interaction that included child abuse was significant for *NR3C1* baseline methylation ($n = 78$; F (1, 56) = 4.26; $p = 0.044$; $ß = 0.276$; Cohen's $f = 0.277$; $R^2 = 0.33$; adj. $R^2 = 0.087$; post-hoc power = 0.67) and showed a trend towards significance for *FKBP5* baseline methylation ($n = 79$, F (1, 57) = 2.81; $p = 0.099$; $ß = 0.215$; Cohen's $f = 0.222$; $R^2 = 0.28$; adj. $R^2 = 0.017$;post-hoc power = 0.38).

More specifically, CRF_1 receptor antagonist-treated, abused patients with high baseline *NR3C1* methylation levels showed the strongest PSS percent change and therefore the best treatment outcome overall (Fig. 2a, b). A post-hoc analysis revealed that the interaction of baseline *NR3C1* methylation and child abuse was significantly associated with PSS percent change after CRF_1 receptor antagonist treatment ($n = 38$; F (1, 20) = 4.58; $p = 0.045$; $ß = 0.331$; Cohen's $f = 0.478$; $R^2 = 0.67$; adj. $R^2 = 0.39$; post-hoc power= 0.81) (Fig. 2a) but not placebo ($n = 40$; $p > 0.05$) (Fig. 2b). Results from the same analysis using CAPS score %-change as treatment outcome showed the same direction of effects but did not reach significance (three-way interaction: $n = 79$; $p > 0.05$) (Fig. 2c, d).

For FKBP5, abused patients with high baseline methylation and treated with the CRF_1 receptor antagonist experienced the strongest CAPS percent change ($n = 79$, F (1, 57) = 2.81; $p = 0.099$). The post-hoc analysis, stratifying patients by treatment and testing the interaction effect of baseline methylation by child abuse on PTSD symptom change, did not reach significance in neither one of the treatment groups ($p > 0.05$ for all) (Fig. 3a–d).

Pre- to post-treatment methylation change by treatment interaction effects and three-way interaction effects including SNP or child abuse on PTSD symptom change

To examine the association between FKBP5/NR3C1 methylation change from baseline to post-treatment and symptom improvement, we tested for interaction effects of treatment by pre- to post-methylation change on %-change of PTSD symptoms from pre- to post-treatment. For NR3C1 and FKBP5, 57 subjects were included in the analysis due to missing methylation data in two baseline samples and one post-treatment sample. None of the tested interactions reached significance (FKBP5: $n = 57$, $p > 0.5$; NR3C1: $n = 57$, $p > 0.5$). Further, including either rs110402 or child abuse in our analysis to test for two three-way interactions (rs110402 × treatment × pre- to post-methylation change or child abuse × treatment × pre- to post-methylation change) on symptom reduction also did not show significant effects (FKBP5: $n = 57$, $p > 0.5$; NR3C1: $n = 57$, $p > 0.5$).

Discussion

The objective of this study was to investigate epigenetic marks of PTSD-related genes in association with PTSD symptom changes after CRF_1 receptor antagonist (GSK561679) treatment in female PTSD patients. In a first analysis, we observed significant differences in *CRHR1* methylation levels after treatment among patients with probable CRF hyperactivity who previously demonstrated the greatest clinical benefit from the CRF_1 receptor antagonist [16]; this effect was not present among those who received placebo. This subgroup of patients who had experienced child abuse and were

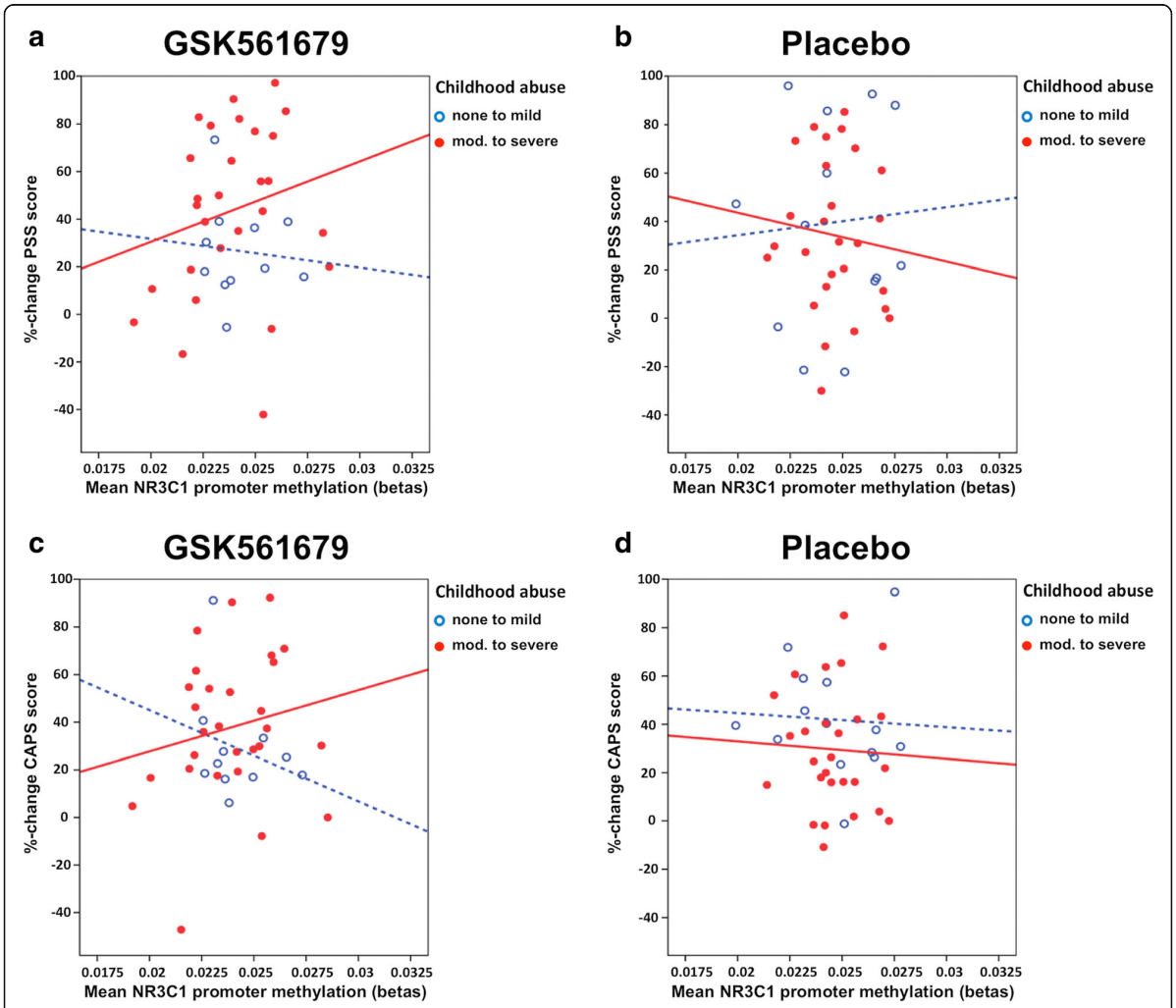

Fig. 2 The scatter plots describe the association between the mean percent change of PTSD symptoms and mean NR3C1 methylation dependent on child abuse in patients treated with GSK561679 (**a**, **c**) or placebo (**b**, **d**). Higher symptom percent change corresponds to improvement (reduction) in PTSD symptoms from baseline to endpoint. Abused patients are shown in red (solid line) and non-abused patients in blue (dashed line). Three-way interaction of NR3C1 baseline methylation × treatment × child abuse was significantly associated with PSS %-change ($n = 79$; $p = 0.044$) (**a**, **b**) but not with CAPS %-change ($n = 78$; $p > 0.05$) (**c**, **d**). After treatment stratification, there was a significant interaction effect of baseline methylation and child abuse on PSS %-change in subjects treated with GSK561679 ($n = 38$; $p = 0.045$) (**a**) but not with placebo ($n = 40$; $p > 0.05$) (**b**). For CAPS %-change, the effect pointed in the same direction without reaching significance (**c**, **d**)

homozygous for the rs110402 GG allele were the only individuals showing a significant increase in *CRHR1* methylation from baseline to the post-GSK561679 treatment time point. All other subjects either showed no change or a reduction in methylation over the time of treatment. On the other hand, baseline *CRHR1* methylation did not predict treatment outcome, suggesting that this epigenetic change may only serve as a potential tracker of symptom changes. The maximum difference in mean *CRHR1* methylation between the subgroups was more than 3%, a change comparable to or even larger than other studies examining peripheral blood DNA methylation and psychiatric disorders or psychiatric treatment response. In fact, when examining the 11 CpGs composing the *CRHR1* variable methylation score, the maximal effects were observed in CpGs cg27410679 and cg04194664. In the subgroup of patients with child abuse and homozygous for the rs110402 GG allele, these CpGs showed an increase in methylation of up to 3.9% and a maximum methylation difference between the four subgroups of 9.9% (cg04194664) and 7.7% (cg27410679). Future studies should evaluate these optimized markers in larger samples.

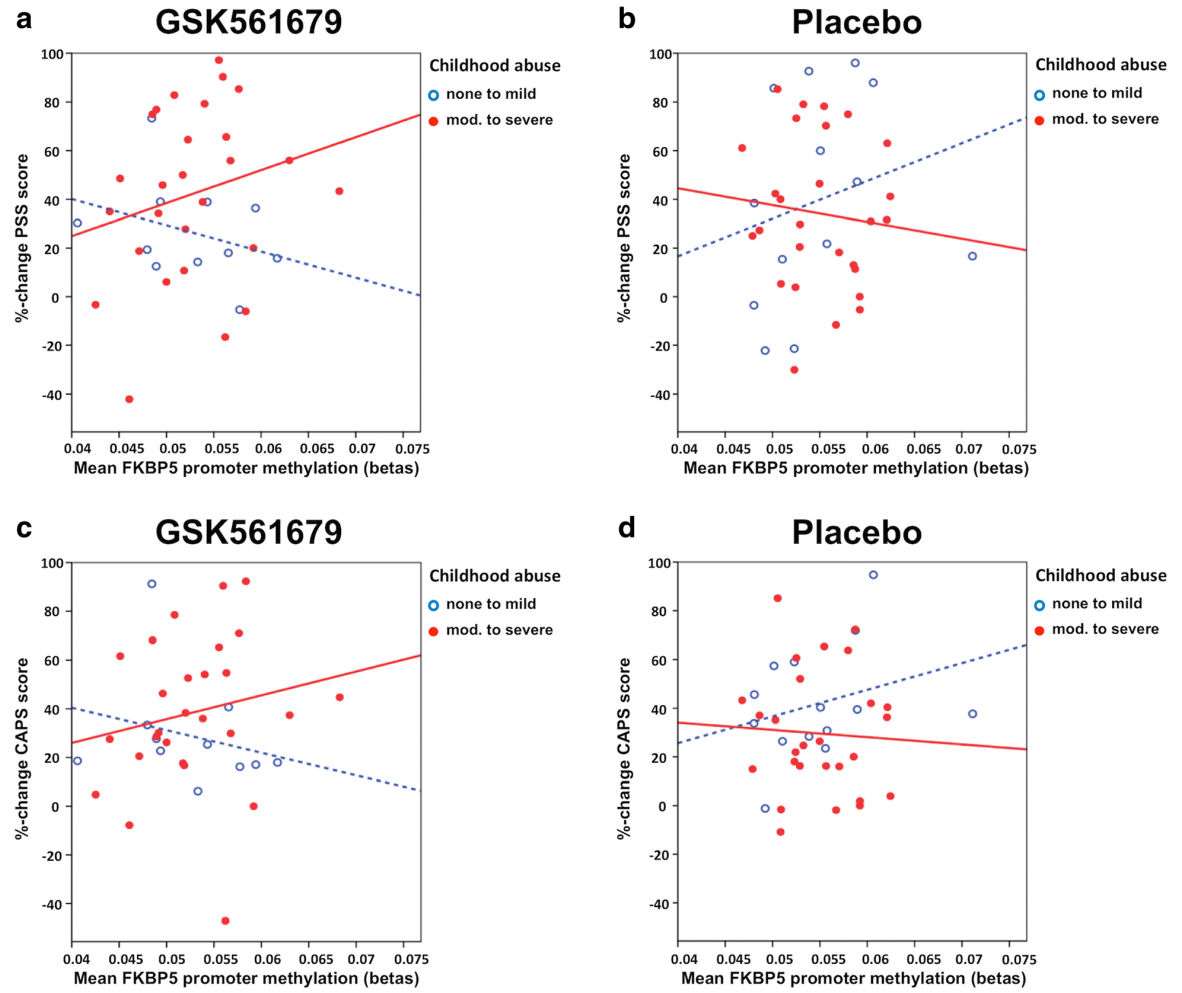

Fig. 3 The scatter plots describe the association between the mean percent change of PTSD symptoms and mean FKBP5 methylation dependent on child abuse in patients treated with GSK561679 (**a**, **c**) or placebo (**b**, **d**). Higher symptom percent change corresponds to improvement (reduction) in PTSD symptoms from baseline to endpoint. Abused patients are shown in red (solid line) and non-abused patients in blue (dashed line). The three-way interaction testing FKBP5 baseline methylation × treatment × child abuse on CAPS %-change had a p value of $p = 0.099$ with an $n = 79$ (**c**, **d**) and $p > 0.05$ with PSS %-change ($n = 78$) (**a**, **b**). After treatment stratification, there was no significant interaction effect of baseline methylation by child abuse on PTSD symptom %-change in neither one of the treatment groups ($p > 0.05$ for all) (**a**–**d**)

A number of factors can contribute to changes in DNA methylation. In a mixed tissue such as peripheral blood, the most likely contributor is the changes in immune cell subtype composition. Changes in immune responses have been reported in PTSD (reviewed by [23]), and symptom normalization may be associated with a change in immune function and cell type proportion [24–26]. We attempted to account for this using a bioinformatics deconvolution method for blood cell types from genome-wide methylation data [27] and adding the estimated cell type proportions as covariates. In addition, there has been increasing evidence suggesting that dynamic methylation changes, as observed in our study, may be mediated by certain transcription factors [28–30]. Several studies have reported on the potential role of the glucocorticoid receptor as one of these transcription factors mediating glucocorticoid-induced DNA demethylation [31, 32]. CRF_1 receptor antagonists influence the regulation of the HPA axis and by that, ultimately, modulate GR activity. Our previously identified subgroup of patients with rs110402 GG genotype and a history of child abuse displayed a significant increase in *CRHR1* methylation after GSK561679 treatment. Previous studies have shown that this combination of environmental and genetic risk is associated with specific disruptions of HPA axis regulation, including an enhanced cortisol response to the

Trier Social Stress Test and the combined dexamethasone suppression/CRF stimulation test [11–14]. A combination of increased CRF activity and GR activation may exist in this subgroup and normalize with specific CRF_1 receptor antagonist treatment. In fact, a number of studies have also reported GR supersensitivity with PTSD [33, 34] and its normalization with effective treatment [35, 36]. Such a reversal of GR supersensitivity in the subset of patients with response to the antagonist may also lead to changes in GR-mediated DNA methylation. In fact, active GR response elements are shown in the ENCODE project for the *CRHR1* locus [37]. Finally, GSK561679 itself could directly impact *CRHR1* methylation. However, the *CRHR1* expression is low in peripheral blood cells (https://gtexportal.org/), suggesting that the epigenetic regulation of the locus indirectly via receptor blockade and adaptive transcriptional regulation is an unlikely mechanism for inducing this effect.

In our second analysis, we investigated peripheral blood DNA methylation of two genes, for which a previous study had found an association with improvement of PTSD symptoms after prolonged exposure therapy [22]. In a small cohort of combat veterans diagnosed with PTSD, the authors reported that pre-treatment *NR3C1* methylation significantly predicted treatment outcome, with higher *NR3C1* methylation at baseline associated with better response to psychotherapy. The authors also observed a decrease in *FKBP5* promoter methylation over treatment in patients showing clinical improvement [22].

Similar to Yehuda et al. [22], we also find that higher baseline methylation of *NR3C1* is associated with better treatment outcome with the antagonist. However, in our analysis, this is only seen in patients who had also experienced child abuse. No association was found for *FKBP5*, neither for baseline levels predicting treatment outcome nor for change in *FKBP5* methylation being associated with symptom improvement, as reported in Yehuda et al. [22]. While exploratory, our results support the conclusion that peripheral blood DNA methylation of *NR3C1* is associated with PTSD treatment response.

The major limitation of this study is the small sample size, particularly after biological subgrouping. Power calculation for our main hypothesis (change of CRHR1 methylation over treatment and prediction of treatment outcome), however, revealed that power would be sufficient to detect medium to large effect sizes, whereas smaller effect sizes would have been missed. A post-hoc power analysis for the specific effect sizes detected in our study showed that power ranged between 0.673 and 0.999. Further, due to the exploratory nature of our study, we did not apply a systematic correction for multiple testing, increasing the risk for false-positive associations. To identify smaller effects, confirm our results, and reduce the risk of a type I and type II error, much larger sample sizes will be required for future studies.

An additional limitation to this study, which represents a general issue in DNA methylation analyses of mixed tissues, is to rule out cell type composition variation as a potential confounding factor contributing to the observed epigenetic changes. As described, we applied a commonly used bioinformatics cell-type deconvolution method [27] to address this issue. However, this method only accounts for six different cell types in the blood, so that changes in subtypes not covered by this algorithm may still contribute to the observed changes in DNA methylation.

Conclusion

Overall, our results indicate that markers for PTSD likely will need to be an index, comprised of several combination markers. Here, we describe the association of *CRHR1* DNA methylation with treatment response, but only in a specific subset of patients defined by genetic and environmental risk factors. While our association of baseline *NR3C1* methylation with PTSD treatment outcome is supportive of previous findings, both studies are small. Given the exploratory nature of the study and the small sample size, larger studies that stratify patients by potential biomarker status will be needed to fully establish the clinical value of these measures.

Methods

Study overview

Detailed descriptions of the trial design and the study results were published previously [15, 16] and are summarized in the following.

Cohort

Patients were recruited at four academic sites (Emory University, Icahn School of Medicine at Mount Sinai, Baylor College of Medicine, University of California San Francisco/San Francisco Veterans Affairs Medical Center) in the USA. The institutional review boards at each study site approved the study. The cohort used for this study consisted of 88 female patients between 18 and 65 years of age. Males were excluded due to potential reproductive organ toxicity of the investigational medication. All subjects were free of psychotropic medication (except non-benzodiazepine hypnotics) for at least 2 weeks prior to randomization. Subjects had to fulfill criteria for a primary psychiatric diagnosis of DSM-IV-defined PTSD of at least 3 month's duration since the index trauma. PTSD status at the baseline (randomization) visit had to be of at least moderate severity, defined as Clinician-Administered PTSD Scale (CAPS) for DSM-IV [38] past-month and past-week total scores ≥ 50. Important exclusion criteria included current or past diagnosis of a psychotic disorder,

bipolar disorder, or obsessive-compulsive disorder. Subjects with a positive test for drugs of abuse at the screening visit, or who met criteria for substance abuse or dependence within 3 months of the randomization visit, or who presented with significant current suicidal ideation were excluded. Pregnant or lactating women and subjects with an unstable medical condition were also excluded.

Study design

Subjects participated in a parallel-group, double-blind, placebo-controlled randomized clinical trial of a novel CRF_1 receptor antagonist (GSK561679). After randomization, patients were either treated with a nightly dose of 350 mg GSK561679 or placebo over 6 weeks. At the baseline visit (prior to treatment phase), numerous data including demographics, vital signs, and several psychiatric measures were assessed, e.g., level of childhood maltreatment was tested using the Childhood Trauma Questionnaire (CTQ). CAPS score and PTSD Symptom Scale-Self-Report (PSS-SR) [39] were assessed at weeks 1, 2, 4, and 6 after randomization to assess PTSD symptom severity, and the percent change of these scores from pre- to post-treatment were used to determine the degree of improvement in PTSD symptoms. For biological assessments (e.g., methylation levels, genotyping), whole blood was collected at baseline ($n = 88$) as well as after 5 weeks of treatment ($n = 60$ with both baseline and post-treatment) and DNA extraction was performed.

DNA extraction

DNA isolation from whole blood was performed with a *magnetic bead*-based technology on the chemagic 360 extraction robot using the chemagic DNA Blood Kit special (PerkinElmer Inc., Waltham, MA, USA). Quality and quantity of the extracted DNA were assessed using the Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

Genotyping

Genome-wide SNP genotyping was performed for all subjects using Illumina HumanOmniExpress-24 BeadChips according to the manufacturer's protocol. We excluded the relatives of individual subjects from the whole sample ($n = 3$, Pihat ≥ 0.0625) based on mean identity by descent (IBD) in PLINK [40]. Eighty-five subjects remained for further QC. For the genome-wide analyses that were used to correct for population stratification, we only included individuals with a sample-wise call rate ≥ 0.98 and SNPs with call rate ≥ 0.98, Hardy Weinberg equilibrium test (HWE) p value $\geq 1 \times 10^{-5}$ and MAF ≥ 0.05, allowing for a total of 575,455 markers in 85 individuals. To correct for population stratification in an ethnically mixed sample, principal components (PC) for the genetic background were calculated from all genotypes for each of the individuals using genome-wide complex trait analysis (GCTA) [41].

Methylation analysis

DNA methylation levels were assessed using the Illumina 450k array. After bisulfite conversion with the Zymo EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA. USA), genome-wide DNA methylation levels were assessed for 84 baseline samples and 60 matching post-treatment samples using Illumina 450K DNA methylation arrays (Illumina, San Diego, CA, USA) as previously published [42].

Quality control of DNA methylation

Minfi Bioconductor R package (version 1.10.2) was used to perform quality control of methylation data including normalization, intensity readouts, cell type composition estimation, and beta and M value calculation. A detection p value larger than 0.01 in at least 75% of the samples led to an exclusion of the probe. Probes that were located close (10 bp from query site) to a SNP which had a minor allele frequency of ≥ 0.05 in any of the populations represented in the sample were removed as well as X chromosome, Y chromosome, and non-specific binding probes. The data were then normalized using functional normalization, which is an extension of quantile normalization included in the minfi R package. The Bioconductor R package shinyMethyl version 0.99.3 was used to identify batch effects by inspecting the association of the first principal component of the methylation levels with plate, sentrix array, and position using linear regression and visual inspection of PCA plots. A linear regression model was fitted in R with the M values for each probe as the dependent variable and plate, sentrix array, and row as the independent variables as factors to remove batch effects. Two baseline samples and one post-treatment sample did not pass quality control, which resulted in 82 baseline samples and 57 matching pairs with 450K methylation data.

Statistical analyses

Statistical analysis was carried out using SPSS v.18.0 (IBM Corp., Armonk, NY, USA) and R software v 3.2 (https://www.r-project.org/). Genotype analysis (SNP rs110402): the intronic SNP rs110402 has been shown to be associated with HPA axis hyperactivity [11, 14, 43]. This may result in a different response to antagonizing the CRF system, depending on a patient's rs110402 genotype. We therefore focused on rs110402 genotype stratification in our analysis. Direct genotypes were taken from the HumanOmniExpress-24 array (rs110402 MAF = 0.401, HWE test p value = 0.52). According to our previous study [16], patients were categorized by rs110402 A-allele carrier status (GG = 33 carriers and 53

A-allele carriers, of which 38 patients had the AG genotype and 15 were homozygous for the A-allele). Grouping individuals carrying one or two copies of the minor A-allele of rs110402 has been used in previous studies [9, 11, 44] and helps to preserve power. Additive effects of that SNP have previously been reported [45]. Methylation analysis: *CRHR1*: From the *CRHR1* gene locus covered by 33 CpGs on the 450k array, the top tertile (11 CpGs) of the CpGs with the most variable methylation change from pre to post-treatment was selected (Additional file 1: Table S1). The mean methylation of these 11 CpGs was calculated and used for further analysis. *NR3C1*: Mean methylation of 5 CpG sites within the 1F promoter and exon present on the Illumina 450K array was used for the analysis (Additional file 1: Table S2). DNA methylation in the 1F promoter and exon had been shown to predict PTSD treatment outcome [22]. *FKBP5*: Mean methylation level of 3 CpG sites within the exon 1 promoter present on the Illumina 450K array was used for the analysis (Additional file 1: Table S3). DNA methylation of this locus was shown to track with symptom improvement [22]. Childhood trauma status was defined as previously described by categorizing individuals as having experienced either no or only mild abuse versus those having experienced at least one type of moderate to severe abuse (emotional abuse ≥ 13, physical abuse ≥ 10, sexual abuse ≥ 8) (57 = abused, 31 = non-abused) using the CTQ [45]. We performed linear regression models adjusted for age, smoking, ancestry PC, and estimated blood cell count to test for main/two-way and three-way interaction effects on methylation changes as well as main/two-way and three-way interactions effects on PTSD symptom %-change. For each of the analysis, only individuals with complete phenotype, methylation data, genotypes, and any additional covariates were included in the model. We calculated power post-hoc using G Power 3.1 [46]. Alpha was set to 0.05, and the number of groups, degrees of freedom, and eta squares were set according to the test-specific calculations performed in SPSS. Statistical significance was considered at $p < 0.05$. Due to the exploratory nature of the study, no correction for multiple testing was applied. As a measure of effect size, Cohen's f was calculated and interpreted as follows: $f < 0.25$ = small effect size; $0.25 < f < 0.4$ = medium effect size; $f > 0.4$ = large effect size [47].

Additional file

Additional file 1: Figure S1. The boxplots describe the mean % change of PSS total score in abused and non-abused patients treated with the CRHR1 antagonist or placebo. GG carriers are shown in blue (plain boxes) and AA/AG in red (striped boxes). rs110402 A carrier status by childhood abuse exposure showed a significant interaction effect on PSS score % change over treatment in subjects treated with the CRHR1 antagonist ($n = 43$; F (1, 31) = 4.42; $p = 0.043$) (a) but not in subjects treated with placebo ($n = 42$, $p > 0.05$) (b). rs110402 GG carriers exposed to child abuse displayed the highest % change of PSS symptoms following CRHR1 treatment. (From Biological Psychiatry; Dunlop et al., 2017). **Table S1.** CRHR1: List of CpGs used for analysis. **Table S2.** NR3C1: List of CpGs used for analysis. **Table S3.** FKBP5: List of CpGs used for analysis. (DOC 977 kb)

Abbreviations

ACTH: Adrenocorticotropic hormone; CAPS: Clinician-Administered PTSD Scale; CpG: Cytosine-phosphate-guanine; CRF1: Corticotropin-releasing hormone receptor 1; *CRHR1*: Corticotropin-releasing hormone receptor 1 (gene); CTQ: Childhood Trauma Questionnaire; *FKBP5*: FK506-binding protein 51 kDa gene; GR: Glucocorticoid receptor; HPA: Hypothalamic-pituitary-adrenal; *NR3C1*: Nuclear receptor subfamily 3 group C member 1; PSS: PTSD symptom scale; PTSD: Post-traumatic stress disorder; QC: Quality control; *SKA2*: Spindle and kinetochore-associated complex subunit 2; SNP: Single nucleotide polymorphism

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EB and JP designed the research and coordinated the experimental work. BD, HM, CN, BR, DI, SM, and TN were responsible for the clinical trial and the different recruitment sites. JP performed the experimental work. JP, TC, and DC performed the statistical analysis. JP and EB prepared the initial manuscript. BR, CN, DC, AZ, DI, SM, TN, HM, and BD revised and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study is being conducted in accord with the latest version of the Declaration of Helsinki. Each site's Institutional Review Board (IRB) approved the study design, procedures, and recruitment strategies, with Emory University serving as the lead site (Emory University IRB, IRB number 00022717; Mount Sinai School of Medicine IRB, IRB number 04-0900 0001 03; Baylor College of Medicine IRB, IRB number H-30433 and the Michael E DeBakey Veterans Affairs Medical Center Research and Development Program, ID number 12G19, HBP; University of California San Francisco IRB, and the San Francisco Veterans Affairs Research and Development Committee, IRB number 12-09929). The study is registered at Clinicaltrials.gov: NCT01018992. All participants provided written informed consent before the study.

Consent for publication

Not applicable

Competing interests

Dr. Mayberg reports grants from NIMH, grants, and other from GSK, during the conduct of the study; personal fees from Abbott Labs (previously St Jude Medical Inc), outside the submitted work.
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Dr. Binder, Dr. Rothbaum, Dr. Neylan, Dr. Pape, Dr. Carrillo-Roa, Dr. Czamara and Dr. Zannas have nothing to disclose.

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Paper II

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Biological Psychiatry

Priority Communication

Corticotropin-Releasing Factor Receptor 1 Antagonism Is Ineffective for Women With Posttraumatic Stress Disorder

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ABSTRACT

BACKGROUND: Medication and psychotherapy treatments for posttraumatic stress disorder (PTSD) provide insufficient benefit for many patients. Substantial preclinical and clinical data indicate abnormalities in the hypothalamic-pituitary-adrenal axis, including signaling by corticotropin-releasing factor, in the pathophysiology of PTSD.

METHODS: We conducted a double-blind, placebo-controlled, randomized, fixed-dose clinical trial evaluating the efficacy of GSK561679, a corticotropin-releasing factor receptor 1 (CRF_1 receptor) antagonist in adult women with PTSD. The trial randomized 128 participants, of whom 96 completed the 6-week treatment period.

RESULTS: In both the intent-to-treat and completer samples, GSK561679 failed to show superiority over placebo on the primary outcome of change in Clinician-Administered PTSD Scale total score. Adverse event frequencies did not significantly differ between GSK561679- and placebo-treated subjects. Exploration of the CRF_1 receptor single nucleotide polymorphism rs110402 found that response to GSK561679 and placebo did not significantly differ by genotype alone. However, subjects who had experienced a moderate or severe history of childhood abuse and who were also GG homozygotes for rs110402 showed significant improvement after treatment with GSK561679 (n = 6) but not with placebo (n = 7) on the PTSD Symptom Scale–Self-Report.

CONCLUSIONS: The results of this trial, the first evaluating a CRF_1 receptor antagonist for the treatment of PTSD, combined with other negative trials of CRF_1 receptor antagonists for major depressive disorder, generalized anxiety disorder, and social anxiety disorder, suggest that CRF_1 receptor antagonists lack efficacy as monotherapy agents for these conditions.

Keywords: Adrenocorticotropic hormone, Child abuse, Clinical trial, Dexamethasone, Pharmacogenetics

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Posttraumatic stress disorder (PTSD) is a common psychiatric syndrome affecting individuals who have been exposed to traumatic events (1). The symptomatology of PTSD is multiplex, encompassing components of intrusive re-experiencing of the traumatic event, avoidance of reminders of the event, negative or reduced range of mood, and hyperarousal and excessive reactivity to the environment. The pathophysiology of PTSD is broad, including abnormalities in fear processing (2), sympathetic nervous system hyperactivity (3), and disturbed hypothalamic-pituitary-adrenal (HPA) axis functioning (4). Excessive fear processing is targeted by two established forms of PTSD treatment: exposure-based psychotherapies and selective serotonin reuptake inhibitors. Excessive sympathetic nervous system activity, as measured by systolic blood pressure, is targeted by prazosin and perhaps by atypical antipsychotics, which have some efficacy for certain PTSD symptoms (5). However, response rates to existing interventions are <60%, with only 20% to 30% of patients achieving remission with medication (6), indicating the need for additional therapeutic options. A wealth of studies implicating HPA axis disruption in PTSD pathophysiology suggests that directly targeting this system may be a fruitful approach (7).

Activation of the HPA axis in response to stress begins with the release of corticotropin-releasing factor (CRF) from the hypothalamus. CRF is a 41–amino acid peptide neurotransmitter that mediates the stress response via its effects on neuroendocrine, immune, autonomic, and behavioral systems (8). CRF binding to CRF type 1 receptors (CRF_1 receptors) in the pituitary gland stimulates the release of adrenocorticotropin (ACTH), which enters the systemic circulation and induces release of cortisol from the adrenal cortex. In healthy subjects, the acute actions of cortisol produce negative feedback to the HPA axis via glucocorticoid receptors in the pituitary and hypothalamus. Abnormalities of the HPA axis in patients with PTSD include low circulating levels of ACTH and cortisol and hypersuppression of these hormones after low-dose dexamethasone administration (4). Elevated CRF concentrations are present in the cerebrospinal fluid of PTSD

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patients (9–11), though mildly ill patients may not show this abnormality (12).

Outside the hypothalamus and anterior pituitary, CRF_1 receptors are expressed widely in the cortex and cerebellum, hippocampus, amygdala, and bed nucleus of the stria terminalis (13). Activation of CRF receptor binding in the amygdala induces fear responses (14), and administration of CRF in animal models produces PTSD-relevant anxiety behaviors, including heightened acoustic startle response, sleep disturbance, and increased conditioned fear response (15). Early life stress in animal models produces hyperactivity of CRF neurons and chronic activation of limbic brain regions (16,17).

Several CRF_1 receptor antagonists studied in animal models have demonstrated potential therapeutic value for stress-related disorders (18). An early human trial suggested efficacy of CRF_1 receptor antagonism for major depression (19), and another CRF_1 receptor antagonist produced anxiolytic effects in healthy adults given 7.5% carbon dioxide (20). However, larger trials examining several CRF_1 receptor antagonists in clinical populations have not found efficacy for the treatment of major depression, generalized anxiety disorder, or social anxiety disorder (21).

GSK561679 is an orally active, selective CRF_1 receptor antagonist that demonstrates anxiolytic effects in animal models (22). The investigator brochure for GSK561679 reports that in healthy adults, GSK561679 dose-dependently suppressed ACTH response to stress in the Trier Social Stress Test and after intravenous administration of CRF, but only inconsistently reduced cortisol responses in these challenge tests. In patients with social anxiety disorder, a single 400-mg dose of GSK561679 reduced reactivity in the amygdala after exposure to facial expressions, similar to a single dose of alprazolam. The drug achieves good brain penetration in rodents and is not a substrate for p-glycoprotein transport. The primary route of metabolism is through cytochrome P450 3A4. Preclinical studies found GSK561679 caused changes to the testes and seminiferous epithelium in male animals, thereby limiting human clinical trials to female participants.

We aimed to determine whether GSK561679 was efficacious for PTSD. Secondary aims were to evaluate the tolerability of GSK561679 and its effects on depressive symptoms. We also examined the potential moderating impact of HPA axis–related genes implicated in the development of PTSD. The clinical trial reported here is a component of the National Institute of Mental Health National Cooperative Drug Discovery/Development Groups program, which aims to facilitate partnerships between academic clinical and preclinical researchers and industry to support the discovery of drug development tools and apply "first in human, first in patient testing."

METHODS AND MATERIALS

Study Overview

A detailed description of the study rationale, methods, and design was previously published and is summarized here (23). The study design was a randomized, double-blind, placebo-controlled, parallel-group clinical trial of GSK561679 that enrolled patients between January 2010 and June 2014. After a screening phase lasting 1 to 4 weeks, patients entered a 6-week double-blind treatment phase, followed by a 1-month off-drug follow-up phase to monitor safety and durability of any clinical changes. Four academic sites conducted the study: Emory University, Icahn School of Medicine at Mount Sinai, Baylor College of Medicine, and the University of California San Francisco. Approval to conduct the study was obtained from the institutional review board of each university and its affiliated Veterans Affairs Hospitals, if applicable. The study was conducted in accordance with the Helsinki Declaration of 1975 and its amendments and is listed as NCT01018992 at ClinicalTrials.gov.

Participants

All participants provided written informed consent before the study. Recruitment was conducted by advertising and clinic referral. Eligible participants were women 18 to 65 years of age who met DSM-IV-TR criteria for chronic PTSD, determined using the Structured Clinical Interview for DSM-IV (24) and confirmed through a clinical interview with a study psychiatrist. For patients with multiple DSM-IV–qualifying traumas, we defined the "index" trauma as the trauma currently causing the greatest distress or impairment to the patient, identified from parts one and two of the Posttraumatic Diagnostic Scale (PDS) (25). PTSD had to be at least moderately severe at the screening and baseline visits, defined as Clinician Administered PTSD Scale for DSM-IV (CAPS) (26) past-month and past-week total scores $\geq$50. Important exclusion criteria included the following: any current or past diagnosis of schizophrenia or other psychotic disorder, bipolar disorder, or obsessive compulsive disorder; current substance abuse or dependence; use of a psychotropic agent, other than a nonbenzodiazepine hypnotic; use of a systemic steroid medication; significant uncontrolled medical conditions, or current clinically significant suicidal or homicidal ideation; current participation in a structured psychotherapy targeting PTSD symptoms; and any current or planned litigation regarding the traumatic event.

Randomization

Randomization to GSK561679 or placebo was 1:1 with permuted blocks generated separately for each site by a statistician who was not involved in the analysis of the data (23). The investigational pharmacist assigned the eligible patient to the treatment indicated by the randomization list at the baseline visit.

Study Medication

The selected dose of 350 mg/day of GSK561679 was based on the tolerability and biological activity observed during phase 1 testing. Study medication was dispensed in two bottles containing 100 mg or 50 mg white tablets of GSK561679 or matching placebo. Patients took three 100-mg tabs and one 50-mg tab each evening between 6:00 PM and 8:00 PM and recorded the time in a dosing diary.

Study Visits and Assessments

The Posttraumatic Diagnostic Scale and CAPS were administered at screening to assess trauma severity. Patients completed the PTSD Symptom Scale–Self-Report (PSS-SR) (27), the Childhood Trauma Questionnaire (28), the Montgomery–Åsberg Depression Rating Scale (MADRS) (29,30), the Quick Inventory of Depressive Symptomatology,

Self-Report (31), the Clinical Global Impression of Severity (32), the Sheehan Disability Scale (33), and the clinician-administered version of the Columbia Suicide Severity Rating Scale (34). An electrocardiogram, laboratory testing, urine drug screen, medical history, and a physical examination were conducted to ensure medical appropriateness for the study. Adverse events were captured by open-ended questions and via the Patient Rated Inventory of Side Effects (35) at each postscreening visit.

On the day before the baseline (randomization) visit, patients underwent phlebotomy for measurement of ACTH and cortisol, underwent baseline laboratory tests, and took 0.5 mg of dexamethasone at 11:00 PM for the low-dose dexamethasone suppression test. Patients returned the next morning to repeat phlebotomy for post-dexamethasone ACTH and cortisol concentrations. Ratings visits occurred at baseline and weeks 1, 2, 4, and 6 postrandomization, with administration of past-week CAPS, MADRS, and the self-report symptom measures. Neuroendocrinological testing was repeated during the fifth week postrandomization. Plasma samples for GSK561679 concentrations were collected at weeks 1, 2, 4, and 6. Methods for DNA genotyping are presented in the Supplement.

The primary outcome was change in past-week CAPS total score from baseline to week 6, assessed at weeks 1, 2, 4 and 6. CAPS raters were initially trained through use of a scoring guide and watching a training video interview. Interrater reliability was assessed annually via independent scoring of standardized videotaped CAPS interviews. Raters whose scores were >4 points from the median for each interview underwent additional training until reliability was achieved.

Statistical Analyses

All analyses used R software (version 3.2; available at https://www.r-project.org). Generalized linear models evaluated the effects of treatment on univariate outcomes; multilevel models examined treatment effects on longitudinal outcomes. Analyses evaluated treatment effects with and without adjustment for site effects. Inclusion of site as a covariate failed to alter any conclusion derived from models without site, and so the results are based on the more parsimonious unadjusted models. Primary analyses used intention-to-treat principles with multilevel models maximizing the use of all available data using restricted maximum likelihood estimation, and dichotomous outcomes imputed as negative/nonresponsive to treatment.

CRF_1 receptor SNP rs110402 was the main focus of the genetic analysis. Direct genotypes were taken from the HumanOmniExpress-24 array (Illumina Inc., San Diego, CA) (rs110402 minor allele frequency = 0.401, Hardy-Weinberg equilibrium test p value = .52), with patients categorized according to rs110402 A allele carrier status (GG = 33 carriers and 53 A-allele carriers, of which 38 patients had the AG genotype and 15 were homozygous for the A allele; Supplemental Table S2). To assess A-allele carrier main effects and interaction of the carrier status with childhood abuse on change in psychiatric symptoms, we performed linear regression models adjusted for age, baseline symptom severity, and ancestry PC (Supplemental Figure S8), with the percent change in CAPS, PSS, and MADRS scores as outcomes. Individuals were categorized as having experienced either no or only mild abuse versus having experienced at least one type of moderate to severe abuse (56 = abused, 30 = nonabused) as previously described using the Childhood Trauma Questionnaire (36). To conserve power, we refrained from testing three-way interactions of SNP by child abuse by treatment on symptom changes but instead analyzed two-way interactions of SNP by child abuse on outcome, stratified by treatment status. Significance was considered at $p < .05$, and owing to limited power, all genetic analyses are considered exploratory only, so no correction for multiple testing was applied.

RESULTS

The CONSORT diagram (Supplemental Figure S1) depicts the overall participant flow for the trial, with $N = 267$ enrolled, $n = 128$ participants randomized, and $n = 96$ completing treatment. The mean age of the sample was 40.5 ± 12.1 years; only three participants identified combat as their index trauma. The baseline demographic and clinical characteristics of the sample are presented in Table 1.

Table 1. Demographic and Clinical Variables at Baseline

Variable	Placebo, $n = 65$	GSK561679, $n = 63$
Race, *n* (%)		
White	32 (49)	40 (64)
African American	28 (43)	18 (29)
Other	5 (8)	5 (8)
Hispanic	5 (8)	8 (13)
Current Major Depression, *n* (%)	43 (66)	41 (65)
Education ($n = 125$), *n* (%)		
<High school	4 (6)	7 (11)
High school degree/some college	29 (45)	24 (38)
College degree	15 (23)	19 (30)
Graduate degree	16 (25)	11 (18)
Current Smoker, *n* (%)	17 (26)	12 (19)
Time Since Primary Trauma ($n = 125$), *n* (%)		
≤6 months	5 (8)	6 (10)
6 months–3 years	15 (24)	11 (18)
3–5 years	11 (18)	5 (8)
≥5 years	32 (51)	39 (64)
Age, Years, Mean (SD)	40.4 (12.3)	40.6 (11.8)
No. of Traumatic Events, Lifetime, Mean (SD)	3.7 (2.2)	3.5 (1.6)
CAPS Past Month Total, Mean (SD)	79.8 (15.6)	82.0 (12.5)
CAPS Past Week Total, Mean (SD)	74.8 (17.6)	77.5 (14.3)
PSS-SR Total, Mean (SD)	30.0 (9.3)	31.1 (7.1)
MADRS, Mean (SD)	25.1 (8.3)	26.5 (7.0)
QIDS-SR, Mean (SD)	13.6 (4.5)	13.3 (4.1)
CTQ Total, Mean (SD)	75.9 (23.9)	79.3 (27.2)
SDS, Mean (SD)	16.3 (7.1)	15.5 (7.1)
CGI-S, Mean (SD)	4.7 (0.7)	4.7 (0.7)

CAPS, Clinician-Administered PTSD Scale; CGI-S, Clinician Global Impression-Severity; CTQ, Childhood Trauma Questionnaire; MADRS, Montgomery–Åsberg Depression Rating Scale; PSS-SR, PTSD Symptom Scale–Self-Report; QIDS-SR, Quick Inventory of Depressive Symptomatology–Self-Report; SDS, Sheehan Disability Scale.

Retention and Treatment Compliance

Kaplan-Meier survival curves failed to demonstrate differential attrition as a function of treatment group (χ^2_1= 0.2, p = .647). Among individuals (n = 91) who completed treatment and who demonstrated compliance with the medication regimen (verified via serum levels at the week 6 or early termination visit only in the GSK561679 condition), retention did not differ as a function of treatment group (placebo: n = 49; GSK561679: n = 42; χ^2_1 = 1.183, p = .278). The mean week 6 concentration of GSK561679 among compliant patients receiving the active drug was 923 ± 603 ng/mL.

CAPS Outcomes

Evaluation of the CAPS past-week total score as a function of time, treatment, and their interaction found no differential change over time between GSK561679 and placebo (t_{435} = 0.713, $p \leq .477$) (Figure 1). The three CAPS-derived symptom clusters of re-experiencing, avoidance, and hyperarousal also found no differential change over time for GSK561679 compared with placebo (all $p > .05$).

Response rates did not differ between treatments, whether defined as a 50% decrease from baseline (placebo: 18 [27.7%]; GSK561679: 14 [22.2%]; χ^2_1 = 0.543, p = .305) or 30% decrease (placebo: 34 [52.3%]; GSK561679: 28 [44.4%]; χ^2_1 = 0.384, p = .238).

MADRS Outcomes

Longitudinal modeling of MADRS scores found no differential change over time between the treatments (t_{425} = –0.693, $p \leq .489$) (Supplemental Figure S2).

Completers and Compliers

Reanalysis of symptom outcomes (i.e., CAPS and MADRS) using all completers, as well as the completers and compliers sample, failed to substantively alter any conclusions. Among the completers and compliers sample who received GSK561679, the mean week 6 serum concentrations between responders (≥30% improvement from baseline) and nonresponders did not differ (responders: 852 ± 427 ng/mL; nonresponders: 706 ± 419 ng/mL; $F_{1,35}$ = 1.1, $p \leq .301$).

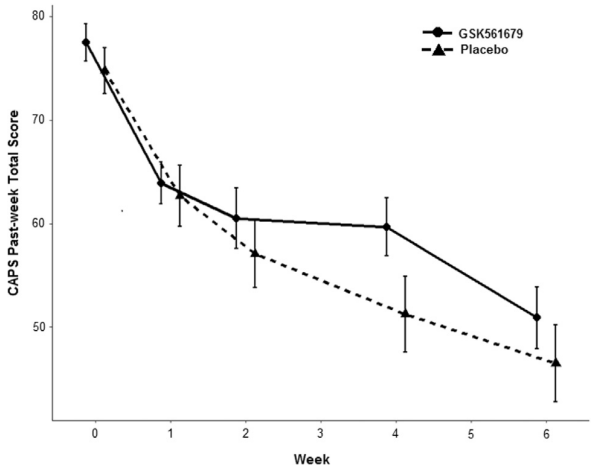

Figure 1. Change in Clinician-Administered PTSD Scale (CAPS) past-week total scores by treatment group. Bars represent ± 1 SE.

Secondary Outcomes

Multilevel modeling evaluated several secondary outcomes as a function of time, treatment, and their interaction. Change in PSS-SR Total scores over time did not reveal a treatment by time interaction (t_{436} = –0.022, p = .983). Similar null results were found for the re-experiencing (t_{438} = –0.016, p = .987), hyperarousal (t_{437} = 0.300, p = .764), and avoidance (t_{436} = –0.263, p = .793) subscales of the PSS-SR. The Quick Inventory of Depressive Symptomatology, Self-Report (t_{427} = 0.748, p = .455), Clinical Global Impression of Severity (t_{411} = 1.126, p = .207), and Sheehan Disability Scale (t_{188} = –0.440, p = .660) also failed to show differential change for GSK561679 over placebo.

Treatment Outcome Moderators

We conducted a post hoc exploratory evaluation of potential clinical moderators to account for potential heterogeneity in treatment response. We found no significant moderation of the results by patient age, time since traumatic event, comorbid major depressive disorder, CAPS score at screening, or Childhood Trauma Questionnaire total score.

Genotype by Childhood Abuse Interaction on Symptom Change Stratified by Treatment

We first tested the interaction effect of SNP rs110402 carrier status and childhood abuse on the percent change of CAPS score, as well as PSS-SR score, separately in GSK561679-treated and placebo-treated patients. rs110402 carrier status showed no significant main effect on the percent change of PTSD symptoms from pre- to posttreatment ($p > .05$) or on CAPS score change over treatment ($p > .05$) in either treatment group. However, childhood abuse and the interaction of genotype by child abuse significantly predicted PSS-SR percent change in the GSK561679 group (abuse: β = 1.534, p = .021; SNP by abuse: β = –1.904, p = .043) but not in the placebo group (abuse: β = –.629, p = .53; SNP by abuse: β = .421, p = .68). More specifically, GG genotype carriers who had experienced childhood abuse showed the highest PSS-SR percent change after GSK561679 treatment (Figure 2). Plotting PSS-SR scores by group over time showed that among the patients with childhood abuse, GG homozygotes who received GSK561679 had consistently lower symptom scores over all 5 postbaseline timepoints (Figure 3A, B).

Interestingly, the interaction of genotype by child abuse on PSS total score was most pronounced for the two PSS-SR subscales of re-experiencing and arousal. Significant interaction effects for the re-experiencing (GSK561679: β = –2.472; p = .006; placebo: β = .075; p = .92) and arousal subscales (GSK561679: β = 2.034; p = .019; placebo: β = .054; p = .94) emerged in subjects treated with GSK561679, but not for the PSS-SR avoidance subscale (GSK561679: β = –0.945; p = .36; placebo: β = .565, p = .44) (Supplemental Figures S3–S5).

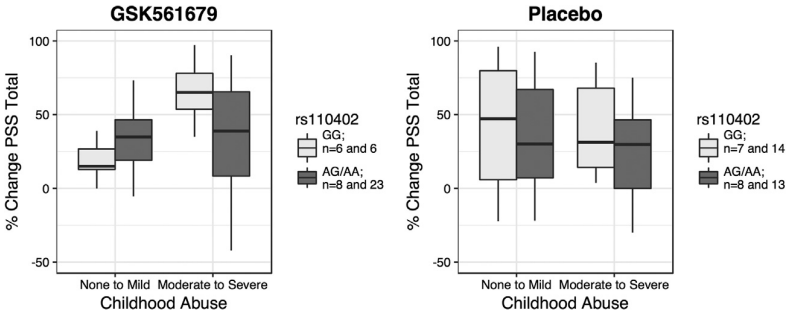

Figure 2. Significant interaction effect of rs110402 and childhood abuse on percent change in PTSD Symptom Scale–Self-Report (PSS-SR) score. The boxplots describe the mean percent change of PSS-SR total score in abused and nonabused patients treated with the GSK561679 or placebo. GG homozygotes are shown in light gray, and carriers of the A allele (AG heterozygotes and AA homozygotes) in dark gray. Higher PSS-SR percent change corresponds to improvement (reduction) in posttraumatic stress disorder symptoms from baseline to endpoint. rs110402 A carrier status by childhood abuse exposure showed a significant interaction effect on PSS-SR score percent change over treatment in subjects treated with GSK561679 (β = –1.904, p = .043) but not in subjects treated with placebo (β = .421, p = .68). rs110402 GG carriers exposed to child abuse displayed the highest percent change of PSS-SR symptoms after GSK561679 treatment.

Genotype by Childhood Abuse Interaction on Depressive Symptoms Stratified by Treatment

Because of the implications of rs110402 for depression after childhood abuse, we also tested the interaction effect of rs110402 and child abuse on the percent change in MADRS scores. There was no main effect of child abuse, nor was there an interaction effect of genotype by abuse, in either of the treatment groups ($p > .05$ for all).

Analysis of Treatment or Genotype Effect on Blood Cortisol and ACTH Levels and Interaction Effects of Treatment by Cortisol/ACTH Levels on Psychiatric Symptom Change

We tested for main effects of GSK561679 as well as rs110402 A-allele carrier status on change in cortisol concentrations over treatment time. There was no significant effect of GSK561679 compared to placebo on morning basal plasma cortisol concentrations after 5 weeks of treatment ($p > .05$) (Supplemental Figure S6). There was also no significant effect on cortisol suppression following the dexamethasone suppression test at baseline, nor a significant difference in cortisol suppression at baseline compared to week 5 ($p > .05$) (Table 2; Supplemental Figure S7). Genotype analyses of rs110402 carrier status showed similar null results ($p > .05$ for all). Neither the interaction of treatment by morning cortisol levels at baseline, nor treatment by change of morning cortisol levels from prestudy to 5 weeks were correlated with pre- to postpercent change of psychiatric symptoms (CAPS, PSS, and MADRS). In addition, there was no interaction effect of treatment with changes in the dexamethasone suppression test from prestudy to 5 weeks on percent change of psychiatric symptoms ($p > .05$ for all). For ACTH analyses, we used the same models replacing cortisol by plasma ACTH concentrations. No significant main or interaction effects were observed ($p > .05$ for all).

Safety and Tolerability

One serious adverse event occurred in each treatment arm, and both were considered unrelated to the study medication. Evaluation of suicidal ideation and behavior using the Columbia Suicide Severity Rating Scale did not find differential levels of either ideation or behavior. No adverse events occurred significantly more frequently in the GSK561679 than the placebo arm (Supplemental Tables S3 and S4).

DISCUSSION

This clinical trial found that a potent CRF_1 receptor antagonist provided no benefit for reduction of PTSD symptoms beyond those achieved with placebo. The failure of GSK561679 to demonstrate efficacy is unlikely to be the result of symptom severity or placebo responsiveness of the sample. The baseline CAPS total score of 76 was similar to baseline scores in positive trials of selective serotonin reuptake inhibitors and venlafaxine, and the mean change in CAPS score of 28 points in the placebo arm was similar to the degree of placebo improvement in those trials, which ranged from 23.2 to 26.2 points (37–41).

One explanation for the trial's failure to show benefit of GSK561679 may be found in our analysis of the rs110402 SNP

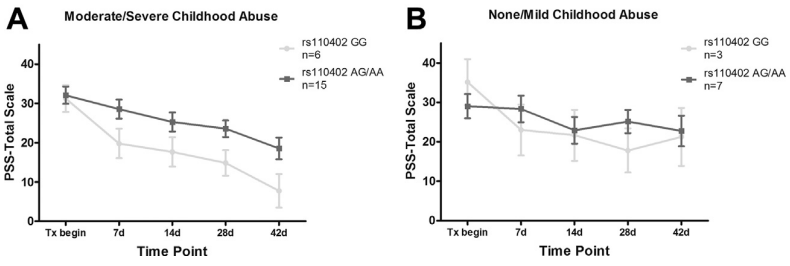

Figure 3. PTSD Symptom Scale–Self-Report (PSS-SR) score change over time among patients treated with GSK561679 by abuse level. Mean (± SEM) PSS-SR total score at all five time points during treatment with GSK561679 in **(A)** patients with a history of childhood abuse and **(B)** patients with mild/no childhood abuse, stratified by rs110402 carrier status. GG homozygotes are shown in light gray, and carriers of the A allele (AG heterozygotes and AA homozygotes) in dark gray. When treated with GSK561679, the GG genotype carriers that experienced childhood abuse showed consistently lower symptom scores over all five time points compared to abused AG/AA carriers, while this genotype effect was not observed in the nonabused group.

Table 2. Morning Plasma Cortisol and Adrenocorticotropin Concentrations Before and After 5 Weeks of Treatment

	Pretreatment				Week 5			
	Pre-Dexamethasone		Post-Dexamethasone		Pre-Dexamethasone		Post-Dexamethasone	
Cortisol, μg/dL ± SD								
Placebo	10.3 ± 3.8	$p = .65$	2.2 ± 2.9	$p = .50$	10.7 ± 4.2	$p = .77$	2.9 ± 2.9	$p = .16$
GSK561679	10.7 ± 3.4		1.8 ± 2.0		10.4 ± 3.1		1.8 ± 3.0	
ACTH, pg/mL ± SD								
Placebo	21.9 ± 15.3	$p = .18$	11.6 ± 7.2	$p = .39$	20.0 ± 12.8	$p = .041$	8.7 ± 4.8	$p = .32$
GSK561679	26.5 ± 14.7		10.2 ± 6.4		27.5 ± 14.4		11.0 ± 11.1	

ACTH, adrenocorticotropin.

of the *CRHR1* gene. Among patients with a history of childhood abuse, GG homozygotes at this locus, in contrast to A-allele carriers, demonstrated significant improvements in self-reported hyperarousal and re-experiencing symptoms with GSK561679 treatment, which were absent in the placebo-treated patients. Thus, the responsiveness of patients to CRF_1 receptor antagonism may depend on their genetic endowment and environmental exposures, which could be linked to an increased activity of the CRF system in these individuals. However, this abuse by allele status analysis was exploratory, the number of patients in each arm was relatively small, and the effect was observed on the PSS-SR, not the primary CAPS scale, so this finding requires replication in larger samples before definitive conclusions about this association can be made.

Some data do not support the model that disruptions in CRF signaling are associated with anxiety disorders, raising the possibility that the negative result is a consequence of poor target selection. Adult wild-type and CRF knockout mice demonstrate similar behavioral responses to stressors, even though CRF knockouts fail to activate the HPA axis in response to stressors (42). In the central nucleus of the amygdala, CRF_1 receptor activation reduces glutamate-mediated excitatory postsynaptic currents and increases excitatory postsynaptic currents in the lateral septum (43). Conflicting data exist on whether chronic antidepressant administration impacts basal CRF messenger RNA expression in the paraventricular nucleus (44–46) or diminishes stress-induced CRF gene expression in the paraventricular nucleus (46). Other data suggest that antidepressants reduce CRF_1 receptor messenger RNA expression in the amygdala (45), but this finding has not been replicated (46). Finally, a small study of PTSD patients who achieved remission with paroxetine found no significant pre- to posttreatment change in cerebrospinal fluid CRF concentrations (12). In combat veterans with PTSD, observation of trauma reminder stimuli resulted in unexpected reductions in cerebrospinal fluid CRF concentrations (47).

Another alternative is that the negative results of this study may be related to the differential anxiolytic and anxiogenic effects of CRF_1 receptor activation by brain region. In the forebrain, CRF_1 receptor increases anxiety by amplifying activity in the hippocampal formation via increased firing frequency of glutamatergic inputs. Stress increases CRF concentrations in the locus ceruleus, which can induce anxiety-like behavior in animals (48), and CRF receptor antagonists applied to the locus ceruleus diminish norepinephrine release to the hippocampus (49) and prefrontal cortex (50). CRF activity at CRF_1 receptor in the dorsal raphe reduces activity of serotonergic neurons (51). In the prefrontal cortex, CRF acting through CRF_1 receptor sensitizes postsynaptic 5-HT_2 receptors that mediate anxiety behaviors in mice (52). In contrast, to these effects, loss of CRF_1 receptor signaling in midbrain dopaminergic neurons increases anxiety by inhibiting dopamine release in the prefrontal cortex (53).

Another consideration for the trial's negative results is the potential sex-specific responses to CRF and CRF_1 receptor antagonists. In contrast to male mice, which show clear behavioral and HPA axis responses to infusion of either CRF or a CRF_1 receptor antagonist into the dorsal raphe, female mice demonstrate modest changes (54). Because participation in the current trial was limited to women, the potential efficacy of GSK561679 in men could not be assessed. Design of future human studies of CRF_1 receptor antagonists should prospectively consider possible sex-specific effects of CRF-modulating drugs.

As part of this National Cooperative Drug Discovery/Development Groups program, a study evaluating the anxiolytic effects of GSK561679 was conducted in healthy adults using a startle paradigm (55). Contrary to expectations, a single 400-mg dose of GSK561679 increased startle in response to a stimulus predictive of electric shock (i.e., increased fear) but had no effect on unpredictable shock (i.e., anxiety), although GSK561679 also reduced baseline startle, which complicates interpretation of the startle potentiation results. In contrast, alprazolam in this study was found to reduce anxiety but did not impact fear. Although these results did not support preclinical rodent data suggesting that CRF_1 receptor antagonism decreases anxiety measures, they were consistent with the rodent data suggesting that CRF_1 receptor antagonism can increase startle responses potentiated by cued fears (56). Taken together, these data suggest that CRF_1 receptor antagonism can inhibit the bed nucleus of the stria terminalis, thereby reducing the "brake" that bed nucleus of the stria terminalis exerts on the reactivity of the central nucleus of the amygdala to fear stimuli (57), but that this inhibitory effect is inadequate to reduce behavioral expressions of anxiety. These different regional actions of CRF_1 receptor antagonism within the central nervous system may have yielded competing effects on patients' anxiety levels. In addition, the current trial used only a fixed dose of 350 mg/day; higher doses may have produced different effects.

Alternatively, if CRF overactivity is truly present in PTSD, the negative study result may indicate that once PTSD is established, a blockade of CRF's extrahypothalamic sensitization effects on anxiety signaling is insufficient to alter the expression of anxiety behaviors. The great majority of animal studies

implicating the role of CRF_1 receptor activation in anxiety responses are based on short-term stressors and drug exposures. For example, in mice, CRF_1 receptor antagonism immediately after a predator stressor successfully blocks the initiation and consolidation of the stressor's effects on startle (58). In human adults affected with depression, PTSD, or anxiety disorders, CRF activation at the time of stress may produce circuit-level changes that, once established, are only weakly responsive to further modulation of CRF signaling. Indeed, chronic overexpression of CRF in adult mice produces only modest effects on behavior (59). Under this model, CRF_1 receptor antagonists may prove more efficacious as preventative treatments immediately posttrauma rather than as monotherapy treatments for established conditions.

While this trial was underway, GSK561679 was found to be ineffective in the treatment of major depressive disorder (60), and one study in social anxiety disorder was completed with undisclosed results (61). The negative result in the current trial suggests that CRF_1 receptor antagonists are unlikely to prove useful for the treatment of anxiety disorders, despite the wealth of suggestive preclinical data (21). Our preliminary data attempting to subtype patients according to possible CRH system hyperactivity suggest, however, that CRF_1 receptor antagonists may be effective in specific biological subgroups of patients. This observation needs to be confirmed by additional, larger studies. Other possible explanations for the failure of CRF_1 receptor antagonists include inadequate central nervous system penetration of the compounds, inadequate treatment duration, abnormal concentrations of CRF-binding protein in the central nervous system (62), competing actions by urocortins (43), or strong compensatory systems that oppose any anxiolytic effect of CRF_1 receptor antagonism (63). The effects of CRF_2 receptor activation in the presence of CRF_1 receptor antagonism are unknown (64), although existing data suggest that preserved CRF_2 receptor signaling in the absence of CRF_1 receptor activation should have provided a protective effect against anxiety (65,66). Despite the failures of CRF_1 receptor antagonists in mood and anxiety disorders, this mechanism of action may find clinical value in other areas of psychiatry.

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Paper III

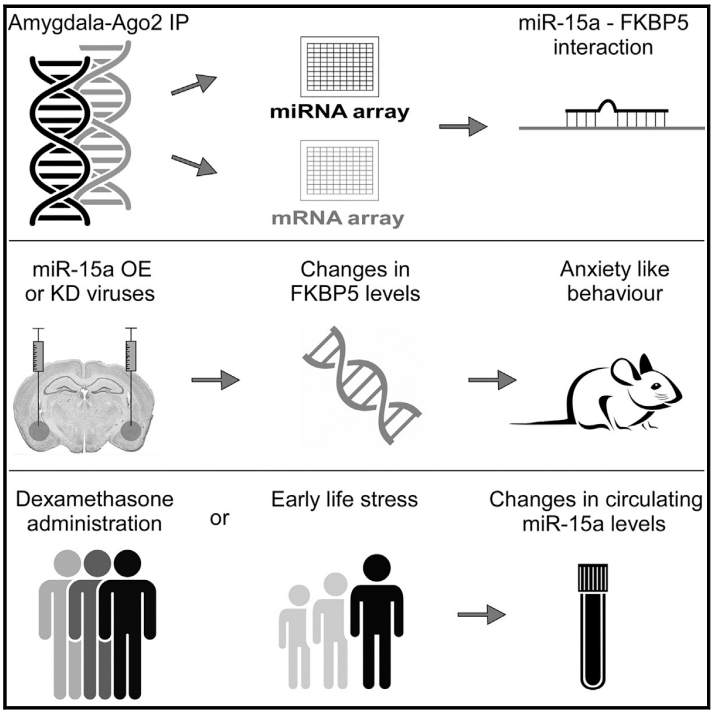
Volk N, **Pape JC**, Engel M, Zannas AS, Cattane N, Cattaneo A, Binder EB, Chen A. (2016). Amygdalar MicroRNA-15a is Essential for Coping with Chronic Stress. ***Cell Reports***, *17*(7), 1882–1891. doi: 10.1016/j.celrep.2016.10.038

Article

Cell Reports

Amygdalar MicroRNA-15a Is Essential for Coping with Chronic Stress

Graphical Abstract



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In Brief

Volk et al. reveal an important role for microRNA-15a in coping with chronic stress, with amygdala-specific manipulation affecting behavioral responses to stressful challenge. Individuals exposed to childhood trauma exhibit increased levels of miR-15a in their peripheral blood, suggesting a target for the treatment of stress-related psychopathologies.

Highlights

- miR-15a levels are elevated in the amygdala-Ago2 complex following chronic stress
- miR-15a targets FKBP51 and affects behavioral responses to stressful challenges
- miR-15a is elevated in peripheral human blood following dexamethasone exposure
- miR-15a is elevated in peripheral human blood of patients exposed to childhood trauma

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Amygdalar MicroRNA-15a Is Essential for Coping with Chronic Stress

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SUMMARY

MicroRNAs are important regulators of gene expression and associated with stress-related psychiatric disorders. Here, we report that exposing mice to chronic stress led to a specific increase in microRNA-15a levels in the amygdala-Ago2 complex and a concomitant reduction in the levels of its predicted target, *FKBP51,* which is implicated in stress-related psychiatric disorders. Reciprocally, mice expressing reduced levels of amygdalar microRNA-15a following exposure to chronic stress exhibited increased anxiety-like behaviors. In humans, pharmacological activation of the glucocorticoid receptor, as well as exposure to childhood trauma, was associated with increased microRNA-15a levels in peripheral blood. Taken together, our results support an important role for microRNA-15a in stress adaptation and the pathogenesis of stress-related psychopathologies.

INTRODUCTION

Recent studies have linked microRNA (miRNA) expression or biogenesis dysregulation to various psychiatric disorders, including anxiety and depression (Dias et al., 2014b; Issler and Chen, 2015; Issler et al., 2014; Lopez et al., 2014; O'Connor et al., 2012; Volk et al., 2014). However, changes in miRNA expression levels do not necessarily reflect their immediate activity; it is only when a specific miRNA, in the canonical pathway, has matured and been incorporated into the RNA-induced silencing complex (RISC) in the presence of argonaute RISC catalytic component 2 (Ago2) that it becomes truly active (Meister et al., 2004) as a result of its association with its target mRNA.

The amygdala plays a pivotal role in regulating the behavioral responses to stressful challenges (Dunsmoor and Paz, 2015; Duvarci and Pare, 2014; Johansen et al., 2011; Lüthi and Lüscher, 2014; Maren and Holmes, 2016). Recently, regulation of some amygdalar functions and stress-related behaviors has been attributed to miRNAs. miR-34c is involved in regulating stress-induced anxiety (Haramati et al., 2011) and miR-34a in fear memory consolidation (Dias et al., 2014a). Furthermore, miR-19b plays an important role in memory consolidation following stress by regulating the adrenergic receptor beta 1 (Volk et al., 2014).

In this study, we investigated Ago2-associated miRNAs and transcripts in the amygdala of mice subjected to a chronic social defeat stress. This chronic social stress paradigm consists of 10 consecutive days of short physical encounters between a C57BL/6 mouse and an aggressive ICR (CD1) mouse (Golden et al., 2011; Krishnan et al., 2007). The repeated exposure to stress is considered a model for the induction of chronic stress (Elliott et al., 2010, 2016; Issler et al., 2014), as well as depression-like behavior (Hollis and Kabbaj, 2014; Malatynska and Knapp, 2005) in mice. Molecular analysis and behavioral studies demonstrate that miR-15a is recruited to the Ago2 complex following chronic stress and is an essential regulator of an intact behavioral response to chronic stress.

RESULTS

miR-15a and FKBP51 mRNA Are Associated with Ago2 in the Amygdala following Chronic Stress

To identify miRNAs that are involved in the regulation of the behavioral response to chronically stressful challenges, we immunoprecipitated the Ago2 complex in tissue obtained from the amygdala of mice 8 days after completion of the chronic social defeat stress. Mice were subjected to the chronic social defeat stress paradigm for 10 consecutive days (Figure 1A), following which they were subjected to a social avoidance test to categorize them as being either "susceptible" or "resilient" to the chronic social defeat stress (Figure 1B). The RNA from the Ago2 complex of three groups—susceptible, resilient, and control—was extracted and analyzed in parallel using two distinct microarray platforms; a miRNA and a mRNA expression

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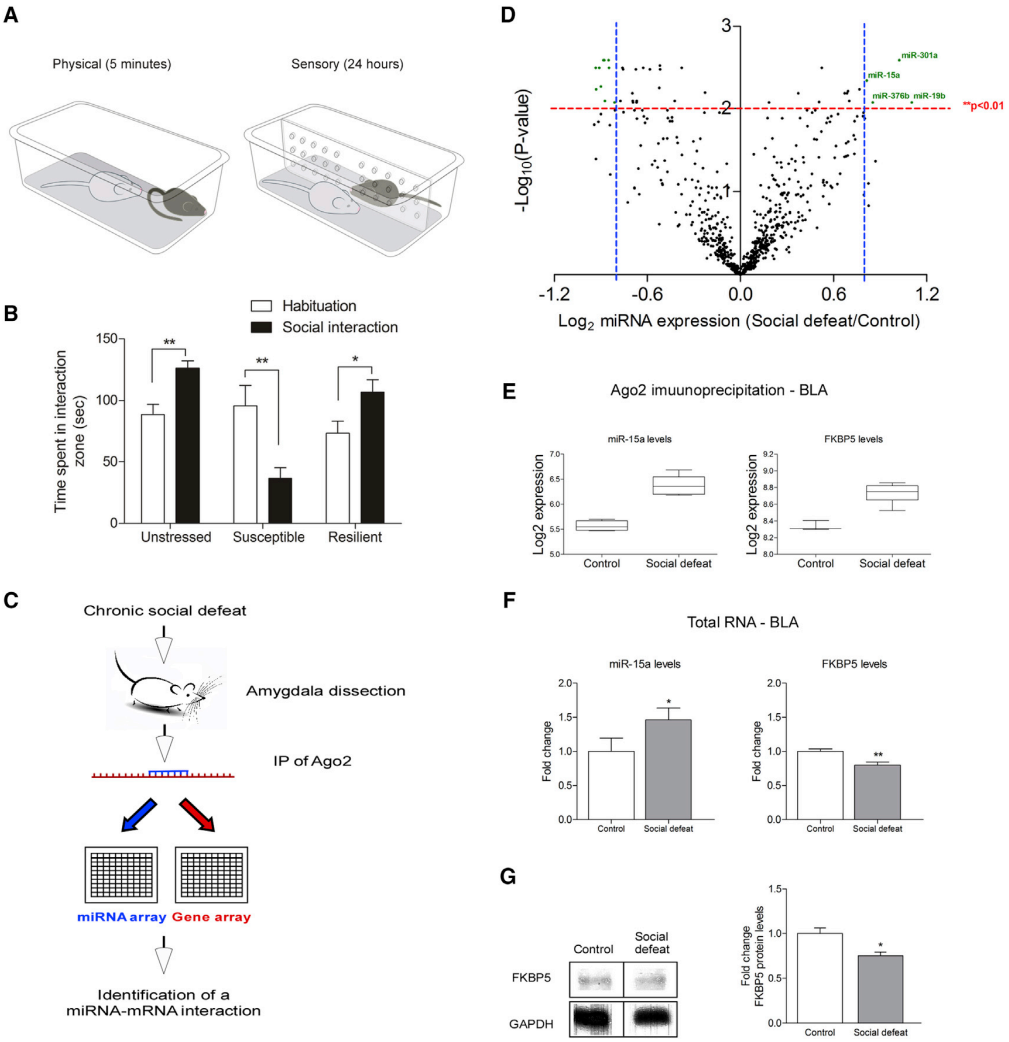


Figure 1. miR-15a Is Elevated following Chronic Stress and Potentially Regulates FKBP51

(A) Schematic illustration of the social defeat paradigm. C57 mice are subjected to 5 min of physical contact (left) with an aggressive ICR mouse, followed by sensory contact for 24 hr (right).

(B) Social avoidance test. Unstressed mice spend more time in the interaction zone following introduction of an unfamiliar mouse, $t(16) = -3.657$, $p = 0.002$. Susceptible mice spend less time in the interaction zone following introduction of an unfamiliar mouse, $t(16) = 3.133$, $p = 0.006$. Resilient mice spend more time in the interaction zone following introduction of an unfamiliar mouse, $t(16) = -2.358$, $p = 0.031$. Data are represented as mean ± SEM.

(C) Extracts of the amygdalae of mice subjected to social defeat were used for immunoprecipitation (IP) with anti-Ago2 antibody. The bound RNA was analyzed on a miRNA array (four control arrays, n = 12 animals; six social defeat arrays, n = 18 animals) and a gene expression array (three control arrays, n = 9 animals; six social defeat arrays, n = 18 animals).

(D) Log_2 miRNA expression analysis. Four miRNAs were elevated, and ten miRNAs were decreased in the amygdala Ago2 complex following social defeat.

(E) miR-15a levels were elevated in the Ago2 precipitate, $t(7) = 7.147$, $p = 0.0002$; as was FKBP51 mRNA, $t(7) = 5.352$, $p = 0.0011$.

(F) miR-15a was also elevated in total RNA extracted from mice amygdalae (n = 5) following social defeat, $t(8) = 2.46$, $p = 0.039$; whereas FKBP51 levels (n = 5) were decreased, $t(8) = 3.531$, $p = 0.008$. Data are represented as mean ± SEM.

(G) FKBP51 protein levels following social defeat. FKBP51 protein levels are downregulated in chronically stressed mice compared to control, $t(6) = 3.049$, $p = 0.014$. Data are represented as mean ± SEM.

Error bars represent mean ± SEM. *$p < 0.05$; **$p < 0.01$.

See also Figures S1 and S2.

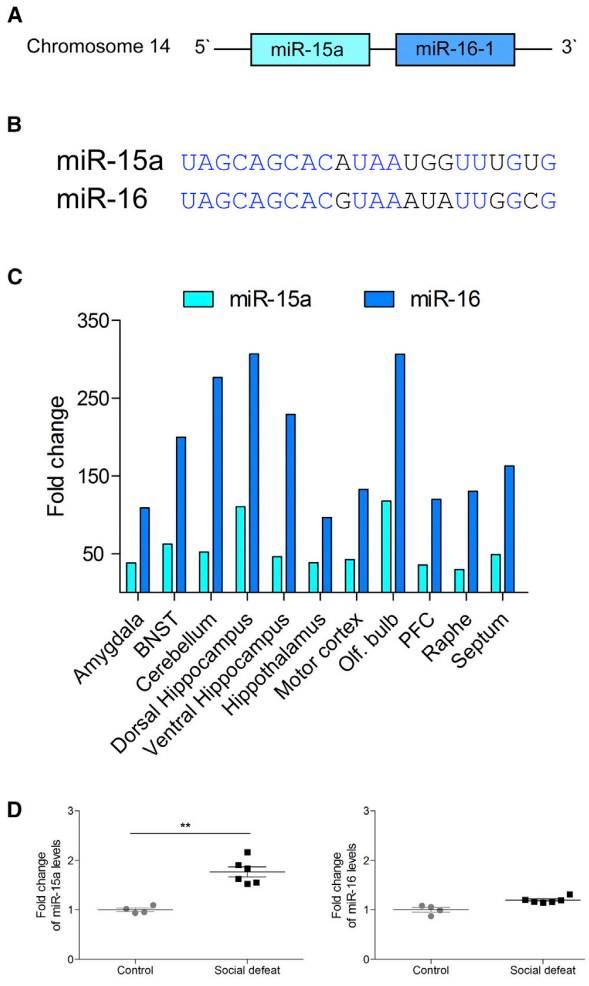


Figure 2. miR-15a and miR-16-1 Are Differentially Expressed following Chronic Stress

(A) Schematic illustration of the miR-15a and miR-16-1 transcript.

(B) Alignment of the mature sequence of miR-15a and miR-16.

(C) The distribution of miR-15a and miR-16 in different brain regions. Olf., olfactory; PFC, prefrontal cortex.

(D) Comparison of the amygdala Ago2 IP results of miR-15a and miR-16. Data are represented as mean ± SEM. **$p < 0.01$.

array (Figure 1C). Initially, we hypothesized that we would observe changes in the miRNA population of the Ago2 complex in the amygdala not only between stressed and control mice but also between susceptible and resilient mice. However, our analysis revealed that no significant changes were detected between the susceptible and resilient mice. For this reason, we combined the two groups of mice into one that is referred to in the text as "social defeat." Analysis of the miRNA array revealed four miRNAs that were upregulated and ten that were downregulated (Figure 1D; Figure S1A). A parallel analysis on the gene array revealed a small number of mRNAs that were either upregulated or downregulated in the Ago2 complex immunoprecipitation (IP) following social defeat (Figure S1B). When we analyzed the mRNAs that were changed following social defeat, we focused on stress-associated genes that were previously described to be expressed in the amygdala. This is the reason we focused on FK506 binding protein 51 (FKBP51) and miR-15a. FKBP51 has been previously linked to the pathogenesis of posttraumatic stress disorder and depression (Binder et al., 2008; Klengel et al., 2013; Lekman et al., 2008; Zannas et al., 2016). miR-15a levels were raised 1.8-fold ($p = 0.0002$) in the array following exposure to chronic stress (Figure 1E). Interestingly, a parallel increase in FKBP51 mRNA ($p = 0.001$), a predicted target of miR-15a, was observed in the Ago2 complex (Figure 1E). Whereas the levels of miR-15a were also elevated ($p = 0.039$) in the total RNA levels of the amygdala tissue, FKBP51 levels were, as expected, decreased ($p = 0.008$) (Figure 1F), supporting the possibility that FKBP51 is directly downregulated by miR-15a in the amygdala. Consistently, the protein levels of FKBP51 were measured, and a reduction of 25% in its levels was observed ($p = 0.014$) (Figure 1G). Interestingly, the levels of miR-15a were also elevated by 60% in the plasma of mice subjected to chronic stress ($p = 0.047$), whereas the levels of miR-124, an abundant brain miRNA, were unchanged (Figures S2A and S2B), implicating miR-15a as a possible marker for chronic stress exposure. These experiments led us to focus on miR-15a and FKBP51 and address their involvement in mediating chronic stress cellular processes.

miR-15a Transcription Regulation

miR-15a is located on chromosome 14 as part of a cluster with miR-16-1 (Figure 2A), indicating that these two miRNAs are co-transcribed. Previous studies have demonstrated that the promoter for miR-15a and miR-16-1 is likely to be the promoter for DLEU2, a non-coding gene that contains the transcript for miR-15a (Zhang et al., 2012). Although both miR-15a and miR-16-1 share a seed sequence, their mature miRNA sequence differs in several nucleotides (Figure 2B). In addition, the total levels of miR-16 in most brain areas appear to be higher than that of miR-15a (Figure 2C), possibly since miR-16 has two copies in the genome (miR-16-1 on chromosome 14 and miR-16-2 on chromosome 3), which both give rise to a similar mature form of miR-16, whose genomic origin is indistinguishable. Importantly, the elevation in miR-15a levels observed in our Ago2 IP is specific for this miRNA and not for miR-16 (Figure 2D), implying miR-15a specificity at the level of the Ago2 complex formation.

FKBP51 Is a Confirmed Target of miR-15a

Consistent with direct targeting of FKBP51 by miR-15a, the seed sequence for miR-15a binding at the 3′ UTR of FKBP51 is highly conserved (Figure 3A). Moreover, a luciferase assay, in which a construct containing luciferase followed by the 3′ UTR of FKBP51 was constructed and transfected into Huh7 cells expressing either miR-15a or a scramble control for it, showed a robust specific reduction in normalized luciferase levels ($p < 0.001$; Figure 3B). Importantly, this reduction was abolished when the miR-15a seed

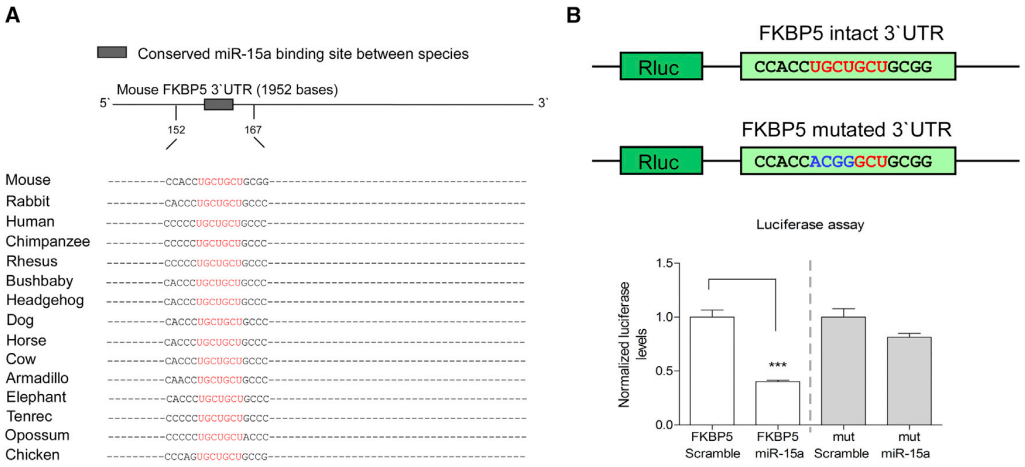


Figure 3. FKBP51 Is Regulated by miR-15a In Vitro

(A) Schematic illustration of FKBP51 3′ UTR indicating the conserved seed match for miR-15a.

(B) Luciferase assay with luciferase fused to the 3′ UTR of FKBP51 containing an intact or a control of a mutated (mut) seed site for miR-15a in the presence of miR-15a or control scramble miR ($n = 6$) showed a 50% decrease in luciferase levels, $t(10) = 9.083$, $p = 0.000$. This decrease was abolished when the intact FKBP51 3′ UTR contained a miR-15a mutated seed. Data are represented as mean ± SEM. ***$p < 0.001$

sequence was mutated (Figure 3B). These results support a regulatory role for miR-15a in directly controlling FKBP51 levels.

Overexpression of miR-15a in the Basolateral Amygdala Does Not Affect Anxiety-like Behavior

To examine whether increased levels of miR-15a in the amygdala are sufficient to mimic the behavioral effects associated with chronic stress exposure, we designed, constructed, and produced lentiviruses overexpressing the precursor of miR-15a or a scramble miR sequence as a control (Figure S3A). The degree of infection and the levels of miR-15a expression were verified using qPCR on RNA samples extracted from amygdala punches obtained from mice injected with these viruses into the basolateral amygdala (BLA). The treated mice showed an approximately 2-fold increase in the level of amygdalar miR-15a compared to scramble control ($p < 0.001$; Figure S3B), which is similar to the elevated levels of miR-15a observed following exposure to chronic stress (Figure 1F). To assess the stress-related behavioral changes of mice expressing higher levels of miR-15a, mice were injected bilaterally into the BLA with either miR-15a-overexpressing or control-scrambled viruses under basal or chronic stress conditions (Figures S3C–S3E). Behavioral assessment of the injected mice indicated no significant changes between mice overexpressing miR-15a or a control scramble miR in the open-field test, or in the elevated plus maze (EPM) test, under baseline (Figures S4A and S4C) or chronic stress (Figures S4B and S4D) conditions. In addition, no changes were observed in the locomotor activity or total time traveled in the open field test of mice overexpressing miR-15a compared to control scramble miR under basal (Figures S4E–S4G) or chronic stress conditions (Figures S4H–S4J). Therefore, we concluded that overexpression of miR-15a in the BLA is not sufficient to mimic the behavioral effects associated with exposure to chronic social defeat.

Reduced Levels of miR-15a in the BLA Increases Anxiety-like Behavior following Exposure to Chronic Stress

Next, we assessed the requirement of endogenous amygdalar miR-15a levels for the behavioral responses under baseline and chronic stress conditions. We designed, constructed, and produced a viral vector containing multiple binding sites for miR-15a (miR-15a Sponge), which enabled the knockdown (KD) of miR-15a levels in the BLA (Figure 4A). The control sponge viral construct was generated by specifically mutating 4 bp on each side of the bulge of the sponge (Figure 4A). Injection of the miR-15a KD or control sponge viruses into the BLA of mice, regardless of their exposure to chronic social defeat, resulted in an approximately 2.5-fold reduction in the levels of miR-15a in the BLA under basal conditions ($p = 0.019$) and following social defeat ($p = 0.012$) (Figures 4B and 4C). Whereas a reduction was observed independently of chronic stress, the absolute levels of miR-15a were higher in the chronic social defeat group compared to controls (Figure 4C). This supports our initial observation regarding elevation in miR-15a levels in the amygdala following chronic social defeat. The BLA of an additional group of mice was injected with miR-15a sponge and a control sponge virus (Figure S5A), and RNA was extracted. The levels of miR-15a were confirmed to be reduced by 40% ($p = 0.009$) using real-time PCR (Figure S5B). These samples were also sequenced using the Illumina TruSeq Small RNA Library Preparation Kit, and no significant changes were observed in the 25 most abundant miRNAs (Figure 4D), thus verifying a specific KD for miR-15a. As expected from our luciferase assay, FKBP51 mRNA levels were elevated in the BLA of mice injected with the miR-15a KD virus ($p = 0.033$; Figure 4E). The protein

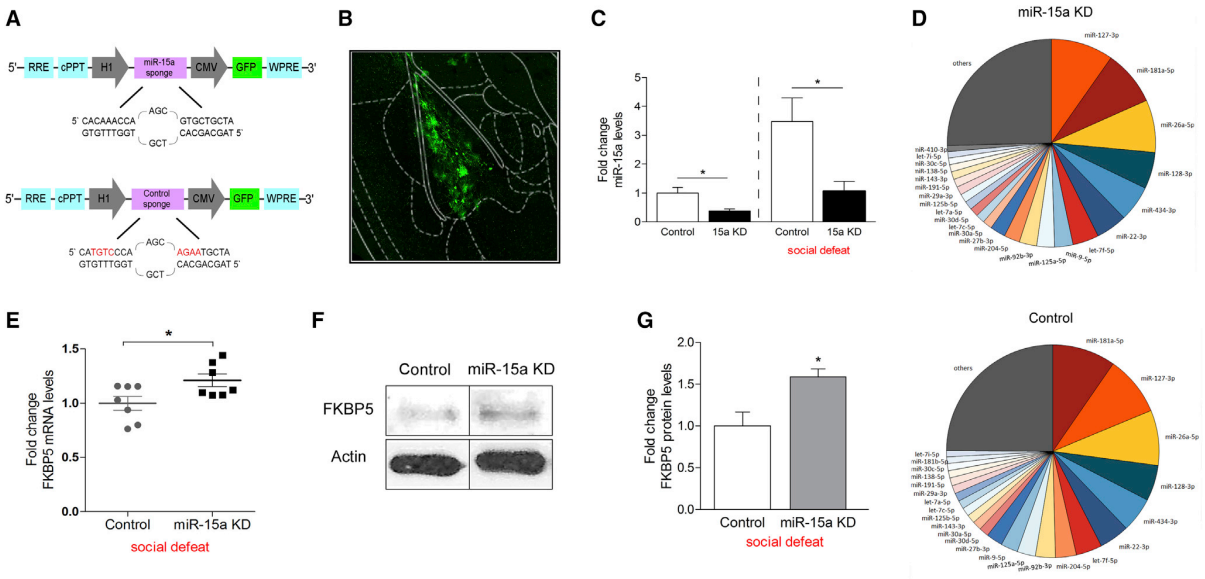


Figure 4. KD of miR-15a in the BLA Results in Increased FKBP51 Levels in the BLA

(A) Schematic illustration of lentiviral GFP-labeled constructs of control and sponge used to knock down miR-15a.

(B) Representative microscope image of virally infected basolateral amygdala (BLA) of a 10-week-old mouse following injection of lentiviral miR-15a KD with enlargement of the BLA region that corresponds to the injection site (Paxinos and Franklin, 2001).

(C) Left: decreased miR-15a levels in the BLA (n = 4) of mice injected with miR-15a KD relative to control under basal conditions, t(6) = 3.175, p = 0.019; or (right) following social defeat, t(6) = 3.528, p = 0.012. Data are represented as mean ± SEM.

(D) miRNA sequencing data. No differences are observed in the top 25 most abundant miRNA from mice injected with miR-15a KD virus compared with the control virus.

(E) Elevated FKBP51 levels in the BLA (n = 7) of mice injected with miR-15a KD relative to control, t(12) = −2.413, p = 0.033. Data are represented as mean ± SEM.

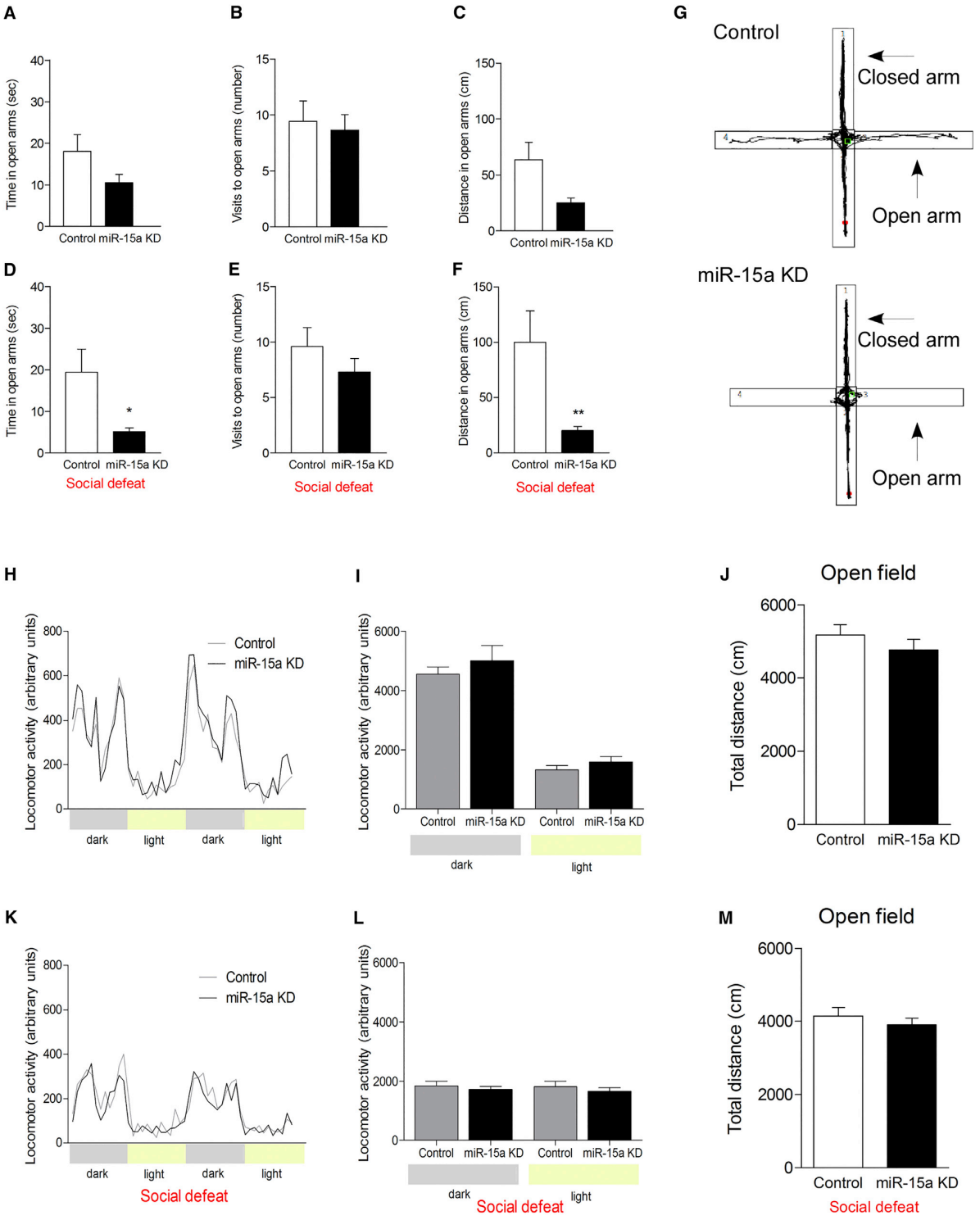
(F and G) FKBP51 protein levels following miR-15a KD virus injection to the BLA. FKBP51 protein levels are upregulated in the BLA of mice injected with miR-15a KD virus compared with control virus, t(6) = −3.060, p = 0.022. Data are represented as mean ± SEM.

*p < 0.05.

See also Figures S3 and S5.

levels of FKBP51 were increased by approximately 50% (p = 0.022; Figures 4F and 4G) following injection of miR-15a KD or control viruses.

Next, we assessed the miR-15a KD mice for anxiety-like behavior using the EPM test. Under baseline conditions (in which mice were not exposed to chronic social defeat), a tendency toward main effect was observed (p = 0.058). No significant changes were observed in the time spent in the open arms, number of visits to the open arms, or distance traveled in the open arms between the miR-15a KD and control groups (Figures 5A–5C). Similarly, the locomotor activity and the total distance traveled in the open field test showed no differences between these groups (Figures 5H–5J). Intriguingly, however, following chronic social defeat, a main effect between the behavior of miR-15a KD and control mice was observed (p = 0.026). Mice with miR-15a KD spent significantly less time in the open arms (p = 0.009) (Figures 5D and 5G) and traveled less distance in the open arms relative to controls (p = 0.002; Figures 5F and 5G, (asterisks indicate significance following correction with Bonferroni correction for multiple testing). No differences were observed in the number of visits to the open arms, the locomotor activity between the groups, or the total distance traveled in the open field test (Figures 5E and 5K–5M). These results demonstrate that KD of miR-15a levels in the amygdala specifically impaired the recovery and behavioral response of mice following their exposure to chronic stress. In the open field test, miR-15a KD mice spent less time in the center of the arena (p = 0.032), but no changes were observed in the distance traveled in the center or the number of visits to the center (Figure S5C). Following chronic social defeat, miR-15a KD and control mice spent similarly less time in the center of the arena, suggesting a "floor effect." However, miR-15a KD mice showed a tendency to travel for less distance in the center of the arena (p = 0.060) and made fewer visits to the center of the arena (p = 0.041) (Figure S5D). These results are in accordance with Hartmann et al. (2015), who observed induced anxiety-related behavior following overexpression of FKBP51 in the BLA. Moreover, Attwood et al. (2011) showed that silencing of FKBP51 levels in the BLA by injection of lentiviral short hairpin RNA led to a reduction in anxiety levels in the EPM. Taken together, the present data suggest that amygdalar miR-15a levels are functionally important in regulating the behavioral response to challenge and suggest that this effect is mediated, at least in part, via a reduction of FKBP51 levels.



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miR-15a Is Regulated by Glucocorticoids and Trauma in Human Samples

To examine the potential parallel role of miR-15a in the human stress response, we first analyzed miR-15a expression levels in RNA extracted from peripheral blood cells of young healthy male subjects following administration of the glucocorticoid receptor (GR) agonist dexamethasone (1.5 mg orally [p.o.]). We observed a significant upregulation of miR-15a at 3 and 6 hr post-treatment (Figure 6A), indicating that miR-15a is potentially regulated by activation of the stress hormone system in humans. In addition, we performed miRNA analyses on peripheral blood cells of subjects with childhood trauma and control subjects matched for age and gender with no history of early life stress. We found that the levels of miR-15a were significantly higher by 32% in subjects exposed to childhood trauma as compared to control subjects who were not exposed ($p = 0.000$, Figure 6B). Taken together, these results support a functional association between the blood levels of miR-15a and psychiatric impairment.

DISCUSSION

The present study reveals an important role for amygdalar miR-15a in regulating the behavioral responses to chronic stressful challenges. miR-15a levels are significantly increased in the amygdala of mice subjected to chronic stress, and amygdala-specific KD of miR-15a changes the behavioral responses to chronic stressful challenges. A target of miR-15a, FKBP51, identified in our studies, has been implicated in a number of stress-related psychiatric disorders (Binder, 2009; Zannas et al., 2016). FKBP51 is part of the immunophilin protein family and is known to play a role in GR transcriptional activation following the elevation of cortisol (Gillespie et al., 2009). Manipulation of FKBP51 levels in the BLA, using small interfering RNA (Attwood et al., 2011), or of its overexpression, using a viral vector (Hartmann et al., 2015), has been linked to changes in anxiety-like behavior. FKBP51, which is strongly implicated in a number of stress-related psychiatric disorders and is currently a leading target for pharmacological manipulation for the treatment of various psychopathologies, is robustly regulated both in vitro and in vivo by miR-15a. Importantly, miR-15a is upregulated by pharmacological activation of the stress response in humans by dexamethasone treatment, as well as exposure to early adverse life events. Therefore, miR-15a might represent an important target for the treatment of stress-related psychopathologies.

Our results imply that, in the chronic stress response, miR-15a and its target FKBP51 represent major components for the following reasons: Although miR-15a is bioinformatically predicted to target other stress- and depression/anxiety-related transcripts, such as GILZ or Sgk1 (Anacker et al., 2013; Thiagarajah et al., 2014), the mRNA levels of these genes were unchanged in our Ago2 IP array (data not shown), supporting the specificity of the assay. In the present study, we focused exclusively on miR-15a regulation of FKBP51 due to the reported involvement of this gene in stress-response regulation and stress-linked psychopathologies (Hartmann et al., 2012, 2015; Scharf et al., 2011). Furthermore, FKBP51 mRNA was detected in the Ago2 complex, implicating a direct binding to the RNAi machinery. Finally, a significant decrease in FKBP51 levels was observed in the total RNA samples that concomitantly exhibited elevated miR-15a levels. Nevertheless, it is important to note that FKBP51 is not the only predicted target of miR-15a and that the changes observed in anxiety-like behavior after knocking down the levels of miR-15a in vivo are not mediated merely by affecting the levels of FKBP51.

Although BLA-specific overexpression of miR-15a resulted in no significant behavioral changes, knocking down miR-15a in the BLA caused an anxiogenic phenotype following exposure to chronic stress. The regulation of miR-15a following exposure to chronic stress and the observed anxiogenic phenotype in the BLA-miR-15a KD mice following chronic stress exposure may suggest that miR-15a is specifically involved in regulating the behavioral responses to repeated or chronic stressful exposure. Nevertheless, miR-15a may potentially be important also in regulating anxiety levels, regardless of the stress history of the mice, and its effect may be amplified by stress. The lack of an anxiolytic phenotype in the BLA-miR-15a-overexpressing mice could be explained either by lack of spatial specificity, meaning that the overexpression of miR-15a was not induced in endogenously relevant BLA neurons, or by a possible "ceiling effect," in which increasing levels of miR-15a on top of its endogenous stress-induced elevation is not effective because the stress response has already reached its full capacity. However, preventing the elevation of endogenous miR-15a in the BLA by its KD resulted in a failure of the mice to mount the required behavioral response when exposed to a chronic stressful challenge.

Finally, the elevation of miR-15a in two distinct human stress-linked scenarios—namely, administration of dexamethasone

Figure 5. Mice with Virally Mediated Reduced Levels of BLA-miR-15a Exhibit Increased Anxiety-like Behavior

(A–C) Results from the elevated plus maze (EPM) test of mice injected with miR-15a KD or control viruses (ns = 11 and 12, respectively) showing a tendency for differences, $F(3, 19) = 2.971$, $p = 0.058$. No significant differences were observed in the time spent in the open arms (A), the number of visits to the open arms (B), or the distance traveled in the open arms (C), according to Bonferroni correction for multiple testing. Data are represented as mean ± SEM.

(D–F) Mice injected with miR-15a KD or control viruses (ns = 10 and 9, respectively) that were also subjected to social defeat showed different behavior in the EPM, $F(3, 15) = 4.08$, $p = 0.026$; with a significant decrease in the time (D) ($U = 13$, $p = 0.009$) and distance (F) ($U = 7$, $p = 0.002$) spent in the open arms of the EPM (corrected according to Bonferroni correction for multiple testing). No changes were observed in the number of visits to the open arms (E). Data are represented as mean ± SEM.

(G) Representative tracking in the EPM of control mice (upper panel) relative to miR-15a KD mice (lower panel).

(H–M) No changes were observed in the locomotor activity and total distance traveled in the open field test between miR-15a KD and control mice under basal conditions (H–J) or following social defeat (K–M). Data in (I)–(J), (L), and (M) are represented as mean ± SEM.

*$p < 0.05$; **$p < 0.01$.

See also Figures S4 and S5.

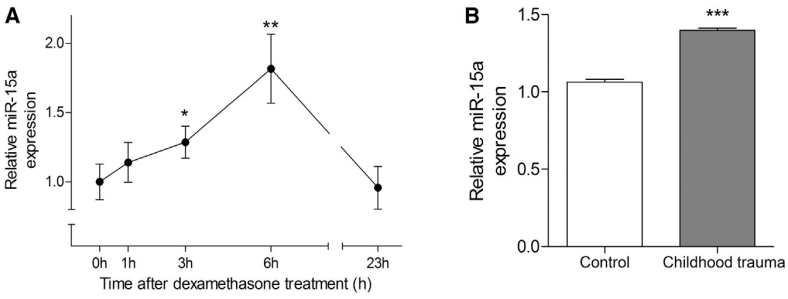

Figure 6. miR-15a Level in Human Peripheral Blood Is Higher following Acute Dexamethasone Treatment and Exposure to Childhood Trauma

(A) Relative miR-15a levels in peripheral blood cells of young healthy male subjects following 1.5 mg dexamethasone treatment. Repeated-measures ANOVA: $F(4, 22) = 4.42$, $p = 0.009$. *$p < 0.05$; **$p < 0.01$. A significant upregulation of miR-15a was observed 3 hr post-treatment, $t(25) = -2.240$, $p = 0.034$; and 6 hr post-treatment, $t(25) = -3.487$, $p = 0.002$.

(B) Levels of miR-15a in blood of subjects exposed to childhood trauma as compared to subjects not exposed, $t(29.715) = -13.776$, $p = 0.000$. Data are represented as mean ± SEM.

to healthy subjects as well as individuals exposed to childhood trauma—strongly suggests its involvement in human stress conditions. Collectively, the preclinical and human translational results presented in the present study strongly suggest that alterations in miR-15a levels are associated with the behavioral response to chronic or repeated stressful challenges and may be relevant in the pathogenesis of adverse life events and stress-linked psychiatric disorders such as anxiety. Targeting miR-15a levels might prove to be beneficial in the treatment of these conditions.

EXPERIMENTAL PROCEDURES

See also the Supplemental Experimental Procedures.

Chronic Social Defeat Stress

10-week old C57BL/6J male mice were subjected to a chronic social defeat stress protocol, as previously described (Krishnan et al., 2007). Briefly, the mice were placed randomly in a home cage of an aggressive ICR mouse and allowed to physically interact for 5 min. During this time, the ICR mouse attacked the intruder mouse and the intruder displayed subordinate posturing. A perforated clear Plexiglas divider was then placed between the animals, and they remained in the same cage for 24 hr to allow sensory contact. The procedure was then repeated with an unfamiliar ICR mouse for each of the next 10 days. The animal protocols were approved by the Institutional Animal Care and Use Committee (IACCU) of the Weizmann Institute of Science.

IP of Ago2 Protein, RNA Purification, and Microarray

Pools of three amygdalae taken from three mice from the same treatment group (either social defeat, n = 18, or control, n = 12) were immunoprecipitated using magnetic protein G beads (Dynabeads, Invitrogen/Life Technologies) and Ago2 monoclonal antibody (WAKO Chemicals).

RNA from the Ago2 IP samples was isolated and analyzed on an Affymetrix miRNA 2.0 array (enriched RNA protocol) and an Affymetrix Mouse Gene 1.0 ST array .

Cloning of 3′ UTRs into Psicheck2 Luciferase Expression Plasmid

The 3′ UTR sequence of FKBP51 was PCR amplified from mouse genomic DNA. This mutation replaced the first 4 nt in the miR-15a seed sequence of FKBP51.

Design, Construction, and Validation of miR-15a Lentiviruses

The miR-15a overexpression vector was cloned following the human synapsin promoter. The miR scramble control was purchased from GeneCopoeia. The H1-miR-15a sponge KD and its control were designed according to Lin et al. (2011).

Stereotactic Intracranial Injections

A computer-guided stereotaxic instrument and a motorized nanoinjector (Angle Two Stereotaxic Instrument, myNeuroLab, Leica Biosystems) were used as described previously (Elliott et al., 2010; Kuperman et al., 2010; Regev et al., 2012).

Behavioral Assessments

All behavioral assessments were performed during the dark (active) phase following habituation to the test room for 2 hr before each test.

Open-Field Test

The open-field test was performed in a 50 × 50 × 22-cm white box, lit to 120 lux. The mice were placed in the box for 10 min. Locomotion in the box was quantified using a video tracking system (VideoMot2; TSE Systems).

EPM Test

The apparatus in this test is designed as a plus sign and contains two barrier walls and two open arms. During the 5-min test, which is performed in relative darkness (6 lux), data are scored using a video tracking system (VideoMot2, TSE Systems).

Homecage Locomotion

Homecage locomotion was assessed using the InfraMot system (TSE Systems). Measurements of general locomotion consisted of two light and two dark cycles in the last 48 hr, collected at 10-min intervals.

Statistics

Data are expressed as mean ± SEM and were performed using the Statistical Package for the Social Sciences (SPSS) software.

Human Studies: Dexamethasone

Dexamethasone-unstimulated peripheral blood samples were drawn at 12:00 p.m. followed by oral administration of 1.5 mg dexamethasone. Subsequently, stimulated samples were collected at 1:00 p.m., 3:00 p.m., 6:00 p.m., and at 11:00 a.m. the following day.

ACCESSION NUMBERS

The accession numbers for the data reported in this paper are GEO: GSE87488, GSE87489, and GSE87533.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.038.

AUTHOR CONTRIBUTIONS

Conceptualization, N.V. and A. Chen; Methodology, N.V. and A. Chen; Investigation, N.V., J.C.P., M.E., A.S.Z., and N.C.; Writing – Original Draft, N.V., J.C.P., E.B.B., and A. Chen; Writing – Review and Editing, N.V., J.C.P.,

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Discussion

Due to limited efficacy of pharmacological interventions, pharmacotherapy of post-traumatic stress disorder remains a great challenge. Directly targeting players involved in the pathophysiology of the disorder is a possibly fruitful approach in novel drug development. As a regulator of the HPA axis, the CRF system represents a promising drug target in PTSD. Although antagonizing this system with CRF1 antagonists has shown very promising results in preclinical studies with animal models of stress related psychiatric disorders, clinical trials with large human cohorts have so far failed to show efficacy in the treatment of e.g. MDD or GAD. These contradictory results are likely based on the heterogeneity of the recruited patients in these trials. The pathophysiology of stress related psychiatric disorders including PTSD is a complex interplay between genetic and environmental factors. Different interactions between multiple genetic and environmental factors will affect different biological pathways resulting in distinct pathophysiological subtypes of PTSD, which consequently will respond differently to a certain treatment. Here, biological markers are of great value. Treatment response biomarkers can either serve as predictive markers to stratify patients into different subgroups of potential responders and non-responders to a targeted therapy or track an individual's response to a therapeutic intervention.

This thesis addresses the above statements by evaluating pharmacological CRF1 receptor antagonism in PTSD, identifying differentially responding subgroups of patients and examining potential DNA-methylation markers of treatment response in a cohort of PTSD-diagnosed women, treated with a CRF1 antagonist. Participants were assessed for genotypes and genome-wide DNA methylation from peripheral blood, as well as detailed information on stress-related phenotypes (Figure 2). Additionally, the presented work

intends to translate promising stress related microRNA findings from mice to human to possibly identify further epigenetic biomarkers in PTSD.

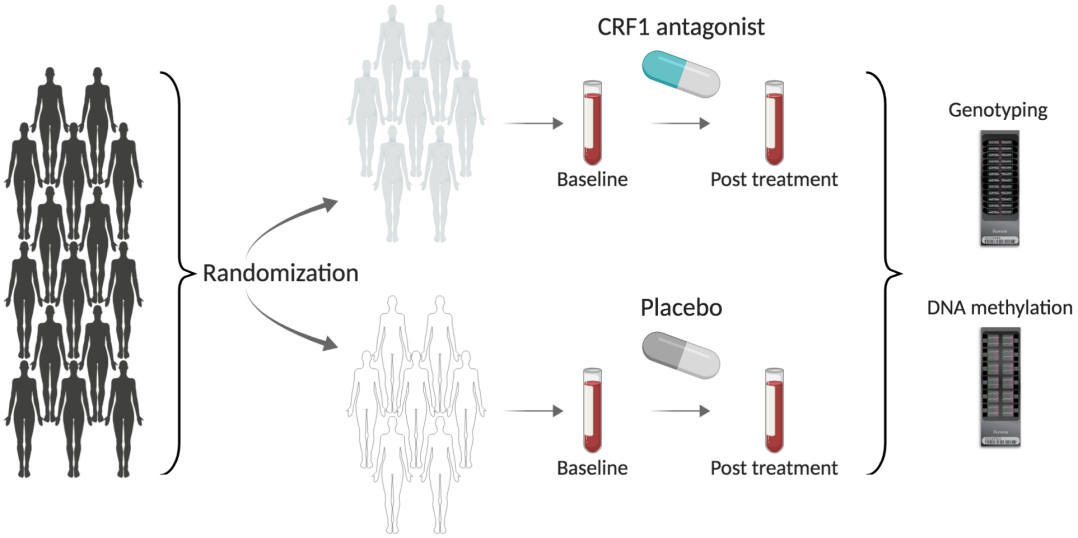

Figure 2: Schematic workflow regarding the placebo-controlled randomized clinical trial:
PTSD diagnosed women between 18 and 65 years of age were assessed for multiple stress-related psychological measures. After randomization, subjects were either treated with the CRF1 receptor antagonist or placebo. For biological assessments whole blood was collected at baseline and again after treatment. From all samples, DNA was extracted and measurement of genotypes as well as genome-wide DNA methylation analysis were performed.

Treatment effects of a CRF1 receptor antagonist in PTSD

In a first analysis, our collaborators investigated the effect of a novel CRF1 receptor antagonist (GSK561679) on PTSD symptom severity in a double-blind placebo controlled clinical trial. A cohort of currently untreated adult female patients with a diagnosis of posttraumatic stress disorder was treated with GSK561679 or placebo over six weeks. The underlying hypothesis was that the CRF1 receptor antagonist would show stronger PTSD symptom reduction than placebo.

This hypothesis was based on considerable evidence from preclinical and human studies (discussed in detail in the introduction). 1) A hyper-activated CRF system in animals leads to anxiety-related behavior. 2) Concentrations of CRF, the ligand of the CRF1 receptor, are elevated in PTSD patients. 3) CRF1 receptor antagonist in animal models have shown promising results for the treatment of stress related disorders.

However, the outcome of the study showed that there was no significant differential change in PTSD symptoms over treatment time between the antagonist and placebo. Therefore, the CRF1 receptor antagonist GSK561679 was not superior over placebo in the treatment of PTSD.

One of several possible explanations for the diverse results between animal and human studies may be the inadequate central nervous system penetration as well as insufficient receptor occupancy of the antagonist in humans. In animals, ex vivo studies assessing receptor occupancy can partially address and answer these questions (Kehne and Cain, 2010). In humans, finding the right dosage of the CRF1 receptor antagonist, to reach adequate CRF1 receptor occupancy and by that, achieve efficacy, remains difficult. To fully evaluate CRF1 receptor occupancy in the human brain an appropriate, high affinity positron emission tomography (PET) ligand will be required. Despite the identification of

several promising ligand candidates, so far none of them has shown efficacy for imaging brain CRF1 receptors in vivo (Lodge et al., 2014, Sanders and Nemeroff, 2016).

Another potential reason for the insufficient treatment effects of CRF1 receptor antagonists in humans, particularly addressing the poor translation of promising preclinical research, is the time point the CRF system is targeted by the antagonist. Most animal studies in this context examine short-term stress exposure and immediate drug exposure right after the external stressor. In contrast, clinical trials testing the efficacy of CRF1 receptor antagonists in humans mainly assess adult patients with stress related disorders that might suffer from these conditions for significantly longer periods of time. In these patients the CRF1 system might have undergone long term and profound changes on the circuit level and may not be sensitive to external modulation by CRF receptor antagonists anymore. Therefore, administration of a CRF1 receptor antagonist right after exposure to a traumatic event might hold the potential to prevent the development of chronic PTSD. However, to clearly answer this question further clinical trials specifically testing this model are necessary (Kehne and Cain, 2010).

A possible bidirectional role of CRF in stress related psychiatric disorders, represents another explanation for the ineffectiveness of the CRF1 receptor antagonist. Increasing evidence suggests that the effect of the CRF system on behavioral responses to stress is not only brain region-specific, but even dependent of the neurotransmitter-specific neuronal circuit. By specifically deleting the *CRHR1* gene in different neuronal subtypes Refojo and colleagues revealed differential behavioural effects in mice. While animals lacking *CRHR1* in glutamatergic neurons of the forebrain showed reduced anxiety-like behavior, *CRHR1* knock out in midbrain dopaminergic neurons resulted in anxiogenic effects (Refojo et al., 2011). In line with these findings Dedic and colleagues demonstrated another distinct CRF/CRF1 neuronal circuit responsible for specific behavioral effects.

Chronic CRF depletion from GABAergic projection neurons of the extended amygdala leads to anxiolytic behavior in mice (Dedic et al., 2018). These findings suggest different CRF/CRF1 circuits as antagonistic modulators of the physiological stress response. Therefore, simultaneously antagonizing CRF1 in these different neurotransmitter-specific neuronal subpopulations might have a neutralizing effect attenuating the efficacy of the applied compound.

Last, the failure of the evaluated drug can be explained by differential treatment response of different subgroups of patients. Particularly relevant to our study is a possible sex-specific response to CRF1 receptor antagonists. This has been shown in animal studies. After CRF1 receptor antagonist infusion into the dorsal raphe, male mice demonstrated strong HPA axis and behavioral responses compared to female animals, which only show very limited changes (Howerton et. al., 2014). However, due to male-specific side effects of GSK561679 only women could be included in the study and treatment effects of GSK561679 on male patients were not tested.

Distinct biological subgroups of patients show differential response to CRF1 receptor antagonist treatment

In a second analysis, we aimed to identify more distinct biological subgroups of patients that show differential response to CRF1 receptor antagonist treatment.

As discussed above, the issue of adequate patient selection represents another potential contributing factor to the failure of CRF1 receptor antagonist treatment in stress related psychiatric disorders. CRF1 receptor antagonists might be particularly relevant for certain subpopulations of patients with distinct biological backgrounds. Several authors suggest that individuals with an enhanced central CRF system represent one of these

subgroups and might benefit most of the drug (Griebel and Holsboer, 2012; Kehne and Cain, 2010; Sanders and Nemeroff, 2016; Spierling and Zorrilla, 2017). We therefore specifically investigated treatment response of subsets of patients with probable differential CRF system activity. More specifically, subjects were stratified according to childhood trauma status and their genotype of the *CRHR1* SNP rs110402.

Our results showed that patients who had experienced child abuse and were homozygous for the rs110402 GG allele exhibited significantly stronger PTSD symptom reduction than other patients, after CRF1 receptor antagonist treatment. Interestingly, particularly these individuals with a history of early trauma and genetic variants in the CRF1 receptor gene (*CRHR1*) are hypothesized to show central CRF system overactivation.

Multiple studies, in both animal and human, have shown long term effects of early life trauma on central CRF system activity. Rats for instance, that experienced postnatal maternal separation, showed elevated CRF concentrations as well as increased CRF mRNA in adulthood. (Ladd et al., 2000; Plotsky and Meaney, 1993; Plotsky et al., 2005). Similar results have been described in adult nonhuman primates. Monkeys that have been exposed to adverse early life rearing conditions exhibited a persistent increase of cerebrospinal fluid concentrations of corticotropin-releasing factor (Coplan et al., 1996 and 2001). Also in humans findings point in the same direction. Several studies have revealed a strong correlation between early adverse life events and CRF system activity. Work by Carpenter and colleagues demonstrated early life adversity to be a significant predictor of CRF concentrations in cerebrospinal fluid in depressed patients (Carpenter et al., 2004). Further, Lee and colleagues showed a positive correlation between CSF CRF levels and the total score on the Childhood Trauma Questionnaire as well as a negative correlation between parental care and CSF CRF concentrations in a cohort of patients with personality disorder (Lee et al., 2005 and 2006).

Genetic variants in the CRF receptor 1 gene have also been reported to associate with neuroendocrine alterations including central CRF system activity. (Chichetti et al, 2011; Heim et al, 2009; Mahon et al, 2013; Sumner et al, 2014; Tyrka et al, 2009). By using the combined dexamethasone/CRF challenge test studies by Tyrka and colleagues as well as Heim and colleagues specifically examined the functional effects of *CRHR1* SNP rs110402, childhood maltreatment and the interaction of the two. Findings showed that both, the rs110402 GG genotype itself (Heim et al., 2008) as well as the interaction with early life trauma (Tyrka et al., 2009), was significantly associated with an enhanced cortisol response in the DEX/CRF test, which has been linked to central CRF system hyperactivity (Dunlop and Wong, 2018; Griebel and Holsboer, 2012).

Taken together, these data, showing an elevated activity of the CRF system in individuals with a history of early trauma as well as in rs110402 GG genotype carriers, suggest a probable CRF system overactivity in individuals with a combination of the two.

Therefore, as previously mentioned, several authors have hypothesized that CRF1 receptor antagonism might be particularly relevant for this biological subgroup. Our findings strongly support this hypothesis by showing significantly better response to CRF1 receptor antagonist treatment in this specific subset of patients. However, the number of patients is small, specifically after subgrouping, and larger sample sizes will be required to entirely establish clinical relevance of theses findings.

Potential CRF1 receptor antagonist treatment markers in PTSD

Gene by environment interactions predicting treatment response

Precision medicine, tailoring the optimal therapy to each individual patient remains a challenging task in stress related psychiatric disorders such as PTSD. In this context, treatment markers represent a helpful tool. They can either serve as stratification markers to subgroup patients matching them to the most effective treatment or as tracking markers to monitor effectiveness of a specific therapeutic intervention.

As discussed above our results suggest that CRF receptor antagonists might be particularly effective in a subset of PTSD patients, showing a history of childhood trauma and being carriers of the GG genotype of the *CRHR1* SNP rs110402. These specific traits therefore have the potential to serve as predictive markers stratifying patients into subgroups of possible responders and non-responders prior to CRF receptor antagonist treatment.

Compared to other stress-related psychiatric disorders like MDD, studies investigating gene by environment interactions in the prediction of treatment response are rare in PTSD. In fact, this is, to our knowledge, the first study looking at G x E interaction effects on therapeutic response after CRF1 receptor antagonist treatment. Due to the complex pathophysiology of stress related disorders, that consists of complex interactions of genetic and environmental factors, G x E interactions studies represent a promising approach in the prediction of treatment response (Klengel and Binder, 2013). However, even in the field of MDD where this concept has been widely studied and multiple studies have shown promising results, no clinically applicable treatment marker has been

developed so far (Keers et al., 2011; Mandelli et al., 2009, Xu et al., 2011 and 2012). Therefore, large prospective studies specifically exploring similar or the same environmental measures are desperately needed to fully establish suitable stratification markers for stress related psychiatric disorders (Klengel and Binder, 2013).

Epigenetic modifications as potential stratification and tracking markers

CRHR1 methylation levels as tracking marker for CRF1 antagonist treatment in PTSD?

As highlighted in the introduction epigenetics represent another layer in the complex pathophysiology of stress related psychiatric disorders. Modifications of the epigenome are candidate mechanisms in mediating both, short term and long-lasting effects of the environment on DNA function without altering the underlying genetic code (Klengel et al., 2014; Zannas et al., 2016). Due to their decisive role in the pathogenesis and the fact that they are accurately and reproducibly measurable across individuals, epigenetic alterations hold considerable promise as biomarkers in PTSD (Voyias et al., 2016).

In our previous analysis, we identified a distinct subset of PTSD patients possibly benefitting more from CRF1 receptor antagonist treatment than others. As discussed above, treatment biomarkers can help stratifying patients into specific subgroups of differential treatment response, but they can further help to monitor the course of the disorder under the given treatment intervention. Epigenetic markers are of special interest since they can serve as both, treatment stratification markers but also, due to their potentially dynamic nature, as tracking markers (Klengel et al., 2014, Wu and Zhang,

2014). Compared to, for example, genetic marks, which are persistent and therefore suitable as trait markers, epigenetic marks hold the potential to change over time, therefore reflecting the current state of the disease (state markers) (Hacimusalar and Eşel, 2018). We were therefore particularly interested whether certain epigenetic modifications would be associated with PTSD symptom change after CRF1 receptor antagonist treatment in our previously identified subgroup of responsive patients. Due to the fact that the drug targets the CRF1 receptor, we specifically focused our analysis on methylation levels of the *CRHR1* gene. Our findings demonstrated significantly different changes of *CRHR1* methylation levels in our distinct subgroup over treatment time compared to the other patients. These individuals were the only ones showing increased *CRHR1* methylation from pre- to post-treatment, while all other subjects either showed no change or a reduction in methylation over the six weeks. Notably this effect was not observed in the placebo group. Such specific methylation changes over treatment time hold the potential to track treatment response to a given therapeutic intervention.

NR3C1 and *FKBP5* methylation levels as treatment markers independent of type of therapy?

Studies investigating epigenetic biomarkers of treatment response in PTSD are extremely rare. In fact, work by Yehuda and colleagues from 2014 represents the first and so far only study in this context. Here, the authors aimed to determine whether methylation levels of *NR3C1* and *FKBP5* would predict or associate with treatment outcome in a small cohort of combat veterans diagnosed with PTSD. The therapeutic intervention applied to the patients in this study was prolonged exposure psychotherapy over twelve consecutive weeks. Findings included that pre-treatment *NR3C1* methylation levels significantly

predicted treatment outcome. More specifically, higher *NR3C1* methylation at baseline correlated with better response to twelve weeks of psychotherapy. The authors further observed a decrease in *FKBP5* methylation associating with better treatment outcome (Yehuda et al., 2013). In summary this study suggests *NR3C1* and *FKBP5* methylation levels as potential markers to subgroup patients as well as monitor the course of treatment using psychotherapy in PTSD.

In our final analysis we were therefore specifically interested whether epigenetic marks of these same loci would also serve as stratification or tracking markers for pharmacological treatment interventions in PTSD. More specifically we examined whether peripheral blood-based DNA methylation levels of *NR3C1* and *FKBP5* are associated with treatment response after CRF1 receptor antagonist treatment in our cohort of PTSD diagnosed women. In contrast to Yehuda and colleagues, we found no association between *FKBP5* methylation levels and CRF1 antagonist treatment outcome in our analysis. For *NR3C1* our findings went in line with the results from Yehuda's previously discussed study. Higher baseline methylation of *NR3C1* was associated with better treatment response to the antagonist. Interestingly this effect was only observed in patients with early trauma. No correlation was found between change in *NR3C1* methylation over treatment and symptom improvement. Taken together, these results suggest NR3C1 baseline methylation as a potential stratification marker independent of the type of treatment.

Potential mechanisms for the observed differences in DNA methylation levels

One possible mechanism inducing these methylation changes is thought to be transcription factor mediated (Kirillov et al., 1996; Feldmann et al., 2013; Weaver et al., 2007). A specifically interesting transcription factor, in the context of stress related

disorders, is the glucocorticoid receptor (GR). We and others have previously shown that glucocorticoid-induced activation of the GR can lead to dynamic changes in DNA methylation (Thomassin et al., 2001; Wiechmann et al., 2019; Wiench et al., 2011). Therefore, differences in GR activity may result in different methylation levels. Dysregulation of the HPA axis plays a central role in the pathogenesis of PTSD. Several studies have shown PTSD-typical HPA axis abnormalities including enhanced activity of the CRF system, alterations in baseline cortisol levels and hypersensitivity of the GR. However, these differences in HPA axis activity are not exclusively relevant between PTSD patients vs. healthy controls but also play a role between different biological subgroups of PTSD, which might explain differences in *NR3C1* and *FKBP5* baseline methylation levels correlated with differential CRF1 receptor antagonist treatment response within our cohort.

Further, pharmacological modification of HPA axis activity might result in dynamic DNA methylation changes. Here, biological subgroups with distinct HPA axis abnormalities might show different neuroendocrine alterations in response to treatment possibly reflected by differential changes in DNA methylation levels. Our previously identified subgroup of patients, who had experienced child abuse and were homozygous for the rs110402 GG allele, has been associated with a specific HPA axis profile in several studies, namely a combination of increased CRF activity and GR sensitivity. This highly active system might be particularly sensitive to CRF1 receptor antagonist treatment. Many studies have shown that GR supersensitivity in PTSD normalizes with symptom improvement after therapy. GR sensitivity normalization in the given subgroup after CRF1 receptor antagonist treatment may result in GR mediated methylation changes.

Due to high tissue and cell type specificity of DNA methylation patterns, cell composition effects may be responsible for an observed epigenetic signal (Breeze et al., 2016; Byun et

al., 2009; Ghosh et al., 2010; Kozlenkov et al., 2016; Varley et al., 2013; Zhang et al., 2013). Therefore, change in cell type distribution, e.g. immune cells, represents another potential contributor to DNA methylation differences in our sample. Increasing evidence suggests a close relationship between PTSD and biological alterations in the immune system. Multiple studies describe imbalances towards a pro-inflammatory state including a shift in immune cell composition in patients suffering from PTSD (Aiello et al., 2016; Jergović et al., 2014; Morath et al., 2014; Wang and Young, 2016; Zhou et al., 2014).

Further, there are several reports about a possible association between PTSD symptom recovery and normalization of immune function (Gill et al., 2013; Morath et al., 2014; Tucker et al., 2004). Particularly interesting in the context of the current work is a study by Morath and colleagues. The authors were able to demonstrate a normalization of originally reduced regulatory T cells correlating with symptom improvement in PTSD patients after twelve sessions of narrative exposure therapy (Morath et al., 2014). Such a shift in immune cell composition after CRF1 receptor antagonist treatment could possibly drive the observed methylation effects in our defined biological subgroup. To control for this potential confounding factor, we used a reference-based deconvolution method for blood cells. After estimating the proportions of the underlying immune cell types, they were included as covariates (Houseman et al., 2012; Teschendorff and Zheng, 2017). However, this algorithm only estimates cell proportions of six main cell types whereas subtypes are not included and therefore remain potential confounders of DNA methylation analyses.

A direct effect of the CRF1 receptor antagonist on epigenetic mechanisms is another potential explanation for the observed findings. Increasing evidence suggests that psychotropic substances partially act through epigenetic effects. Modulation of epigenetic machinery, resulting in DNA methylation changes, has been shown for a number of

antidepressant drugs (Lisoway et al., 2018). Paroxetine, and different tricyclic antidepressants, for example have been demonstrated to alter methylation levels by inhibiting DNA methyltransferase I (DNMT1) in rat astrocytes (Perisic et al., 2010; Zimmermann et al., 2012). However, due to significant pharmacodynamic differences between these antidepressants and CRF1 receptor antagonists, a direct epigenetic effect can only be hypothesized and future pharmaco-epigentic studies are needed to strengthen this assumption.

A general challenge of methylation studies in stress related-psychiatric disorders is the issue of cross tissue correlation. Given the difficulty of assessing human brain samples, which represent the tissue of interest in psychiatric studies, epigenetic analyses in these studies are often limited to peripheral tissues like blood or saliva. The question remains whether stress-induced DNA methylation changes correlate across different types of tissue throughout the organism and to what extent methylation levels of e.g. blood can be used as appropriate markers for the brain (Klengel et al., 2014; Klengel and Binder, 2015; Pape and Binder; 2014). Over the last years, there have been some very interesting reports about cross tissue effects from blood to brain which suggest at least some specific methylation changes/signaling pathways in the brain to be reflected in peripheral blood samples (Bakulski et al., 2016; Klengel et al., 2013; Provencal et al., 2012).

Despite these promising results, multiple epigenome wide studies have shown highly tissue-specific DNA methylation profiles, specifically in brain and blood, with a very limited overlap of CpG sites showing similar methylation patterns (Lokk et al., 2014; Walton et al., 2016). These data clearly suggest, that no general conclusions should be drawn from peripheral methylation changes to pathomechanisms in the brain. Here, further studies specifically examining the relation of epigenetic signatures between

peripheral tissue and brain are of great need. Nonetheless, these statements should not diminish the potential of using peripheral tissue to identify epigenetic modifications as biomarkers for stress-related psychiatric disorders. To serve as a suitable biomarker, it is irrelevant whether these epigenetic changes merely depict disease-associated modifications, independent of the disorder's pathophysiology or actually mirror PTSD-specific epigenetic changes in the brain. (Zannas et al., 2015)

MicroRNA-15a as a potential future biomarker in PTSD?

MicroRNAs represent another epigenetic mechanism with high biomarker potential in stress related disorders like PTSD (Issler and Chen, 2015). As mentioned in the introduction, the number of studies published in this line of research is still limited and so far only a few studies have been completed in humans. Our group identified miR-15a to be differentially regulated in mice after stress exposure. More specifically, animals that were subjected to chronic social defeat demonstrated significantly increased miR-15a levels in the amygdala. Simultaneously it's predicted target *FKBP5* showed decreased mRNA levels suggesting miR-15a as a direct regulator of FKBP5. Moreover, knockdown of miR-15a in the amygdala affected the animals' stress response, resulting in increased anxiety-like behavior following stress exposure. These results strongly suggest an involvement of miR-15a in stress reactivity and by that possibly in PTSD and other stress related disorders. As a next step, we were therefore specifically interested whether these findings were translatable to humans. To examine its potential role in the human stress response, we analyzed miR-15a expression levels in peripheral blood of young healthy male subjects after dexamethasone treatment. We saw a significant up-regulation of miR-15a after three hours and a further increase six hours post treatment. A second analysis

aimed to examine the effect of early life stress on miR-15a levels. Therefore, microRNA expression analysis in peripheral blood was carried out in a cohort of 20 subjects exposed to childhood trauma and 20 controls without early life stress. Results revealed significantly elevated miR-15a expression levels in patients with a history of early life trauma compared to controls.

These translational findings strongly suggest miR-15a's involvement in the human stress response. Further, the fact that altered miR-15a levels in human peripheral blood are present a few hours, but also up to several years later, makes this particular microRNA a promising biomarker candidate in stress related psychiatric disorders like PTSD.

Concluding remark

This cumulative thesis addresses the ongoing challenge of appropriate treatment in stress related disorders like PTSD. To overcome this current therapeutic stagnation new treatment concepts are desperately needed. The established psychiatric diagnostic classification systems lack objectivity, being based on clinical evaluation instead of considering the underlying psychobiology of the given disorder. Future therapeutic strategies should focus on individualized treatment, matching the right therapy to the right patient, by using objective measures such as genetics or blood-based biomarkers. By identifying specific biological subgroups showing differential treatment response to a CRF1 antagonist in female PTSD patients this thesis emphasizes the importance of precision medicine in this context (Figure 3). The presented work further highlights the potential use of epigenetic changes in these disorders as e.g. diagnostic or treatment prediction biomarkers. While these findings strengthen the promising concept of precision medicine, they need to be validated in future studies using much larger sample

sizes. Further, given the multifactorial etiology and the complexity of the underlying pathophysiology of psychiatric disorders it is most likely that clinically applicable markers will be an index, comprised of several combination markers. Therefore, future studies in this field should take into account different kinds of moderators associated with a given psychiatric disorder like genetics, epigenetics, neuroimaging as well as clinical features.

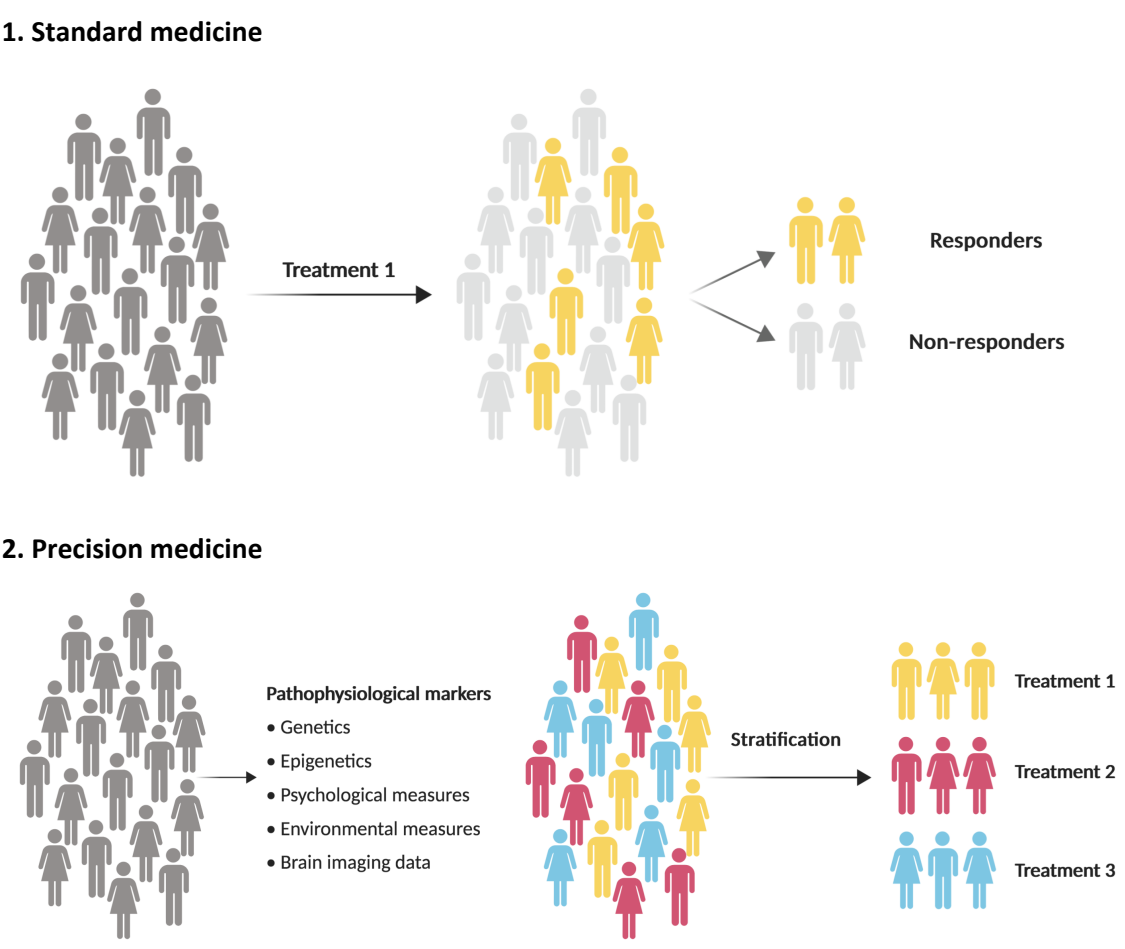

Figure 3: Representation of the standard treatment approach (trial and error) vs. precision medicine
1. According to their psychiatric diagnosis (using the established psychiatric diagnostic classification systems ICD-10 or DSM-5) patients are treated with the respective first line treatment. Usually only a subset of patients will show beneficial treatment outcome. For nonresponder an often lengthy trial and error process of treatment follows.
2. Precision medicine aims to tailor the optimal therapy to each individual patient. Using pathophysiological markers like genetics, epigenetics, psychological measures etc. patients are stratified in differential biological subgroups and individual therapies can be applied. (Menke et al., 2018)

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DNA methylation levels are associated with CRF1 receptor antagonist treatment outcome in women with post-traumatic stress disorder

Supplemental Information

Figure S1

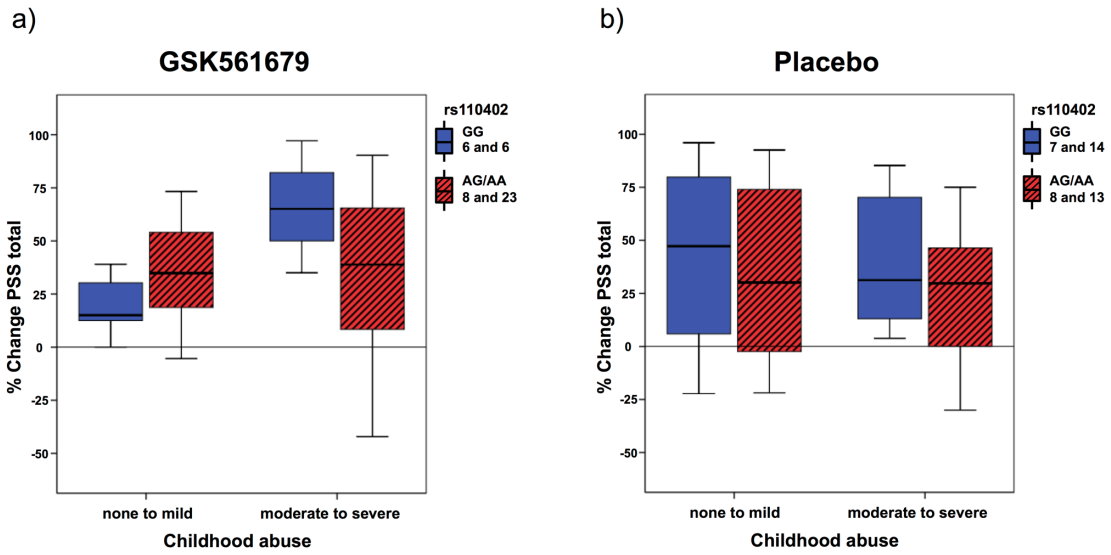

Figure S1 The boxplots describe the mean % change of PSS total score in abused and non-abused patients treated with the CRHR1 antagonist or placebo. GG carriers are shown in blue (plain boxes) and AA/AG in red (striped boxes). rs110402 A carrier status by childhood abuse exposure showed a significant interaction effect on PSS score % change over treatment in subjects treated with the CRHR1 antagonist (n=43; $F(1, 31)=4.42$; $p=0.043$) **(a)** but not in subjects treated with placebo (n=42, $p>0.05$) **(b)**. rs110402 GG carriers exposed to child abuse displayed the highest % change of PSS symptoms following CRHR1 treatment. (From Biological Psychiatry; Dunlop et al., 2017)

Table S1. CRHR1: List of CpGs used for analysis

CpG	Chr.	Genomic location (hg19)
cg18090064	17	43716542
cg04194664	17	43716618
cg16228356	17	43848958
cg08929103	17	43860356
cg04856689	17	43862033
cg24063856	17	43863304
cg13947929	17	43863356
cg27410679	17	43866279
cg16642545	17	43878770
cg00022871	17	43884359
cg00025823	17	43909151

Table S2. NR3C1: List of CpGs used for analysis

CpG	Chr.	Genomic location (hg19)
cg17860381	5	142783570
cg04111177	5	142783608
cg15910486	5	142783621
cg15645634	5	142783639
cg18068240	5	142783844

Table S3. FKBP5: List of CpGs used for analysis

CpG	Chr.	Genomic location (hg19)
cg16012111	6	35656758
cg07843056	6	35656848
cg01294490	6	35656906

Corticotropin-Releasing Factor Type 1 Receptor Antagonism Is Ineffective for Women with Posttraumatic Stress Disorder

Supplemental Information

SUPPLEMENTARY TEXT

DNA extraction and genotyping

DNA isolation from whole EDTA blood was performed with a magnetic bead based technology using the PerkinElmer Chemagic 360 extraction robot. Quality and quantity of the extracted DNA was assessed using the EpochMicroplate Spectrophotometer (BioTek).We excluded relatives of individual subjects from the whole sample (n = 3, Pi_Hat ≥ 0.0625) based on mean identity by descent in PLINK (1). For the genome wide analyses referring the population stratification, we only included individuals with a sample-wise call rate ≥ 0.98 and SNPs with call rate ≥ 0.98, Hardy Weinberg equilibrium test (HWE) p-value ≥ 1×10^{-5} and MAF ≥ 0.05, allowing for a total of 575,455 markers in 86 individuals. To correct for population stratification in an ethnically mixed sample, principal components (PC) for genetic background were calculated from all genotypes for each of the individuals using Genome-wide Complex Trait Analysis (**Figure S8**).

SUPPLEMENTARY TABLES

Table S1. CONSORT Checklist

Section/Topic	Item No	Checklist item	Reported on page No.
Title and abstract			
	1a	Identification as a randomised trial in the title	Prevented by character limit
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2-3
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4-6
	2b	Specific objectives or hypotheses	5-6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	n/a
Participants	4a	Eligibility criteria for participants	6-7
	4b	Settings and locations where the data were collected	6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	8-9
	6b	Any changes to trial outcomes after the trial commenced, with reasons	n/a

Sample size	7a	How sample size was determined	Ref. 34
	7b	When applicable, explanation of any interim analyses and stopping guidelines	n/a
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	7
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	7
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	7
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	7
	11b	If relevant, description of the similarity of interventions	n/a
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	8-9
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	9
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	10
	13b	For each group, losses and exclusions after randomisation, together with reasons	Fig S1
Recruitment	14a	Dates defining the periods of recruitment and follow-up	6
	14b	Why the trial ended or was stopped	n/a
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	27

Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	10
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	10-11
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	10
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	11-13
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	13, Tables S3,S4
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	14-17
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	14-17
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	14-17
Other information			
Registration	23	Registration number and name of trial registry	6
Protocol	24	Where the full trial protocol can be accessed, if available	6
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	17-18

Table S2. Ethnicity and allele frequency of CRF1 SNP rs110402

Race	GG	AG/AA	Total
White	18	33	51
Black	14	14	28
Other	1	6	7
Total	33	53	86

Table S3. Spontaneously reported adverse events

Adverse Event	GSK561679	Placebo	Total
Headache	25	24	49
Nausea	19	11	30
Insomnia	6	11	17
Diarrhea	6	9	15
Upper Resp. Tract Infection	8	7	15
Sedation	5	8	13
Dizziness	7	4	11
Rash	2	8	10
Vomiting	4	6	10
Dyspepsia	4	5	9
Constipation	2	5	7
Dry Mouth	5	2	7
Irritability	3	4	7
Pruritis	4	3	7
Abdominal Pain	1	5	6
Arthralgia	5	1	6
Cough	2	4	6
Depression Worsening	2	3	5
Neck Pain	3	2	5
Rhinitis Allergic	2	3	5
Sinusitis	1	4	5
Vision Blurred	2	3	5
Contusion	0	4	4
Disturbance in Attention	1	3	4
Hypersensitivity	1	3	4
Migraine	3	1	4
Muscle spasm	1	3	4
Myalgia	1	3	4
Palpitations	2	2	4
Abdominal Distension	0	3	3
Flatulence	0	3	3
Hot Flush	0	3	3
Non-Cardiac Chest Pain	0	3	3
Oropharyngeal Pain	0	3	3
Tinnitus	3	0	3

All $p > .05$

Table S4. Patient Rated Inventory of Side Effects (PRISE) symptom counts

Symptom	Placebo n (%)	GSK561679 n (%)	p-value
Anxiety	50 (77)	54 (86)	p < 0.29
Blurred Vision	21 (32)	15 (24)	p < 0.38
Chest Pain	13 (2)	9 (14)	p < 0.53
Constipation	16 (25)	17 (27)	p < 0.92
Decreased Energy	54 (83)	51 (81)	p < 0.93
Diarrhea	20 (31)	22 (35)	p < 0.62
Difficulty Sleeping	59 (91)	58 (92)	p < 0.99
Difficulty Urinating	1 (2)	0 (0)	p < 0.99
Dizziness	31 (47)	24 (38)	p < 0.27
Dizziness on Standing	23 (35)	19 (3)	p < 0.66
Dry Mouth	22 (34)	22 (35)	p > 0.99
Dry Skin	29 (45)	25 (4)	p < 0.70
Fatigue	55 (85)	47 (75)	p < 0.15
Frequent Urination	21 (32)	16 (25)	p < 0.50
General Malaise	25 (38)	22 (35)	p < 0.82
Headache	51 (78)	45 (71)	p < 0.47
Blurred Vision	21 (32)	15 (24)	p < 0.38
Chest Pain	13 (2)	9 (14)	p < 0.53
Constipation	16 (25)	17 (27)	p < 0.92
Decreased Energy	54 (83)	51 (81)	p < 0.93
Diarrhea	20 (31)	22 (35)	p < 0.62
Difficulty Sleeping	59 (91)	58 (92)	p < 0.99
Difficulty Urinating	1 (2)	0 (0)	p < 0.99
Increased Perspiration	19 (29)	13 (21)	p < 0.36
Itching	28 (43)	27 (43)	p > 0.99
Loss of Sexual Desire	28 (43)	32 (51)	p < 0.49
Menstrual Irregularity	11 (17)	9 (14)	p < 0.87
Nausea/Vomiting	26 (4)	28 (44)	p < 0.74
Painful Urination	6 (9)	1 (2)	p < 0.11
Palpitation	18 (28)	20 (32)	p < 0.76
Poor Concentration	53 (82)	53 (84)	p < 0.88
Poor Coordination	16 (25)	17 (27)	p < 0.76
Rash	9 (14)	8 (13)	p > 0.99
Restlessness	42 (65)	40 (63)	p > 0.99
Ringing in Ears	18 (28)	15 (24)	p < 0.76
Sleeping Too Much	19 (29)	11 (17)	p < 0.17
Tremors	11 (17)	2 (3)	**p < 0.02**
Trouble Achieving Orgasm	11 (17)	7 (11)	p < 0.45

Bolded value is p<.05

SUPPLEMENTARY FIGURES

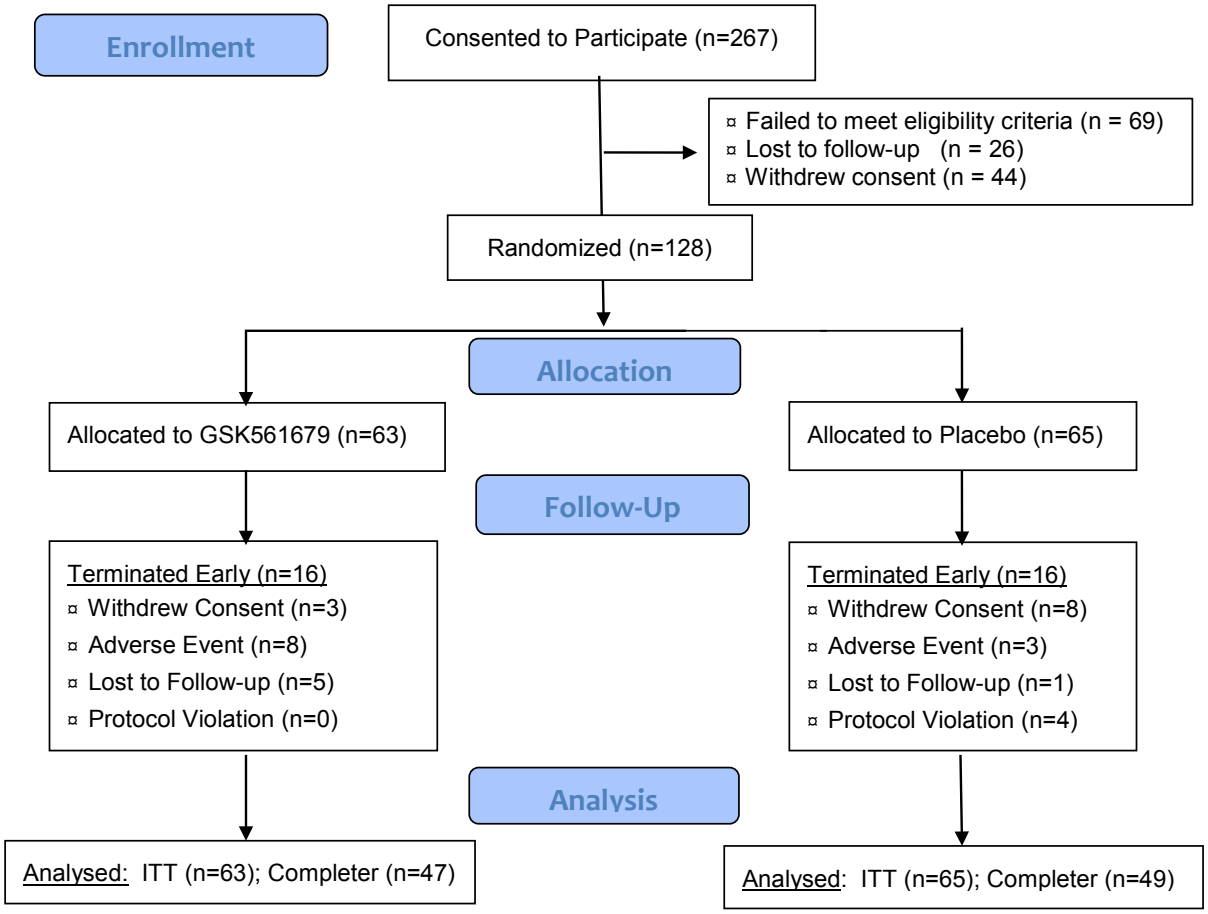

Figure S1: CONSORT flow diagram

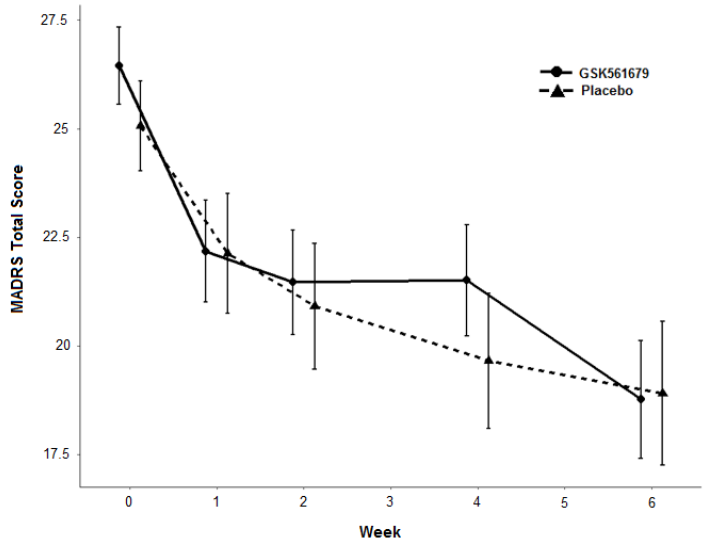

Figure S2: Change in MADRS scores over time
S.E. bars represent ± 1 S.E.

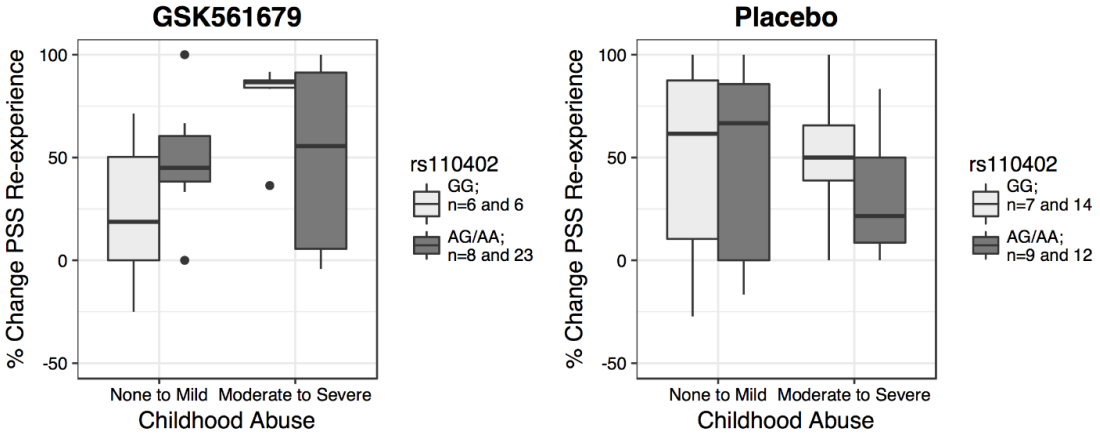

Figure S3: Significant interaction effect of rs110402 and childhood abuse on percent change in PSS re-experiencing score
The boxplots describe the mean % change of PSS re-experiencing score in abused and non-abused patients treated with GSK561679 or placebo. GG carriers are shown in light grey and AA/AG in dark grey. Black dots indicate outliers. rs110402 A carrier status by childhood abuse exposure showed a significant interaction effect on PSS re-experiencing score % change over treatment in subjects treated with GSK561679 (-ß=-2.472; p=0.006) but not in subjects treated with placebo (ß=-0.075; p=0.92). rs110402 GG carriers exposed to child abuse displayed the highest % change of PSS symptoms following GSK561679 treatment.

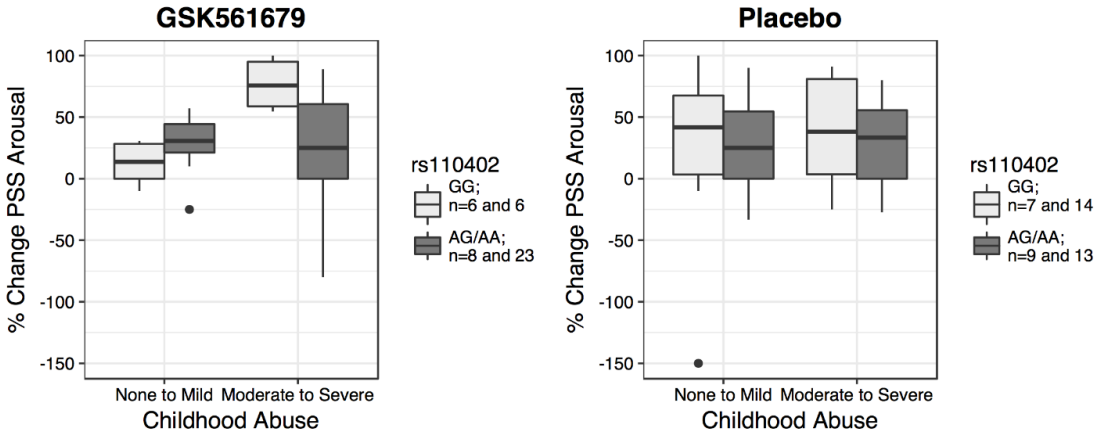

Figure S4: Significant interaction effect of rs110402 and childhood abuse on percent change in PSS arousal score

The boxplots describe the mean % change of PSS arousal score in abused and non-abused patients treated with GSK561679 or placebo. GG carriers are shown in light grey and AA/AG in dark grey. Black dots indicate outliers. rs110402 A carrier status by childhood abuse exposure showed a significant interaction effect on PSS arousal score % change over treatment in subjects treated with the GSK561679 (ß=-2.034; p= 0.019) but not in subjects treated with placebo (ß=0.054; p=0.94). rs110402 GG carriers exposed to child abuse displayed the highest % change of PSS symptoms following GSK561679 treatment.

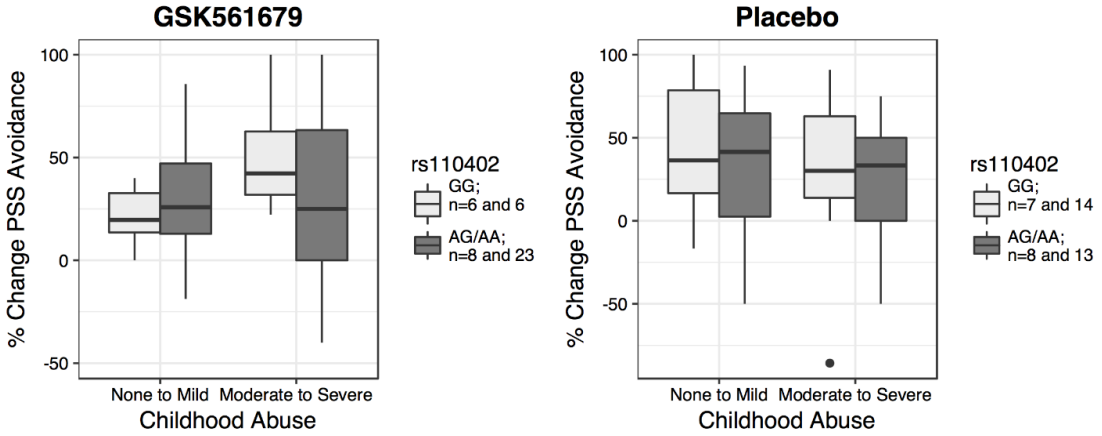

Figure S5: Lack of interaction effect of rs110402 and childhood abuse on percent change in PSS avoidance score

The boxplots describe the mean % change of PSS avoidance score in abused and non-abused patients treated with GSK561679 or placebo. GG carriers are shown in light grey and AA/AG in dark grey. Black dots indicate outliers. rs110402 A carrier status by childhood abuse exposure showed no significant interaction effect on PSS avoidance score % change over treatment in subjects treated with either GSK561679 (ß=-0.945; p=0.36) or placebo (ß=0.565; p=0.44).

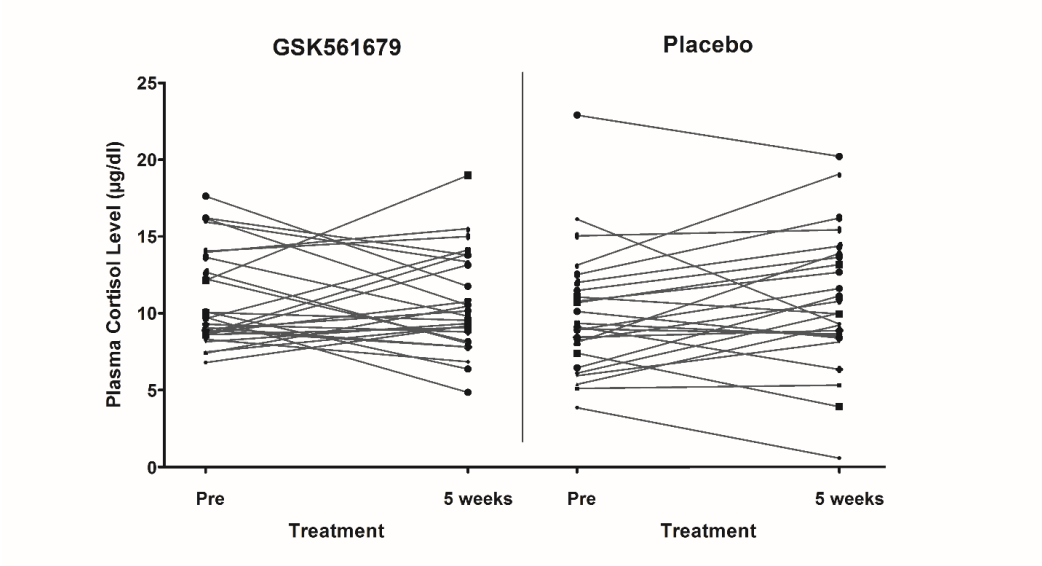

Figure S6. Lack of effect of GSK561679 and placebo on morning cortisol
Non-significant change in morning cortisol from baseline to week 5 between patients treated with GSK561679 or placebo ($p<.05$).

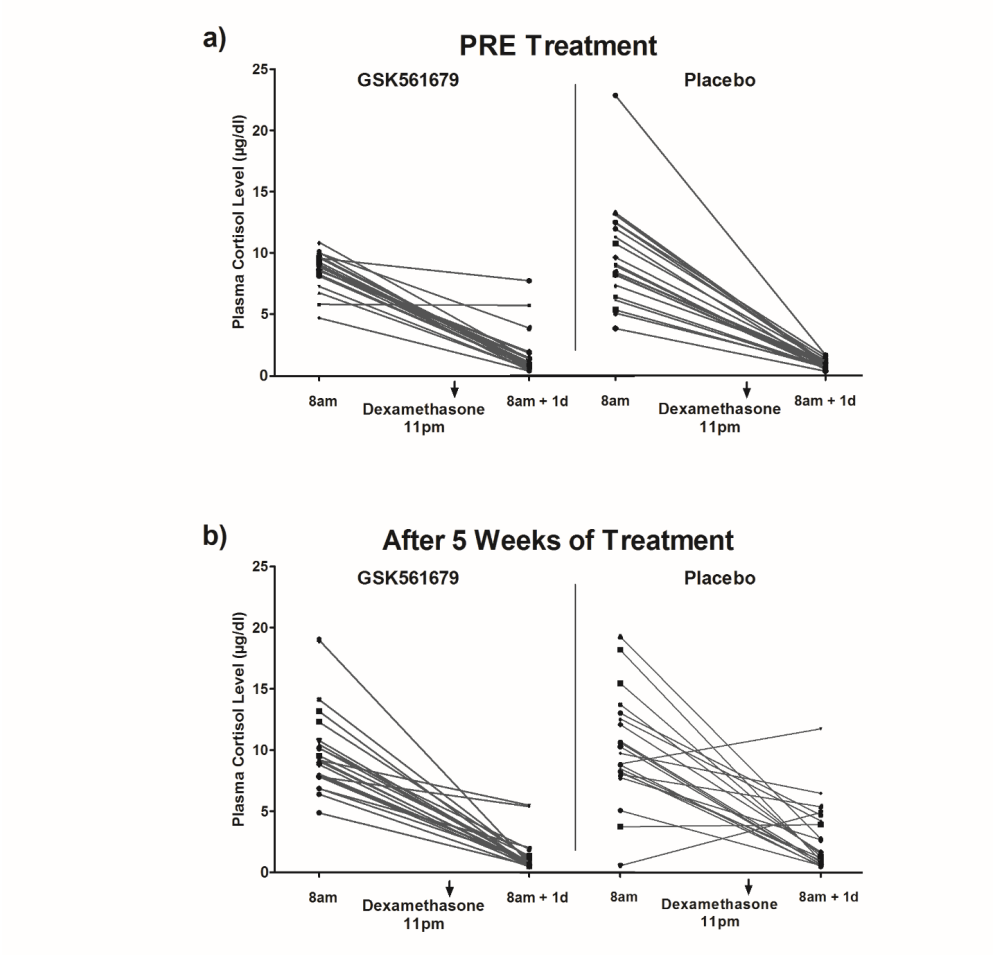

Figure S7: Lack of effect of GSK561679 on change in morning plasma cortisol levels after dexamethasone suppression

Change of 8:00am plasma cortisol levels before and after administration of 0.5mg dexamethasone in subjects treated with the GSK561679 or placebo. a) Pre-treatment; b) after 5 weeks of treatment. At both time points no significant difference was observed between the two treatments groups ($p>0.05$ for all; Pre-treatment: n= 36 GSK561679, 33 placebo; 5 weeks after treatment: n= 29 GSK561679, 26 placebo).

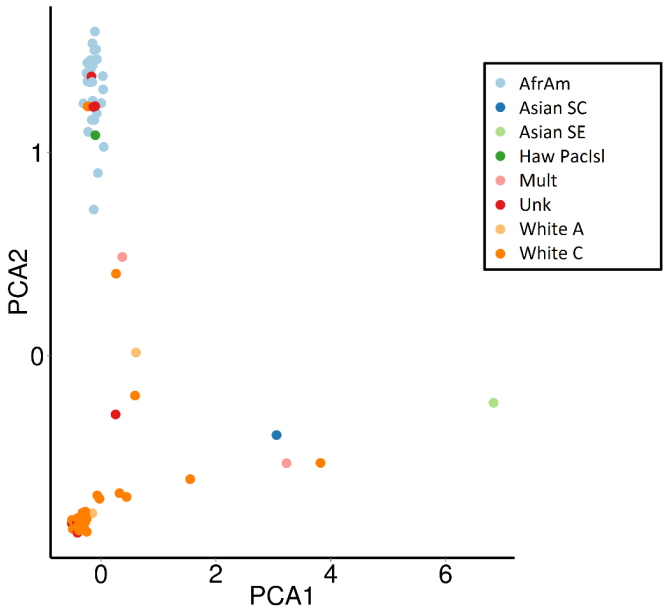

Figure S8: PCA Plot
PCA plot of samples shows good concordance between self-reported ethnicity (legend) and estimated ethnicity by principal component analysis. African-American (AfrAm), Asian South Central (Asian SC), Asian South East (Asian SE), Hawaiian Pacific Islands (Haw PacIsl) Multiple (Mult), Unknown (Unk), White Arabic (White A), White Caucasian (White C).

SUPPLEMENTARY REFERENCE

1. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. (2007): PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81: 559-575.

Supplemental Information

Amygdalar MicroRNA-15a Is Essential for Coping with Chronic Stress

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Cell Reports, Volume 17

A

Upregulated	log2(x)	stepuplog10(y)
mmu-miR-19b_st	1.103437211	2.076686023
mmu-miR-301a_st	1.023376015	2.589425203
mmu-miR-376b_st	0.853955738	2.076686023
mmu-miR-15a_st	0.815886886	2.344322875

Downregulated	log2(x)	stepuplog10(y)
mmu-miR-491_st	-0.811076393	2.076686023
mmu-miR-744_st	-0.847844614	2.495934396
mmu-miR-423-5p_st	-0.849815159	2.589425203
mmu-miR-770-3p_st	-0.874521579	2.093004858
mmu-miR-210_st	-0.878568412	2.589425203
mmu-miR-346_st	-0.883534799	2.589425203
mmu-miR-667_st	-0.899353539	2.271560179
mmu-miR-320_st	-0.909412145	2.495934396
mmu-miR-668_st	-0.929609229	2.235314375
mmu-miR-139-3p_st	-0.93265839	2.495934396

p value (step up)	stepuplog10(y)
0.05	1.301029996
0.01	2
Fold change cut off	log2(x)
1.75	0.807354922
0.571428571	-0.807354922

B

RefSeq	Gene name	Fold change	p value	Full name
NM_175314	Adamts9	1.65039	0.000496	Mus musculus a disintegrin-like and metallopeptidase
NM_010220	Fkbp5	1.30443	0.001009	Mus musculus FK506 binding protein 5
NM_001166737	Vmn1r103	1.56482	0.003005	vomeronasal 1 receptor 103
NM_001005568	Olfr1281	1.32074	0.003176	olfactory receptor 1281
NM_001081064	Pdzd2	1.31115	0.003384	PDZ domain containing 2
NM_001081391	Csmd3	1.38291	0.003796	CUB and Sushi multiple domains 3
NM_001105189	Vmn2r78	1.34524	0.005359	vomeronasal 2, receptor 78
NM_146649	Olfr1160	1.3222	0.00763	lfactory receptor 1160
NM_001011822	Olfr787	1.32773	0.006516	olfactory receptor 787
NM_172800	Sdk2	1.32533	0.008346	Sidekick Cell Adhesion Molecule 2
NM_175473	Fras1	1.40663	0.009796	Fraser syndrome 1

RefSeq	Gene name	Fold change	p value	Full name
XR_032386	Gm4804	-1.33326	0.001287	glyceraldehyde-3-phosphate dehydrogenase pseudogene
ENSMUST00000065297	Lonrf1	-1.31542	0.002133	LON Peptidase N-Terminal Domain And Ring Finger
NM_001177750	Gm10767	-1.39142	0.002899	predicted gene 10767
NR_004051	Btnl5	-1.59032	0.003562	Mus musculus butyrophilin-like 5 (Btnl5), non-coding RNA
XR_001896	Gm8174	-1.68049	0.004361	glyceraldehyde-3-phosphate dehydrogenase pseudogene
XR_033497	Gm3809	-1.37672	0.005489	glyceraldehyde-3-phosphate dehydrogenase pseudogene
XR_031132	Gm5210	-1.35348	0.004779	glyceraldehyde-3-phosphate dehydrogenase pseudogene
NR_029580	Mir194-1	-1.3076	0.006629	microRNA 194-1
XR_033575	Gm7712	-1.34359	0.006891	predicted gene 7712

P value
0.01
Fold change cut off
1.3, -1.3

Figure S1: miRNA and gene array results (related to fig. 1)
(A) A list of miRNAs that were significantly (step up p value<0.01) up and down regulated in RNA from IP of amygdala Ago2 complex following chronic stress in mice. **(B)** A list of genes that were significantly (p value<0.05) up and down regulated in RNA from IP of amygdala Ago2 complex following chronic stress in mice.

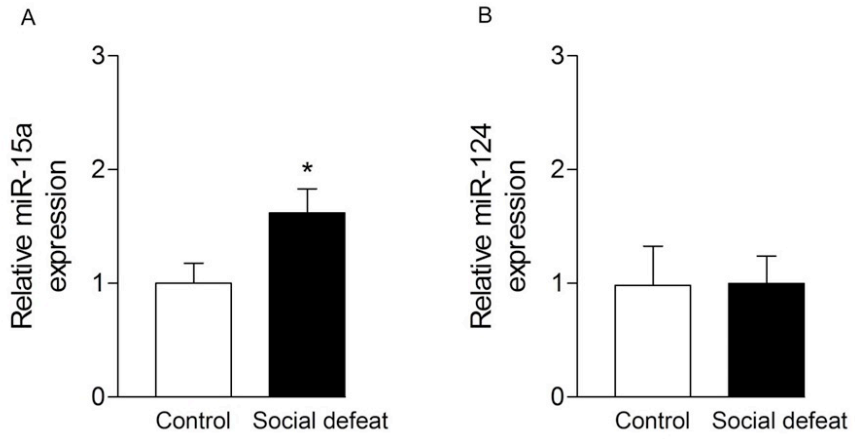

Figure S2: miR-15a levels are elevated in mice plasma following social defeat (related to fig. 1)
Real time PCR analysis. (**A**) The levels of miR-15a (n=6) were increased by 60% (t(10)=-2.265, p=0.047) in plasma of mice subjected to the chronic social defeat paradigm whereas (**B**) the levels of miR-124 (n=7,6) remained unchanged.

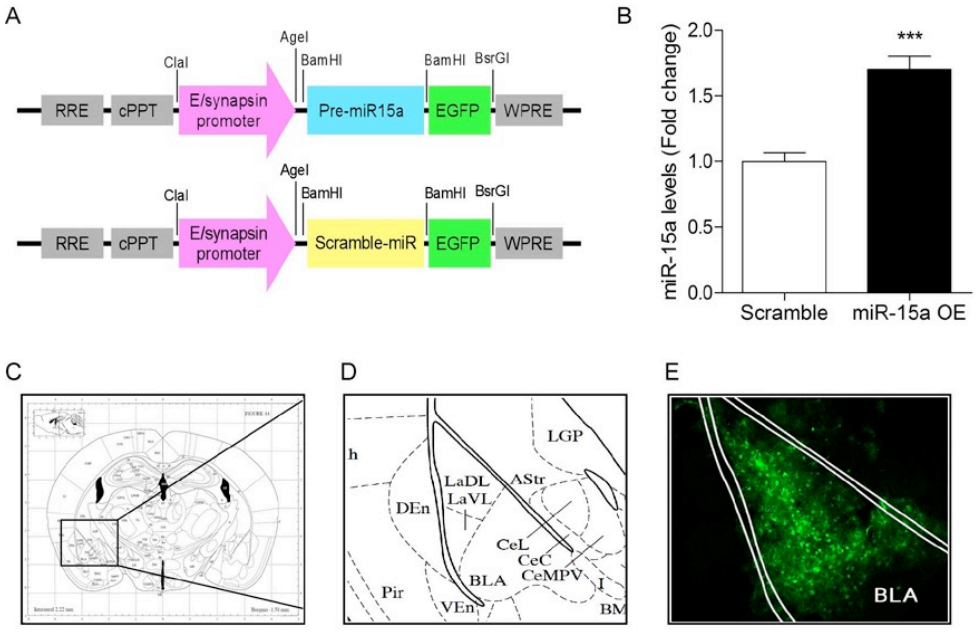

Figure S3: Construction and validation of miR-15a over-expressing lentiviruses (related to fig. 4) (**A**) Schematic illustration of the Syn-miR-15a over-expression (OE) and scramble control lentiviral constructs. (**B**) miR-15a levels in the basolateral amygdala (BLA) (n=6) of mice injected with miR-15a OE or control (scramble) lentiviruses under basal conditions (t(10)=-6.147, p=0.000). (**C**) Schematic representation of the site of delivery. Adapted from Paxinos and Franklin digital mouse brain atlas. (**D**) Enlargement of the BLA region corresponding to the injection site. (**E**) Representative microscope image of a virally infected BLA following miR-15a injection of a 10-week old mouse.

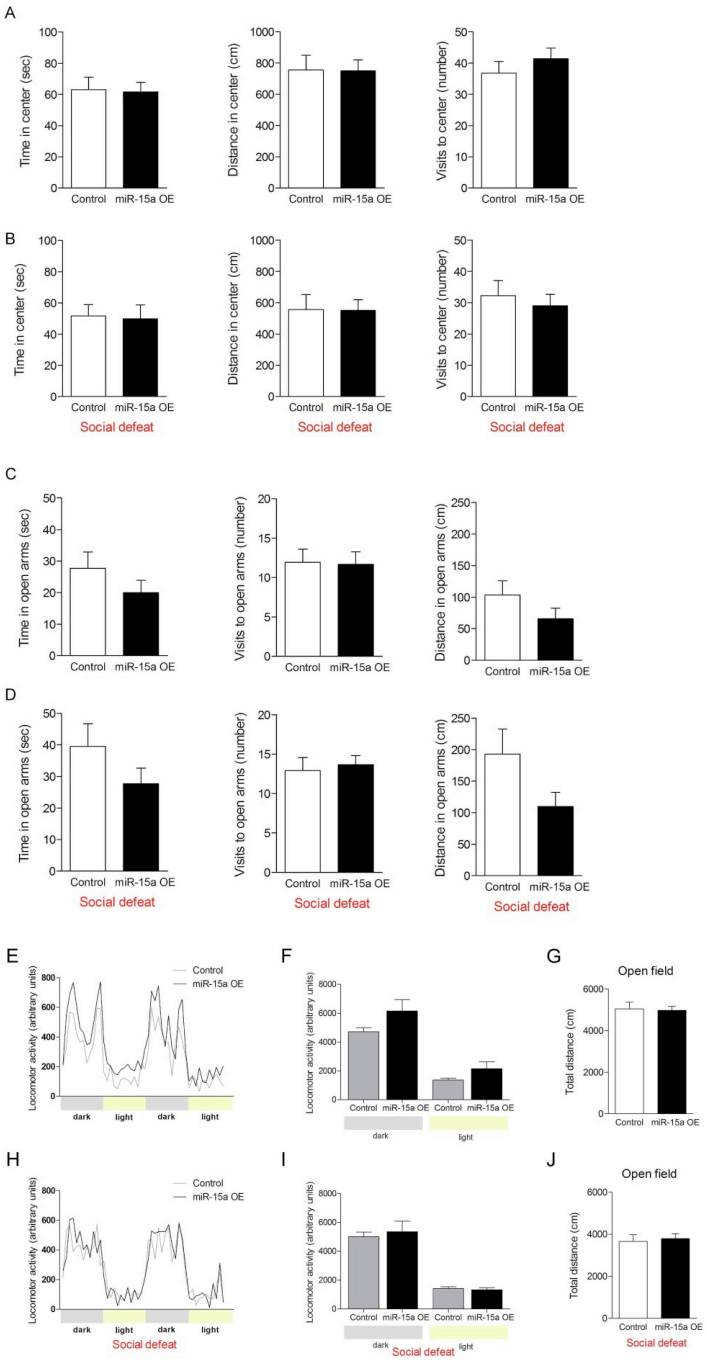

Figure S4: Anxiety and depression-like behavioral tests and locomotor activity in mice over-expressing miR-15a (related to fig. 5) (**A, B**) No significant differences were observed in the open field test between BLA miR- 15a OE and control mice under basal conditions (**A**) or following chronic social defeat (**B**). (**C, D**) No significant differences were observed in the EPM test between BLA miR-15a OE and control injected mice under basal conditions (**C**) or following chronic social defeat (**D**). (**E-J**) No significant differences were observed in the locomotor activity or total distance traveled in the open field test between BLA miR-15a OE and control mice under basal conditions or (**H-J**) following chronic social defeat.

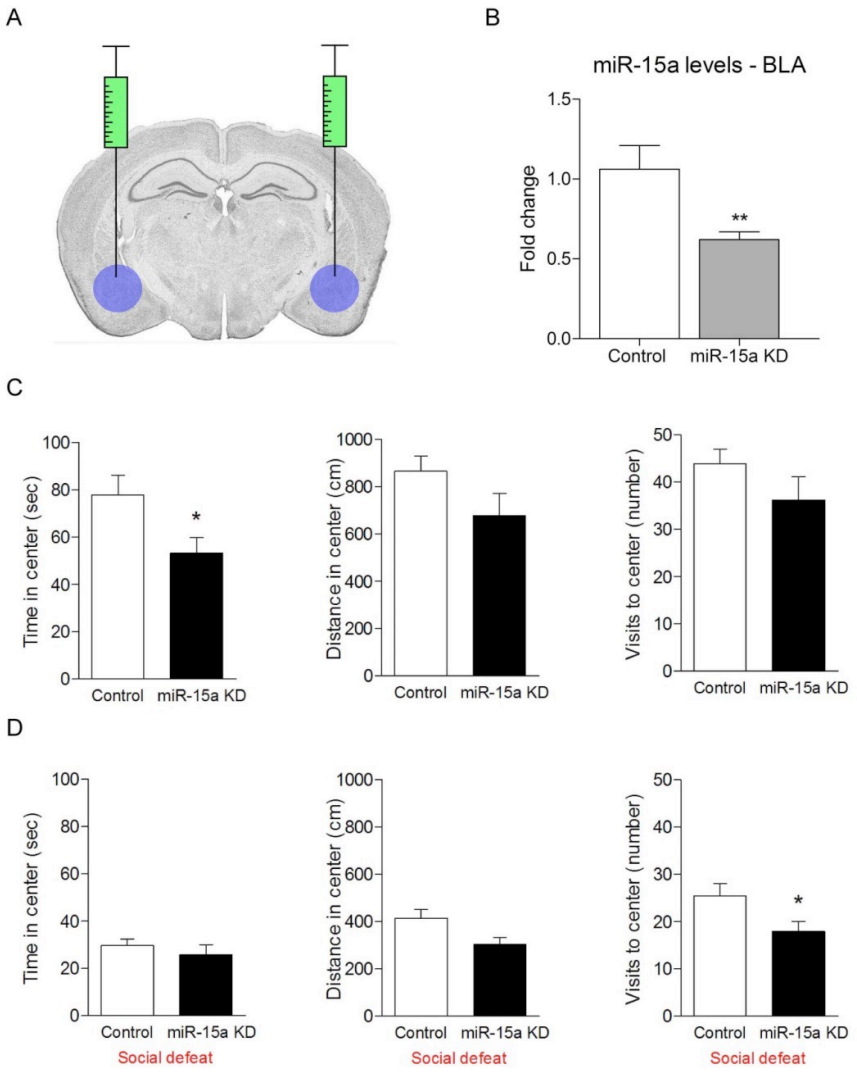

Figure S5: Knockdown of miR-15a results in increased anxiety-like behavior in the open-field test (related to fig. 4 and 5) (A) Illustration of miR-15a KD and control virus injection sites. Blue circles represent the punch area used for RNA extraction. **(B)** Real time PCR analysis of miR-15a levels (n=5). The levels of miR-15a were significantly decreased following injection of miR-15a KD virus compared to control virus ($t(8)=-3.445$, $p=0.009$). **(C)** Open-field test for mice injected with miR-15a KD virus relative to control (n=11, 12). The mice spent significantly less time in the center of the arena ($F(1,22)=5.27$, $p=0.032$). No changes were observed in the distance spent in center or visits to center. **(D)** Open-field test for mice injected with miR-15a KD or control viruses (n=10, 9) and were also subjected to social defeat. There were no differences in the time the mice spent in the center of the arena but they showed a tendency to travel less distance in the center of the arena ($U=22$, $p=0.060$) and had fewer visits to the center of the arena ($U=20$, $p=0.041$).

Supplemental Materials and Methods (related to Experimental Procedures)

Animals

C57BL/6J mice and ICR mice (outbred mice strain, also known as CD1) (Harlan Israel, Kiryat Weizmann, Rehovot) were maintained in a pathogen-free temperature-controlled (22 ± 1°C) mouse facility on a reverse 12 h light-dark cycle at the Weizmann Institute of Science, according to institutional guidelines. Food (Harlan Israel, Kiryat Weizmann, Rehovot) and water were given *ad libitum*. C57BL/6J mice were housed 4 per cage whereas ICR mice were single caged. The total number of animals used for the Ago2 IP was 30 (18 social defeat and 12 controls). The total number of animals used for the lentiviruses experiment was 40 in total (10 per group).

Chronic social defeat

10-week old C57BL/6J male mice were subjected to a social defeat protocol as previously described (Krishnan et al. 2007). Briefly, the mice were placed randomly in a home cage of an aggressive ICR mouse and allowed to physically interact for five minutes. During this time, the ICR mouse attacked the intruder mouse and the intruder displayed subordinate posturing. A perforated clear Plexiglas® divider was then placed between the animals and the mice remained in the same cage for 24 h to allow sensory contact. The procedure was then repeated with an unfamiliar ICR mouse for each of the 10 consecutive days. Control mice were housed in the same room as the social defeat mice but were taken out of the room during the five-minute interaction with the ICR. Control mice were handled daily and housed 2 per cage with a perforated clear Plexiglas® divider placed between the 2 mice. The cage used for the social defeat is a type II long cage for mice (W x D x H) 15.59 x 8.46 x 6.77 inch. The bedding used during the social defeat was Aspen Sami bedding 17304. Mice were not lethally injured although superficial marks were observed. We find that within a period of 5 minutes the mice tend to avoid major injuries.

Microdissection of brain sites for Ago2 IP

Amygdala samples were collected from social defeat and control mice 8 days after the end of the chronic social defeat protocol. Tissue collection and processing was performed as previously described (Lebow et al., 2012; Sztainberg et al., 2010). Briefly, after removing the brain and placing it on an acryl 1 mm brain matrix (Stoelting Co., Wood Dale, IL, cat# 51380), 2 mm slices were taken using standard razor blades (GEM, 62-0165) based on designated anatomical markers. Blunted syringes of different diameters were used to punch out the amygdala from slices removed from the matrix.

Immunoprecipitation of Ago2 protein

Pools of 3 amygdalae taken from 3 mice from the same treatment group (either Social defeat n=18 or Control n=12) were homogenized in NP40 buffer, which was supplemented with RNase inhibitor, protease inhibitor and phosphatase inhibitor (Roche Diagnostics, Indianapolis, IN). The samples were constantly agitated for 2 h at 4°C. Samples were then centrifuged for 20 min at 12,000 rpm at 4°C in a microcentrifuge; the supernatant was placed in a fresh tube, kept on ice and the pellet was discarded. Magnetic protein G beads (Dynabeads, Invitrogen Life Technologies, Carlsbad, CA) were incubated with the Ago2 monoclonal antibody (WAKO chemicals GmbH, Neuss, Germany) with rotation at room temperature for 10 minutes. After several washes, the samples were added to the Ago2-coated protein G beads and incubated overnight at 4°C under agitation. The following day the beads were washed 3 times with PBS. For RNA purification, the beads were homogenized in RLT buffer (RNeasy kit, QIAGEN, Hilden, Germany - miRNA supplementary protocol). For western blot analysis, the beads were boiled in sample buffer to release the protein from the beads.

RNA purification and microarray

RNA from the Ago2 immunoprecipitation samples was isolated using the RNeasy plus kit (QIAGEN, Hilden, Germany) following QIAGEN's supplementary Protocol 1: Purification of total RNA containing miRNA. RNA for all other purposes was isolated from frozen brain punches using the miRNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendation. RNA derived from tissues of stressed mice following Ago2 immunoprecipitation was further analyzed on an Affymetrix miRNA 2.0 array (enriched RNA protocol) and an Affymetrix Mouse Gene 1.0 ST array (Affymetrix, Santa Clara, CA).

Microarray analysis

For the miRNA analysis, we used 4 arrays for the control mice and 6 arrays for the social defeat mice (each array consists of a pool of 3 mice). We used step up correction for multiple testing correction. The fold change threshold was 1.75 with a p value of $p < 0.01$. For the gene analysis, we used 3 arrays for the control mice and 6 arrays for the social defeat mice (each array consists of a pool of 3 mice). The fold change threshold was 1.3 with a p value of $p < 0.05$.

Bioinformatic analysis of microRNA microarray results

miRNAs and genes were tested in 3 different web based programs in search for a seed match between a miRNA and a 3'-UTR: Target Scan (http://www.targetscan.org), Miranda (http://www.microrna.org), (Betel et al., 2008) and Pictar (http://pictar.mdcberlin. de).

Cloning of 3'-UTRs into Psicheck2 luciferase expression plasmid

The 3'-UTR sequence of FKBP51 was PCR amplified from mouse genomic DNA using a forward primer: CCAACTCAGGACTGAACAGT and a reverse primer: GTTCCTTAGGCTGTGGAGAA. The DNA sequence for the mutated form of FKBP51 was generated by site directed mutagenesis using the original cloning primers of FKBP51 and 2 new primers: FKBP51-SDM-F ATGACCACCACGGGCTGCGG and FKBP51-SDM-R CCGCAGCCCGTGGTGGTCAT. This mutation replaced the first 4 nucleotides in the miR-15a seed sequence of FKBP51 from TGCT to ACGG. The 3'- UTR fragments were then ligated into pGem-T easy vector (Promega, Madison, WI) according to the manufacturer's guidelines, and further subcloned into a single *NotI* site at the 3' end of luciferase in the Psicheck2 reporter plasmid (Promega, Madison, WI). Cloning orientation was verified by diagnostic cuts and sequencing.

Transfections and luciferase assay

Huh7 cells were grown on poly-L-lysine coated 48-well plates to 70-85% confluence and transfected using polyethylenimine with the following plasmids: 5 ng of Psicheck2- 3'- UTR plasmid and 215 ng of EGFP over-expressing vector for either a specific miRNA, or a miR-scramble EGFP plasmid. 24 h following transfection, cells were lysed and luciferase reporter activity was assayed as previously described (Chen et al. 2005). Renilla luciferase values were normalized to control luciferase levels (transcribed from the same vector but not affected by the 3'-UTR tested) and averaged across eight-well repetitions per condition.

miRNA RT-qPCR expression analysis

Quantitative miRNA expression was acquired and analyzed using a step one thermocycler (Applied Biosystems, Waltham, MA), using miRCURY LNA Universal RT microRNA PCR primers (Exiqon, Vedbaek, Denmark) or miScript primer assay (QIAGEN, Hilden, Germany). RNA samples were assessed using miRCURY Universal cDNA Synthesis kit II and miRCURY ExiLENT SYBR Green (Exiqon, Vedbaek, Denmark) or miScript II RT kit and miScript SYBRgreen PCR kit (QIAGEN, Hilden, Germany), according to the manufacturer's guidelines. U6, 5S rRNA were used as internal controls. Gene expression was obtained using the High Capacity kit and SYBR green PCR master mix (Applied Biosystems, Waltham, MA). The real-time PCR primers for FKBP51 were: forward: ATGACTACTGATGAGGGCAC and reverse: GACATAAACTTTGTCACCAAAC.

Design, construction and validation of miR-15 lentiviruses

The miR-15a over-expression vector was cloned as follows: the enhanced form of human synapsin I promoter (Hioki et al. 2007) was PCR amplified (forward primer: ttttttatcgatctcgagtagttattaatagtaatc, reverse primer: ttttttaccggtggcgcgcccgccgcagcgcagatggt) from pENTR1A-E/SYN-GFP-WRPE1 (Kindly provided by Dr. Takeshi Kaneko, Department of Morphological Brain Science, Graduate School of Medicine, Kyoto University, Kyoto, Japan) and inserted between ClaI and AgeI restriction sites to replace the CMV promoter in pCSC-SP-PW-GFP (kindly provided by Dr. Inder Verma, The Salk Institute for Biological Studies, La Jolla, CA). Following the Synapsin promoter, the precursor for miR-15a was inserted:

gcacataccagtgttagatttttttcaaacatagattttatgtgttctacttttttcctaaaaagccttttctgtaaattactattgaggtgctaggagttt caaaaccaaccccttggagtaaagtagcagcacataatggtttgtggatgttgaaaaggtgcaggccatactgtgctgcctcaaaatacaaggac ctgatcttctgaagagagtacctgtctttttattcatagctcctatgatagcaatgtc.

The miR scramble control was purchased from GeneCopoeia (Rockville, MD) and subcloned into pCSC-SP-PW-SYN-GFP plasmid. The miR-15a sponge KD and its control were designed according to Lin *et al.* (Lin et al. 2011) and inserted following an H1 promoter in the p156RRL-CMV-GFP viral plasmid. The sequence for miR-15a sponge was:

CGCGGATCTAGCTAGCCACAAACCAAGCGTGCTGCTAATCGCACAAACCAAGCGTGCTGCTAATCGCACAAAC CAAGCGTGCTGCTAATCGCACAAACCAAGCGTGCTGCTAGCTAGATCGATCTTCTAGAAAGATCCAAACCAAG CGTGCTGCTAATCGCACAAACCAAGCGTGCTGCTAATCGCACAAACCAAGCGTGCTGCTAATCGCACAAACCA AGCGTGCTGGCTAGCTAGCCTAGATCCGCGTCGTTAATTAACCTTAGGGCTTAGCGCTAGC.

The sequence for miR-15a sponge control was:

CTAGCTAGCCATGTCCCAAGCAGAATGCTAATCGCATGTCCCAAGCAGAATGCTAATCGCATGTCCCAAGCAG AATGCTAATCGCATGTCCCAAGCAGAATGCTA.

High titer lentiviruses were produced as previously described (Tiscornia et al. 2006). Briefly, recombinant lentiviruses were produced by transient transfection in HEK293T cells. Infectious particles were harvested at 48 and 72 h post-transfection, filtered through 0.45 µm-pore cellulose acetate filters, concentrated by ultracentrifugation, re-dissolved in sterile HBSS, aliquoted and stored at –80°C.

Stereotactic intracranial injections

A computer-guided stereotaxic instrument and a motorized nanoinjector (Angle Two™ Stereotaxic Instrument, myNeurolab, Leica Biosystems, Buffalo Grove, IL) were used as previously described (Elliott et al., 2010; Kuperman et al., 2010; Regev et al., 2012). 10- week old male mice were randomly selected and anesthetized using 1.5% isoflurane and 1 µl of the lentiviral preparation was delivered to each BLA using a Hamilton syringe connected to a motorized nanoinjector system at a rate of 0.2 µl per min (coordinates relative to bregma: AP = –1.58 mm, L = ±3.3 mm, H = –4.6 mm). Following a 2-week recovery period, mice were subjected to behavioral studies and later anesthetized and perfused with 4% PFA. The fixed brains were serially sectioned and immunohistochemically stained in order to confirm the location of the injection site, as previously described (Regev et al., 2011). The antibodies that were used were: Goat biotinylated anti-GFP (Abcam, Cambridge, UK; ab6658) and Alexa Fluor 488 Streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA; 016-540-084). 3 repetitions were used for the immunohistochemical analysis.

Behavioral assessments

All behavioral assessments were performed during the dark (active) phase following habituation to the test room for 2 hours before each test. Behavioral tests were conducted as previously described (Haramati et al., 2011; Lebow et al., 2012) in the following order, from the least stressful procedure to the most and ending with home cage locomotor testing: Open-field, EPM and home cage locomotion.

Open-field test: The open-field test was performed in a 50 x 50 x 22 cm white box, lit to 120 lux. The mice were placed in the box for 10 minutes. Locomotion in the box was quantified using a video tracking system (VideoMot2; TSE Systems, Bad Homburg, Germany).

EPM test: This apparatus in this test is designed as a plus sign and contains 2 barrier walls and 2 open arms. During the 5-minute test, which is performed in relative darkness (6 lux), the number of entries, the distance traveled and the time spent in the open arms is automatically scored using a video tracking system (VideoMot2, TSE Systems, Bad Homburg, Germany).
Homecage locomotion: Homecage locomotion was assessed using the InfraMot system (TSE Systems, Bad Homburg, Germany). Mice were housed individually for 72 h, in which the first 24 h were considered habituation to the individual housing conditions. Measurements of general locomotion consisted of 2 light and 2 dark cycles in the last 48 h, collected at 10 min intervals.

Statistics
Data are expressed as mean ± standard error of the mean (Binder et al.). Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software (SPSS Inc., Chicago, IL). All data sets' distributions were tested for normality using Shapiro-Wilks test in order to determine which statistical tests should be applied. In cases where indices of 2 groups were compared and the data's distribution was normal, a 2-sided students t-test was used, where the data departed from normal distribution, the Mann- Whitney U test was applied. All data sets were also tested for variance similarity between compared groups. For the miRNA array results, a q-value correction was performed. Mice were excluded from the analysis if they had values higher than AVG+2*SD or lower than AVG-2*SD.

Human studies - qPCR analysis
Samples: For this study, 26 males of Caucasian origin aged between 19 and 30 years were recruited (mean age = 25.58 +/- 2.64SD). All participants were free of a history of psychiatric disorders as well as major neurological and general medical disorders. Further exclusion criteria were regular use of medical drugs, as well as excessive alcohol or caffeine consumption. All subjects gave written informed consent. Procedures were approved by the Ethics Committee of the Ludwig Maximilians University, Munich, Germany, in accordance with the Declaration of Helsinki.
Study Design: Unstimulated peripheral blood samples were drawn at 12:00pm followed by oral administration of 1.5 mg of dexamethasone. Subsequently stimulated samples were collected at 1:00pm, 3:00pm, 6:00pm, and at 11:00am the following day. PAXgeneTM (QIAGEN, Hilden, Germany) whole blood RNA collection tubes were used at each time point for whole blood collection. *RNA extraction:* Total RNA was extracted using the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) with the QIAGEN method for column purification of nucleic acids (PreAnalytiX, Hombrechtikon, Switzerland) according to the manufacturer's instructions. For RNA quality and quantity, extracted samples were subsequently run on the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA). All samples had an RNA integrity number (RIN) ≥7.
Quantitative real time polymerase chain reaction: Total RNA was reverse transcribed using the miRCURY LNA™ Universal RT miRNA PCR cDNA synthesis kit (Exiqon, Vedbaek, Denmark). CDNA was diluted and mixed with the Exiqon microRNA LNA PCR primers as well as the Exiqon SYBR Green master mix and assayed in 10 µl reactions. qPCR experiments were performed using the Roche 480 LightCycler system (Roche Applied Science, Roche Diagnostics, Indianapolis, IN) in 384-well plates. Each sample was run in technical triplicates. Normalization of the results was performed against SNORD38 using the delta Ct method.

Western blot analysis
Frozen brain samples were homogenized in RIPA buffer supplemented with proteinase inhibitors (Sigma-Aldrich, St. Louis, MO) and were incubated on ice for 10 min. After 10 min centrifugation, the supernatant was transferred to a new tube and sample buffer was added to the sample, which was then boiled for 5 min and placed on ice. The samples were separated in a 10% polyacrylamide gel electrophoresis. Transfer was performed using an assembly of nitrocellulose membrane and Whatman paper. The transfer was performed at 100v, 280 mAmp for 1h and 40 minutes. After washes with PBST (PBS + 20% Tween 20) membrane was blocked with 10% milk for 1 h. The first antibody was added (goat anti FKBP51 – Santa Cruz 11518, mouse anti-GAPDH – abcam 8245 or

mouse Anti-b-Actin- Sigma-Aldrich, St. Louis, MO; A1978) to PBST and placed on constant shaking at 4°C overnight or 1 h at room temperature. The second antibody (anti-goat HRP – abcam 6885, anti-mouse HRP – cell signal 7076) was added in 10% milk for 1 h. Each step was separated by additional washes with PBST. Finally, ECL was added to the membrane which was then exposed to film.

Social avoidance test

The test is performed in an open field design with a small neighboring chamber that is separated from the open field with a divider with small open slits, allowing full sensory contact between the 2 fields. The mice are allowed to habituate to the open field for 3 minutes, and then an unfamiliar ICR mouse is placed in the neighboring chamber, and they are allowed to interact for 3 minutes. The entire session is videotaped and analyzed with Ethovision software (Noldus, Wageningen, Netherlands). The space next to the small neighboring chamber is deemed the interaction zone. The time a mouse spends in the interaction zone with the unfamiliar ICR mouse is divided by the time the mouse spent in the interaction zone without the unfamiliar ICR mouse and then multiplied by 100. Mice were categorized into 3 groups: Control, “Susceptible” and “Resilient”. Mice that received below 100 in this analysis were characterized as “Susceptible”. Mice that received above 100 in this analysis were characterized as “Resilient”. Only control mice that received above 100 were further used.

miRNA Sequencing analysis

RNA samples were quantified by Qubit and 400 ng RNA used for library preparation using the Illumina TruSeq Small RNA Library Preparation Kit following the standard protocol and size-selected on DNA-PAGE to a size of 145-155nt. QC was carried out using BioAnalyzer High Sensitivity DNA chips and libraries quantified using the KAPA library Quantification Kit for Illumina (Kapa Biosystems Inc., Wilmington, MA) on a Roche Lightcycler480. Libraries were multiplexed in euqimolar pools and sequenced single-end 50nt on an Illumina MiSeq at MPI of Psychiatry, München, using v3 chemistry to a depth of minimum 5Mio reads per sample (% >=Q30 higher then 96). Fastq raw sequence reads were quality-checked using FASTQC* and reads between 15-40nt length containing the Illumina Small RNA Adapter selected and adaptertrimmed using Cutadapt*. Reads were aligned to miRbase v21 and normalized to counts per million using sRNAbench* (library mode using Bowtie*). Differential expressed microRNAs were queried using the Bioconductor - DESeq2 package* applying a minimum cut-off of 5 read counts in all samples.

Subjects exposed to early life trauma

Control subjects were available at the IRCCS Fatebenefratelli Institute, Brescia (Italy). Individuals presenting a history of neurological disease, prior electro-convulsivant treatment, prior traumatic brain injury, or mental retardation (IQ<70) were excluded from the study. Written informed consent was obtained by participants after receiving a complete description of the study, which has received approval by the local ethics committee.

The absence of psychiatric disorder was ascertained via 2 schedules: the Mini International Neuropsychiatric Interview (M.I.N.I. Plus, Bonora et al., 1995), to exclude any psychiatric disorder in Axis I; and the Structured Clinical Interview for DSM disorders (SCID-II, Spitzer et al., 1993) to exclude any psychiatric disorder in Axis II.

The list of traumatic events includes loss of a biological parent due to death or separation for at least 6 months, including being taken into local authority care, severe physical, sexual abuse by a parental figure and neglect. Physical abuse includes incidents that meet at least 2 of the following criteria: a) the abuse consisted of being hit with a belt or stick, or being punched or kicked; b) the abuse resulted in an injury, including broken limbs, black eyes or bruising; and c) the perpetrator was considered to be out of control. Sexual abuse was defined as unwanted or illegal sexual experiences prior to age 17 years with any adult or an individual at least 5 years older than the recipient, not necessarily limited to the immediate family; moreover, these experiences have to meet at least 2 of

the following criteria: a) the perpetrator was known to the individual; b) the perpetrator was a relative; c) the perpetrator lived in the same household; d) the unwanted sexual experience occurred more than once; e) the perpetrator touched the child's genitals; f) the perpetrator forced the child to touch the perpetrator's genitals; and g) the abuse involved sexual intercourse.

Neglect was defined in terms of parents' disinterest in material care (feeding and clothing), health, schoolwork and friendships. Neglect was quantified for both mother and father.

The most conservative cut-off points published by Bifulco et al. (2005) were used to dichotomise these responses into a yes/no answer. For this study, we performed miRNA analyses using qPCR in a group of 20 subjects who reported at least one type of abuse (physical abuse, sexual abuse, physical, parents separation or loss and neglect) and 20 subjects matched for age and gender with no history of early life stress (mean age ± SD: 38.1 ± 6.1 and 37.5 ± 6.7 respectively in the subjects with and without childhood trauma, $p<0.05$); percentage of females of 55% and of 54% respectively in the subjects with and without childhood trauma, $p<0.05$).

Blood samples were collected by using PaxGene Blood Tubes. After collection, blood samples were then kept at room temperature for 2 hours, then at -20°C for 2 days and then at -80°C until their processing.

miRNA isolation from the blood of subjects

Total RNA was extracted from 2.5 mL of blood with the PAXGene Blood miRNA Kit (QIAGEN, CA, USA), designed for the simultaneous isolation of small and large RNAs; RNA concentration and quality were assessed through a NanoDrop spectrophotometer (Thermo Scientific, MA, USA).

Real-time PCR analyses

We looked specifically at the expression of hsa-miR-15a-5p by Real Time PCR (RTPCR). RT-PCR was conducted using TaqMan MicroRNA Assays (Applied Biosystems, CA, USA), following the manufacturer's instructions and the reactions were run on the StepOnePlus instrument (Applied Biosystems, Waltham, MA). The Ct values were normalized according to the deltaCt method on the endogenous controls RNU44 and RNU48.

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Addendum

Review article

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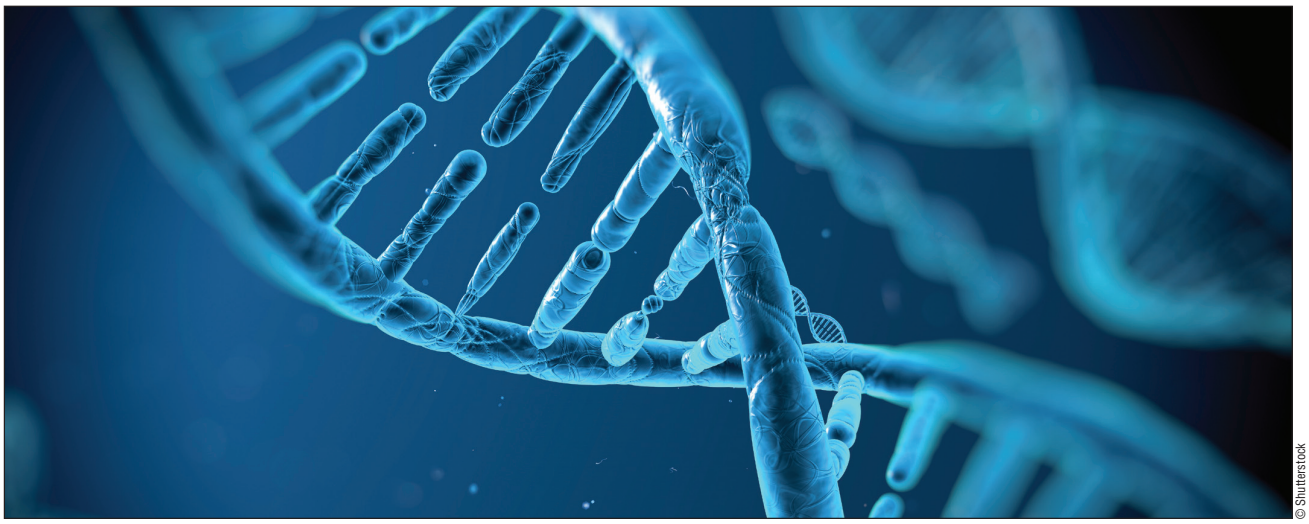
CME Article

The Role of Genetics and Epigenetics in the Pathogenesis of Posttraumatic Stress Disorder

Julius C. Pape, MD; and Elisabeth B. Binder, MD, PhD



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ABSTRACT

Posttraumatic stress disorder (PTSD) represents a common psychiatric disorder that can emerge after a traumatic life event. Despite a high incidence of trauma exposure (40%-90%) in the general population, only a minority (7%-12%) will eventually develop the disorder. As indicated by twin and family studies, genetic factors are an important contributor to PTSD, suggesting an individual genetic vulnerability to the disorder. Studies exclusively focusing on genetic main effects have shown limited results, likely because environmental factors play a key role in this disorder. Gene and environment interaction (GxE) studies may represent a more promising approach to better understand the pathophysiology of this disorder because they jointly consider the genetic predisposition as well as the environmental trigger. On a molecular level, such GxE and long-lasting effects of these interactions on transcriptional regulation may be mediated by epigenetic modifications. A number of studies suggest that the etiology of PTSD is the result of a complex interplay of genetics, environmental factors, and epigenetic regulation. This article reviews current genetic and epigenetic findings in the field of PTSD, focusing both on candidate gene and genome-wide approaches. Although there has been some initial progress, the field still lacks large-scale studies on the genetic level, but some are currently underway within the Psychiatric Genomics Consortium PTSD. Finally, the reviewed studies support that a combination of different approaches, integrating genetic and epigenetic data, will be necessary to better understand the underlying molecular mechanisms of PTSD. **[*Psychiatr Ann*. 2016;46(9):510-518.]**

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With a prevalence of about 5% in the general population and an overall lifetime prevalence of 7% to 12%, posttraumatic stress disorder (PTSD) is a common psychiatric disorder. Per definition, PTSD is a disorder with long-lasting symptoms occurring after exposure to a traumatic life event. These symptoms include intrusive memories, avoidance and numbing, and hyperarousal. Women are twice as likely as men to develop the disease.[1]

Although environmental triggers are well-defined, a key question that remains unanswered is why only a small percentage of people that experience trauma go on to develop PTSD.[2,3] The ratio between a high lifetime trauma incidence and the relatively low prevalence of PTSD suggests that exposure to a trauma does not inevitably lead to development of the disorder.[4] Some of the environmental factors that seem to be responsible for an altered response to traumatic life events are the type and intensity of the trauma, exposure to previous trauma, and living in unsafe neighborhoods. Nonetheless, inter-individual differences in susceptibility to the disease exist and these may be mediated by genetic factors. As indicated by twin and family studies, genetics represent an important factor in accounting for the risk of developing this disorder. Several of these studies have consistently shown the estimated genetic contribution to be between 30% and 40%.[5] It should be noted, however, that heritability research for PTSD is complex, as it is depends on comparable environmental exposures in relatives.

Although there is a clear indication of genetic contribution to this disorder, so far the investigation of the main effects of genetics in PTSD has provided only limited results.[6] As mentioned above, environmental factors, in this case traumatic life events, play a decisive role in the pathogenesis of the disorder. Some of the lasting effects caused by these factors are likely mediated by epigenetic changes. These are changes that do not affect the sequence of the DNA but rather its accessibility to transcription factors or effects mediated by noncoding RNA that shape the transcriptional response of affected tissues.[7] Consequently, research in the field of PTSD has now increasingly focused on the interplay of genetics, environment, and epigenetic factors.

So far the investigation of the main effects of genetics in PTSD has provided only limited results.

One main approach to the study of the genetic component of PTSD was to investigate candidate genes. These genes were selected for their involvement in systems possibly relevant for PTSD. These include genes involved in the serotonergic and dopaminergic system but also more specifically, genes from molecular pathways that are thought to be involved in the pathogenesis of PTSD, such as genes involved in the stress-hormone system or relevant for different aspects of fear conditioning.[8] However, as with other complex disorders, candidate gene studies are fraught with inconsistent replication and the risk of false-positive associations.[9] To overcome this issue, The Psychiatric Genomics Consortium PTSD Workgroup has recently been formed to enable genome-wide association studies (GWAS).[10] It is hoped that large-scale GWAS may provide novel, hypothesis-free, genetic risk variants for PTSD.

This article provides an overview of the genetic and epigenetic mechanisms possibly involved in the pathophysiology of PTSD, as well as recent findings and developments in this field.

CANDIDATE GENES

Because dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis has been shown to play a decisive role in the pathogenesis of PTSD,[11] genes involved in its regulation are of particular interest. The HPA axis represents the most important system regulating the neuroendocrine stress response of an organism.[12] It acts through a complex interplay of direct interactions and negative feedback loops. In response to stress, the release of corticotropin-releasing hormone (CRH) in the hypothalamus sets off a cascade of reactions, promoting the release of a number of hormones from the adrenal glands, including cortisol. Cortisol then acts on two nuclear hormone receptors—the mineralocorticoid and glucocorticoid receptor (GR). A set of downstream effects, including transcriptional ones, allows the organism to adapt to stress exposure, but once the stressor has passed, also decreases HPA activity through negative feedback mediated by the GR. This stress hormone system is dysregulated in PTSD, making genetic variants of key regulators in this system prime candidates in understanding the genetics of PTSD. These candidates include the receptors for CRH, particularly *CRHR1*, the gene encoding the GR (*NR3C1*), a co-chaperone of the GR, (*FKBP5*), as well as the important pituitary peptide *PACAP* (*ADCYAP1* gene) and its receptor.

Although case/control association studies are the main method used for the study of many disorders, they often fail for PTSD due to the strong environmental component. Therefore, a number of genetic studies in PTSD have sought to incorporate interactions between genes and trauma exposure.[7]

CRH and its receptor CRHR1 play a role in several stress-related disorders, including PTSD. In animal studies, in-

tracerebroventricular infusion of CRH led to anxiety and particularly PTSD-like behavior, and these effects were reversed by the administration of CRHR1 antagonists.[13,14] In humans, increased levels of CRH in the cerebrospinal fluid of patients with PTSD compared to controls represents another interesting finding linking CRH to the disorder.[15,16]

Three *CRHR1* polymorphisms that have previously been associated with the development of depression after child abuse have been tested in the context of PTSD. These polymorphisms not only interact with child abuse to predict depression, but also with endocrine dysregulation, with an exaggerated stress response seen in risk-allele carriers with exposure to early trauma.[17,18] Imaging studies also indicate that these polymorphisms moderate neural responses to emotional stimuli.[19,20] Although these specific single nucleotide polymorphisms (SNPs) were not associated with PTSD,[21] two recent studies reported associations of other *CRHR1* SNPs and the disorder. First, rs12944712 was significantly related to PTSD severity in a prospective study of children who had experienced medical trauma.[22] Second, the major alleles of two polymorphisms within the *CRHR1* gene increased the risk for posthurricane PTSD symptoms.[23]

Despite being a main regulator of the HPA axis and, therefore, thought to be crucially involved in the pathogenesis of PTSD, investigations into possible associations of genetic variants of the GR with the disorder have not been successful. For example, Bachmann et al.[24] studied GR polymorphisms in the context of PTSD in a cohort of 118 combat veterans diagnosed with PTSD. The authors did not detect altered frequencies of the tested polymorphisms in cases compared to controls.[24]

FKBP5 is a heat shock protein 90 associated co-chaperone of the GR complex. Among its other functions, *FKBP5* regulates GR sensitivity. Cortisol binding to the complex leads to a change in affinity, which results in an exchange of FKBP5 with other co-chaperones such as FKBP4. Subsequently, the increased binding of FKBP4 promotes the recruitment of dynein, which leads to translocation of the GR to the nucleus, where it acts as a transcription factor. Activation of the GR by glucocorticoids enhances, among many other genes, *FKBP5* transcription, resulting in the formation of an intracellular ultrashort feedback mechanism. Increased levels of FKBP5 inhibit GR activity.[25,26]

Because of its critical role in regulating GR sensitivity, genetic polymorphisms in *FKBP5* are a target of PTSD research. In several different cohorts, a haplotype tagging a functional polymorphism that alters the induction of *FKBP5* mRNA by GR has been associated with PTSD, but only when in combination with childhood trauma exposure.[27,28] One study has also shown that depending on the environment and trauma, the *FKBP5* "risk" allele may also confer protective features, as it has been associated with posttraumatic growth in people who experienced Hurricane Katrina.[29] As detailed later in this article, this interaction may be mediated by allele-specific DNA methylation changes in the *FKBP5* locus that further disinhibit *FKBP5* transcription.[30] A number of animal studies have shown that increased *FKBP5*, especially in the amygdala, is associated with key endophenotypes often related to PTSD, such as stress coping and increased anxiety, but also altered fear extinction.[31-35] In addition, in humans, the genetic polymorphisms associated with increased *FKBP5* expression have not only been associated with PTSD but also with related endophenotypes. For example, the risk allele has been associated with an enhanced GR suppression as measured by the low-dose dexamethasone suppression test.[36] Imaging studies point to an important role of *FKBP5* not only in amygdala reactivity to threat,[37-39] but also structure, function, and connectivity of the hippocampus, a brain region consistently implicated in PTSD.[30,39-41] For example, people carrying two risk alleles exhibited lower structural as well as functional connectivity between the anterior cingulate cortex and the hippocampus, a connection critical for adaptive cognitive and emotional processing.[42] *FKBP5* risk alleles have also been associated with behavioral endophenotypes for PTSD, such as bias toward threat[43] and an increase in intrusions, even in healthy people.[44] They are also associated with peri-traumatic dissociation in children who experienced acute medical injury, a strong predictor for PTSD later in life.[45] Finally, these genetic polymorphisms were also associated with response to PTSD treatment. In a cohort of 43 people exposed to trauma during civil war in Uganda, the authors evaluated the treatment effect of narrative exposure therapy dependent on an *FKBP5* genotype (rs1360780). After 10 months of therapy, people carrying the risk (T) allele had a significant increase in relapse of PTSD symptoms compared to noncarriers.[46]

The pituitary adenylate cyclase activating polypeptide (PACAP) represents another key mediator of the stress response and, therefore, has been studied in the context of PTSD. A study by Ressler et al.[47] examined a civilian cohort of highly traumatized people and patients with PTSD. The authors identified a genetic polymorphism (rs2267735) in the PACAP receptor type 1 gene (*PAC1R*; *ADCYAP1R1*) that was significantly associated with PTSD in women only. In another study using a cohort of similarly traumatized people (n = 1,160), the finding was not replicated, but interestingly an association between PTSD and the interaction of rs2267735 and trauma load was observed, and again the association was restricted to women.[48]

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Further, an interaction of the same SNP and childhood maltreatment was found to be associated with PTSD symptoms in 495 adult women.[49] Finally, there seems to be an *ADCYAP1R1* genotype effect on individual brain function. The activity of PTSD-relevant brain regions, the amygdala and hippocampus, was analyzed in 49 traumatized women after exposure to threatening and neutral stimuli. The authors observed increased amygdala activity after the threat stimuli as well as lower functional connectivity with the hippocampus in carriers of the risk genotype (CC).[50]

Despite some promising findings in PTSD research using the candidate gene approach, a single gene is unlikely to explain the complex phenotype of the disorder. By combining different candidate genetic risk variants to form a cumulative risk score, Boscarino et al.[51] aimed to develop a possible PTSD prediction tool. The authors used a cumulative risk allele count including polymorphisms of different genes (*CRHR1*, *FKBP5*, *COMT*, *CHRNA5*, *CRHR1*) to show an interaction effect of the risk score and trauma exposure level on PTSD symptom severity.[51] In a subsequent study, they incorporated this genetic risk-allele information to their previously developed PTSD screening instrument, which already included data concerning mental health status, substance abuse, and other psychosocial measures. Adding the genetic information to the existing screening tool significantly increased the ability to predict PTSD.[52]

In a pilot study, Rothbaum et al.[53] also found evidence that a composite additive risk score derived from polymorphisms in 10 previously identified genes associated with stress response (*ADCYAP1R1, COMT, CRHR1, DBH, DRD2, FAAH, FKBP5, NPY, NTRK2*, and *PCLO*) predicted the development of PTSD symptoms after trauma exposure in patients recruited in the emergency department and observed prospectively. This risk could be attenuated by early intervention.

GENOME-WIDE ASSOCIATION STUDIES

In contrast to the above-presented candidate gene studies, GWAS represent a hypothesis-free tool to identify the most common genetic variations associated with the disease on a genome-wide level.

In the past 10 years, GWAS studies have decisively broadened our knowledge about new loci associated with susceptibility to common complex disorders not only in psychiatry, but across many medical disorders. To achieve this, international GWAS consortia were established to analyze sample sizes large enough to reach adequate power, which is one of the critical factors for these analyses. The Psychiatric Genomics Consortium represents such a consortium that has been successful in identifying robust genetic risk variants for a number of disorders, in particular schizophrenia.[54] Although a few GWAS results for PTSD have been published, they are mostly limited by the relatively small sample sizes. To overcome this, the first PTSD GWAS consortium (Psychiatric Genomics Consortium-PTSD) was recently formed.[10]

The first PTSD GWAS was conducted in 2012 by Logue et al.[55] The authors found one associated SNP after correction for multiple testing. This SNP was located in the retinoid-related orphan receptor alpha gene (*RORA*). The significant SNP did not reach genome-wide significance in two African-American replication samples, but several other *RORA* SNPs were found to be nominally significant in these samples.[55] The *RORA* gene was previously found to be associated with other psychiatric disorders such as bipolar disorder and major depressive disorder.[56,57] Three other GWAS that were published recently identified genome-wide significant polymorphisms in genes previously connected to neurobiological pathways and processes implicated in PTSD.[58-60] The most recent GWAS found a SNP (rs717947) that was significantly associated with PTSD on a genome-wide level. Having used a small discovery sample (n = 147) the authors were able to replicate their finding in a much larger cohort where the SNP remained significant in women diagnosed with PTSD but not in men (n = 2,006). Interestingly, the discovered SNP correlated with an intermediate neural phenotype (more precisely with altered medial and dorsolateral prefrontal activation to fearful faces) identified using functional magnetic resonance imaging (fMRI) data in a subset of the replication sample.[61] Although these studies brought to light some promising results, they only represent a first step in identifying "true" and replicable genetic risk loci for PTSD. In addition to the challenge faced by all other psychiatric disorders (ie, small genetic effect sizes with the need of samples exceeding several thousand samples and diagnostic heterogeneity), differences in environmental exposure and gene and environment (GxE) interactions also need to be incorporated in the models.

EPIGENETICS

The genetic background plays a decisive role and provides an important contribution to disease pathogenesis. However, the complex phenotype of PTSD cannot be explained by genotype alone. Like most psychiatric disorders, the etiology of PTSD is multifactorial in nature, where environment represents another important contributor to the disease. Although environmental factors do not affect the genetic code itself, they can alter gene function by epigenetic changes such as DNA methylation, histone modification, or noncoding RNAs.[62,63] These modifications are candidate mechanisms for mediating effects by the environ-

ment on the DNA, which most often result in an alteration of gene transcription and protein translation. Such effects can be long lasting but also lead to a rather short-term change (eg, RNA expression). Epigenetic changes can occur at multiple stages throughout life, including in adulthood, and are not limited to early developmental phases as previously assumed. They represent adaptations or maladaptations to a changed environment and these epigenetic consequences can be moderated by genetic variation, thus providing a molecular mechanism for GxE interaction.

In the context of PTSD, the environmental factors affecting a person are, by definition, different kinds of trauma exposure. Several studies have shown that stressful life events can lead to alterations in epigenetic marks. Here, DNA methylation represents the most examined and best known mechanism. Briefly, cytosine bases (mainly in CpG sites) are converted to 5-methylcytosine by covalent modification. This results in decreased transcription factor binding to these loci which, if located in the promoter region of a gene, subsequently suppresses its transcription.[64]

Pioneering work in this field was performed by Weaver et al.[65,66] in animals showing epigenetic modification induced by early life experience. More specifically, the authors observed that low levels of maternal licking and grooming led to hypermethylation of the hippocampal GR gene in pups followed by decreased GR expression. This study is of great relevance for PTSD because early adverse life experience is an important risk factor for the disorder and the investigated animal model reflects PTSD-related phenotypes such as decreased cortisol levels at baseline as well as increased cortisol suppression after the dexamethasone suppression test[65,66] (reviewed by Anacker et al.,[67] Szyf,[68] and Zhang et al.[69]).

These findings were also translated to humans. McGowan et al.[70] analyzed the human GR promoter (NR3C1) in suicide victims with a history of childhood abuse compared to suicide victims without childhood abuse and control subjects. Comparable to the results of the rodent studies, the *NR3C1* promoter showed increased methylation as well as significantly lower GR expression in postmortem hippocampus tissue of suicide victims who were abused as children.[70]

The fact that epigenetic regulation represents a key mechanism in mediating the long-lasting effects of stressful life events might be of particular interest in PTSD. As a result, numerous studies described in more detail below specifically investigating the epigenetic changes affecting genes regulating the HPA axis have been conducted.

In fact, people with PTSD display significantly lower *NR3C1* promoter methylation compared to healthy controls in DNA from peripheral blood,[71] and differential methylation at these sites serves as a predictor of treatment outcome. In a cohort of combat veterans diagnosed with PTSD, patients with higher pretreatment promoter methylation responded significantly better to psychotherapy.[72] Another study demonstrated that increased *NR3C1* promoter methylation in peripheral blood was associated with less intrusive memory of the traumatic event and reduced PTSD risk in men, and also showed that it may be related to differences in recognition memory-related brain activity.[73]

ADCYAP1R1, previously described as being associated with PTSD symptoms, also shows epigenetic modification. In a study examining *ADCYAP1R1* methylation levels in highly traumatized people with or without PTSD, the authors observed a significant positive correlation between methylation of the *PAC1R* locus and PTSD symptom severity.[47]

Another recently identified player involved in the pathophysiology of PTSD at the epigenetic level is the spindle and kinetochore associated protein 2 (SKA2). SKA2 plays a role in the activation of the GR and, therefore, may moderate negative feedback of the HPA axis. Methylation levels of this locus were recently described as a predictor of suicidal behavior. A study by Guintivano et al.[74] examining human postmortem brain tissue showed significantly increased methylation levels of a CpG (cg13989295) located in the *SKA2* gene as well as significantly decreased *SKA2* expression levels in suicide completers compared to controls. Because of its molecular function and the association of increased suicide rates among PTSD patients, *SKA2* methylation was also investigated in patients with PTSD. A combined predictor using *SKA2* methylation at the above-described CpG (cg13989295) and early trauma scores resulted in a significant prediction of PTSD status.[75]

Two further studies investigated associations between SKA2 methylation at cg13989295 and PTSD.[76,77] In a cohort of 200 soldiers exposed to trauma, Sadeh et al.[76] examined CpG 13989295 methylation levels and PTSD symptoms. The authors observed a positive correlation between PTSD symptoms and *SKA2* methylation levels.[76] A study by Boks et al.[77] investigating a Dutch military cohort showed that *SKA2* methylation together with childhood trauma scores were able to significantly predict posdeployment PTSD symptoms, confirming the findings by Kaminsky et al.[75] and again strengthening the role of *SKA2* as a possible PTSD biomarker.

Besides genes involved in the regulation of the HPA axis, several other

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candidate genes (eg, implicated in the serotonergic or dopaminergic system) have been extensively studied in the context of PTSD.[78,79] Detailed description of these studies is beyond the scope of this review but is available elsewhere.[80,81]

Instead of focusing on a single candidate gene, several studies[82-87] examined DNA methylation on a genome-wide level or focused on subsets of genes involved in stress response, immune regulation, or located in repetitive genomic elements. These studies aim to elucidate specific methylation patterns of PTSD patients across multiple genomic loci. Although no single consistent sites have been identified so far, the combined findings suggest that trauma and PTSD are associated with genome-wide changes in DNA methylation and may have a system-wide impact on the organism.[82-87]

Finally, increasing evidence suggests that parental vulnerability to PTSD can be transmitted to the next generation.[88] Here, epigenetics represents a candidate mechanism involved in the transgenerational transmission. Studies by Yehuda et al.[89,90] have specifically investigated the molecular biologic background of this mechanism, demonstrating alterations in methylation levels of GR and FKBP5 in offspring in relation to parental trauma.[89,90]

It is important to note that epigenetic alterations caused by the environment can be dependent on DNA sequence. Therefore, the epigenetic response of an organism to trauma exposure and the associated individual susceptibility to PTSD can be influenced by genetic variation.[91] One such example of genotype-specific epigenetic changes associated with trauma has been described for the *FKBP5* locus. Here, a combination of a genetic variation that leads to an altered transcriptional response of *FKBP5* to glucocorticoids and exposure to trauma during childhood leads to further DNA demethylation in additional glucocorticoids responsive elements. This combination of genetic and epigenetic factors then leads to a disinhibition of *FKBP5* regulation that has been associated with PTSD and related endophenotypes.[30]

It has become clear that a complex interplay of genetics, environment, and epigenetic changes underlies the pathophysiology of PTSD.

Noncoding RNAs, in particular micro RNAs (miRNAs), represent another important epigenetic mechanism in posttranscriptional regulation of gene expression. miRNAs are non–protein-coding single stranded RNAs about 22 base pairs long that are evolutionarily highly conserved. The biogenesis of miRNAs is a complex multistep biochemical process, which begins in the nucleus and results in the cytoplasm where the mature miRNA becomes functionally active. Here they are incorporated into the RNA-induced silencing complex and perform gene silencing. More precisely, they bind and interact with complementary sites in the 3'UTR region of their target mRNA, which leads to translation repression or degradation of their target (for a detailed review see Ha and Kim[92]).

In the past few years, miRNAs have been progressively emerging as pivotal factors involved in the pathogenesis of psychiatric disorders. Most of the studies examining the role of miRNAs in PTSD were performed in animals and used fear conditioning or predator exposure models.[93-95] These models are thought to explain the underlying mechanisms of fear and the occurrence of trauma-related symptoms in PTSD.[96]

For example, a study by Haramati et al.[94] showed that acute stress leads to a significant upregulation of miR-34c levels in the amygdala of rodents. The authors observed that anxiety-like behavior induced by an acute stressor was significantly decreased after virus-mediated overexpression of this miRNA.[97] Interestingly, the *CRHR1* transcript is one of the main targets of miR-34c.

A recent study in humans identified DICER1 as being involved in the pathogenesis of PTSD.[98] DICER1 is an important enzyme in the biogenesis of miRNAs, converting precursor miRNAs to mature miRNAs. In a cohort of 184 mainly African-American patients with or without diagnosis of PTSD with comorbid depression, the authors observed a significantly lower blood expression of *DICER1* in cases versus controls and were able to replicate these findings in two independent cohorts. This was associated with overall lower miRNA levels in patients versus controls. In a follow-up fMRI study, they showed that the decreased *DICER1* expression levels were associated with elevated amygdala activation to fearful stimuli, which represents a neural correlate for PTSD.[98]

Although still in their primary stages, these and other studies examining miRNAs role in the underlying molecular mechanisms of PTSD show some promising initial results, which nevertheless need to be intensively further investigated.

CONCLUSIONS

Over the last few years, it has become clear that a complex interplay of

genetics, environment, and epigenetic changes underlies the pathophysiology of PTSD. Studying the main genetic effects will not be sufficient to explain the complex and multifactorial etiology of the disease, and environmental factors have to be taken into account. Studies assessing GxE interactions will be important. Here, however, many challenges need to be overcome, ranging from sufficiently large sample size for power to consistent and exhaustive measurement of environmental factors, to statistical issues.[99] Epigenetic modifications may represent a candidate mechanism by which environmental factors interact with genetic predisposition to shape risk and resilience to PTSD.

In the end, a combination of GWAS or better genome-wide GxE interactions in large, well-phenotyped cohorts will be necessary and will need to be combined with mechanistic examinations, including epigenetic measures.

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Eidesstattliche Erklärung

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

München, den 15.09.2020 ……..
Julius Pape

Erklärung

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 2014 mit Erfolg der Doktorprüfung im Hauptfach Medizin bei der Medizinischen Fakultät der Ludwig-Maximilians-Universität München unterzogen habe.

☐ ~~dass ich ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.~~

München, den 15.09.2020 ……..
Julius Pape

Declaration of contribution/as co-author

Publication I

Pape JC, Carrillo-Roa T, Rothbaum BO, Nemeroff CB, Czamara D, Zannas AS, Iosifescu D, Mathew SJ, Neylan TC, Mayberg HS, Dunlop BW, Binder EB. (2018). DNA methylation levels are associated with CRF1 receptor antagonist treatment outcome in women with post-traumatic stress disorder. ***Clinical Epigenetics***, *10*(1). doi: 10.1186/s13148-018-0569-x

Author contributions: JP conceived and designed the research, performed and coordinated all experimental work, performed all statistical analyses and wrote the manuscript.

EB conceived and designed the research and coordinated the experimental work. BD, HM, CN, BR, DI, SM, and TN were responsible for the clinical trial and the different recruitment sites. TC and DC contributed to the statistical analysis. EB contributed to manuscript writing. BR, CN, DC, AZ, DI, SM, TN, HM, and BD revised and edited the manuscript. All authors read and approved the final manuscript.

Publication II

Dunlop BW, Binder EB, Iosifescu D, Mathew SJ, Neylan TC, **Pape JC**, Carrillo-Roa T, Green C, Kinkead B, Grigoriadis D, Rothbaum BO, Nemeroff CB, Mayberg HS. (2017). Corticotropin-Releasing Factor Receptor 1 Antagonism Is Ineffective for Women With Posttraumatic Stress Disorder. ***Biological Psychiatry***, *82*(12), 866–874. doi: 10.1016/j.biopsych.2017.06.024

Author contributions: JP performed DNA extraction and genotype measurement, performed all statistical analyses concerning the genetic data, contributed to manuscript writing as well as final revision and editing.

Publication III

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Author contributions: JP contributed conceiving and designing the human studies, performed RNA extraction, qPCR analysis and the respective statistical analyses, contributed to manuscript writing as well as final revision and editing.

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