In the aftermath of trauma

Marks of stress-related psychopathology

Remmelt R. Schür

IN THE AFTERMATH OF TRAUMA
MARKS OF STRESS-RELATED PSYCHOPATHOLOGY

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In the aftermath of trauma

Marks of stress-related psychopathology

In de nasleep van trauma

Sporen van stress-gerelateerde psychopathologie

(met een samenvatting in het Nederlands)

Proefschrift

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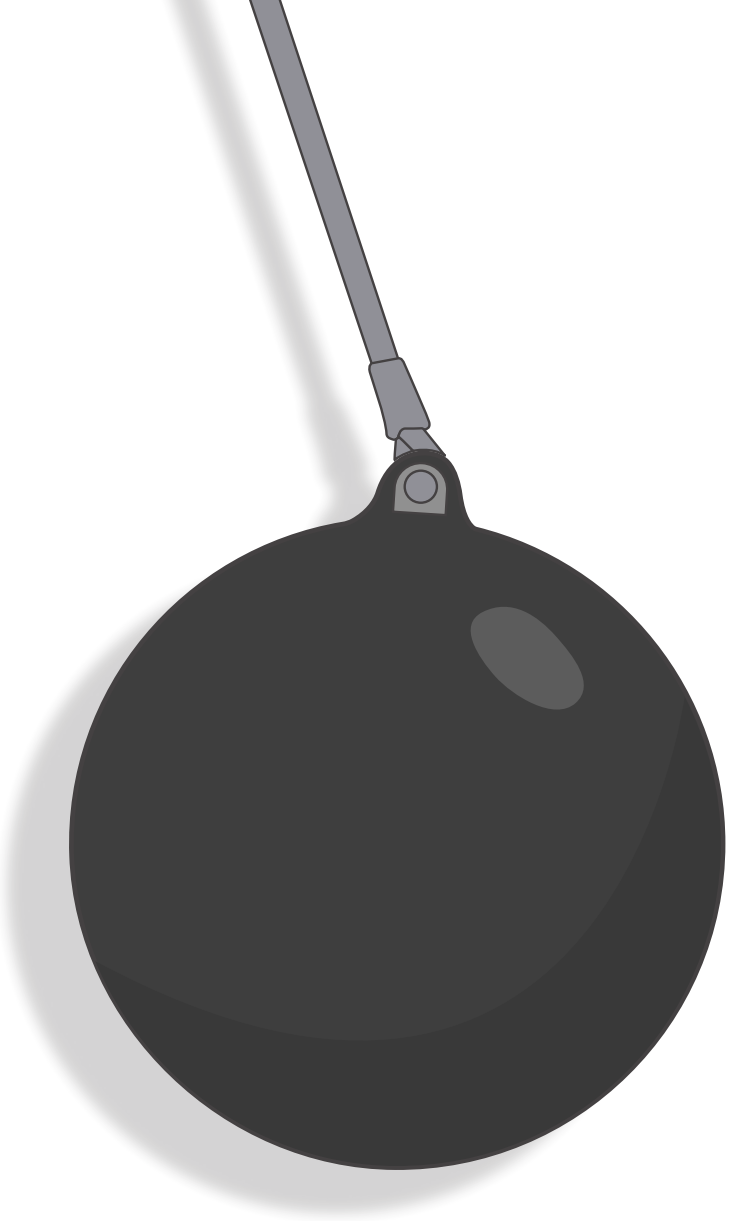
CONTENTS



CHAPTER 1

General introduction

STRESS AND PSYCHOPATHOLOGY

Stress can be defined as the experience of an anticipated or actual threat to an organism's homeostasis (Joëls et al., 2012). Stress represents a key risk factor for the development of a wide range of psychiatric disorders, including posttraumatic stress disorder (PTSD), major depressive disorder (MDD) (Kendler et al., 1999), schizophrenia (Holtzman et al., 2013), bipolar disorder (Agnew-Blais and Danese, 2016) and anxiety disorders (Moreno-Peral et al., 2014). A limited or diminished ability to cope with stressful situations seems crucial for this relationship between stress and psychopathology. Adequate coping with stressful situations requires adaptive physiological changes aimed at attaining stability through change, a process called allostasis (McEwen, 2004). Mediators of such change include steroid hormones (e.g. cortisol, discussed below), neuropeptides, neurotransmitters (e.g. gamma-aminobutyric acid (GABA), discussed below) and cytokines, interacting and functioning in time- and brain region-specific manners (Joëls and Baram, 2009). An inadequate coping ability has been linked to psychiatric disorders and is characterized by a deficit in 1) releasing sufficient mediators in a stressful situation, 2) preventing the mediators from overshooting and/or 3) inhibiting these mediators when the stressful situation has passed (McEwen, 2004). These situations lead to maladaptive changes in the brain and the rest of the body, called allostatic load, and to an increased risk for psychopathology (McEwen, 2004). This thesis focuses on the development of psychopathology in relation to two systems that mediate allostasis: the hypothalamus-pituitary-adrenal (HPA) axis and the GABA system. In the context of stress and psychiatric disorders the HPA axis has received ample attention, whereas the GABA system and particularly its association with the HPA axis has been less explored.

THE HPA AXIS

HPA axis functionality

Following exposure to a perceived threat the hypothalamus releases corticotropin-releasing hormone (CRH), which stimulates the pituitary to secrete adrenocorticotropic hormone (ACTH), which boosts the adrenal glands to produce and release a surge in cortisol (or corticosterone in most rodents) (see Figure 1.1). Cortisol mobilizes glucose storages to provide the organism with energy, refrains the immune system and the sympathetic nervous system (which is the first system to respond to stress), and exerts large effects on the brain (Joëls et al., 2012; Sapolsky et al., 2000). Both rapid (non-genomic) and slow (transcriptional) effects of cortisol on neural activity have been described, mediated by mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (Joëls and Baram, 2009). Genomic effects of cortisol on substructures of the hippocampus and the amygdala (see Figure 1.2) ensure emotional encoding of the stressful situation (Joëls et al., 2012) and may alter an organism's future response to similar situations.

An adaptive cortisol response is characterized by a quick rise in reaction to stress and a rapid decline after the stress has subsided (De Kloet et al., 2005). This decline is the result of GR-mediated negative feedback on the HPA axis, which takes place in the pituitary and to a lesser extent in the hypothalamus. The delay in these feedback (cortisol travels

from the adrenals to the pituitary and the brain) and feedforward (ACTH leads to cortisol secretion) loops gives rise to a fast oscillation of cortisol (the ultradian rhythm) (Walker et al., 2010), which is important for HPA axis functionality and neural functioning (Lightman and Conway-Campbell, 2010). Moreover, cortisol levels depend on the sleep-wake cycle, being high in the morning (to meet metabolic demands during the day) and decreasing during the day (circadian rhythm). As a consequence of these rhythms and superimposed stress, cortisol levels are highly variable over time.

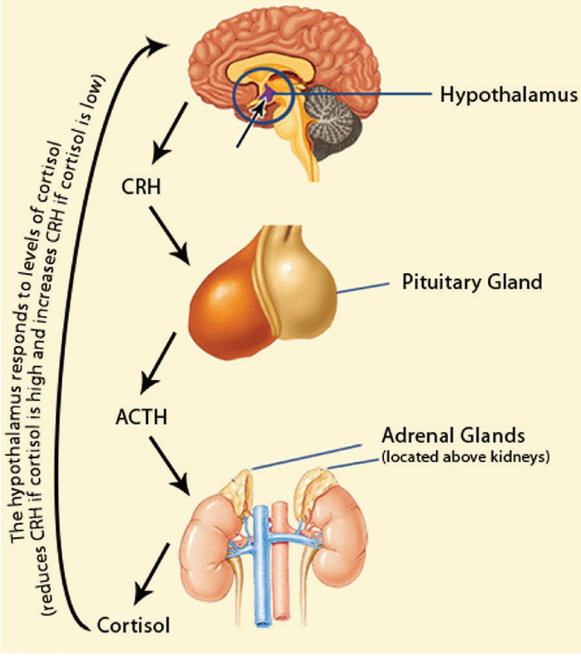


Figure 1.1. The Hypothalamus-pituitary-adrenal (HPA) axis
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Measurement of HPA axis functionality

The high variability of cortisol levels over time necessitates measurement at multiple time points. Cortisol measurements around a stressor, after awakening or in response to dexamethasone (a GR agonist, often combined with CRH) are often employed strategies to probe HPA axis functionality. Psychosocial stress tests, such as the Trier Social Stress Test (Kirschbaum et al., 1993), offer a stress challenge that resembles the real life social situation, as they elicit reactions from suprahypothalamic brain structures (e.g. the prefrontal cortex, the hippocampus and the amygdala (see Figure 1.2)) (Joëls and Baram, 2009), that consequently influence the HPA axis. Many alternatives to cortisol measurement provide additional information about HPA axis functionality (e.g. GR sensitivity or (epi)genetic variation in genes involved in HPA axis functionality).

Effects of traumatic stress on HPA axis functionality

Traumatic stress can have profound effects on HPA axis functionality, an influence that is at least partly mediated by epigenetic mechanisms and present in both humans and rodents (Houtepen et al., 2016; Weaver et al., 2004). Specific sensitive periods during development, especially early in life, seem crucial for HPA axis programming, and during these periods organisms are particularly vulnerable to (traumatic) stress (Lupien et al., 2009).

Prenatally, maternal stress leads to a decrease in GRs and MRs in the rat hippocampus (a crucial structure for negative feedback on the HPA axis) (Lupien et al., 2009). In humans, a link between maternal stress during pregnancy and elevated basal HPA axis activity at the age of 10 years has been established (O'Connor et al., 2005).

HPA axis functionality also seems particularly sensitive to postnatal early life stress. In rats, low maternal care in the first week of life has been associated with decreased GR-mediated negative feedback on the HPA axis in adult life, in part through epigenetic programming of a specific exon in the GR gene (Weaver et al., 2004). In humans, the ortholog of this exon is similarly affected by childhood maltreatment (McGowan et al., 2009). Human studies, however, show contrasting directions of the effect of childhood trauma on HPA axis functionality, with both blunting of the cortisol response to the TSST (Carpenter et al., 2011) and increased cortisol reactivity (Heim et al., 2000). These differences may be partly due to the specific types of abuse (physical abuse and neglect, emotional abuse and neglect, and sexual abuse) and their chronicity (Carpenter et al., 2011), as well their interaction with sex (Juster et al., 2016; Kirschbaum et al., 1999; Kudielka et al., 2004; Stephens et al., 2016) and genetic variation (Shalev et al., 2009; Uhart et al., 2004; Wüst et al., 2009).

Repeated or traumatic stress after childhood seems to have less influence on HPA axis functionality. Nevertheless, stressful life events in adolescence may still impact on HPA axis functionality through epigenetic mechanisms (Van der Knaap et al., 2014). Moreover, rodent studies show morphological changes in hippocampal cells following chronic stress in adulthood (McEwen, 2000). In line with this, Post (Post, 1992) proposed a stress sensitization model, in which recurrent stressors or depressive episodes may lead to progressive neurobiological changes.

HPA axis functionality in relation to psychiatric disorders

Disturbances in HPA axis functionality are thought to partially mediate the well-established relationship between traumatic stress and psychiatric disorders (Kessler et al., 2010). Accordingly, a large body of evidence proposes that HPA axis dysfunctionality is a generic vulnerability factor for psychiatric disorders (Raison and Miller, 2003), such as MDD, PTSD, anxiety disorders (e.g. panic disorder) and schizophrenia. In support, GR knock-out in the limbic system of rodents leads to depressive-like behavior, which normalizes after antidepressant administration (Boyle et al., 2005). In humans, some studies have also shown that antidepressants may restore HPA axis functionality by targeting the hypothalamus and the hippocampus (Holsboer, 2000; Pariante and Miller, 2001). Moreover, some beneficial effects of GR antagonists have been described in the treatment of psychotic depression (Blasey et al., 2011). Further evidence comes from a study demonstrating that healthy members of families highly prevalent in affective disorders showed an HPA axis response to the combined dexamethasone-CRH test in between the response of patients with MDD and healthy individuals without a high genetic load for affective disorders (Modell et al., 1998).

If HPA axis dysfunctionality is causal in the pathogenesis of a range of psychiatric disorders, the question remains to what extent these HPA axis dysfunctionalities are disorder-specific. Most evidence for HPA axis alterations comes from studies on PTSD and MDD, and some hypotheses for disorder-specific HPA axis aberrations have been suggested. In PTSD, hypocortisolism in combination with a sensitized GR (possibly as a result of childhood trauma (Lupien et al., 2009)) may predispose individuals for developing the disorder following a traumatic experience (Van Zuiden et al., 2011; Yehuda, 2001). Similarly, blunting of the cortisol stress response has been proposed as a vulnerability factor for panic disorder (Wintermann et al., 2016). In contrast, MDD seems to be associated with hypercortisolism (Pariante and Miller, 2001) and a desensitized GR (Raison and Miller, 2003), while progressive blunting of the cortisol stress response may be a characteristic of depression chronicity and/or severity (Booij et al., 2013). However appealing these separate hypotheses for MDD and PTSD are, the clinical reality is much more complex, with around 37% of the patients with PTSD also showing sufficient criteria for MDD (Breslau et al., 1991). Moreover, psychiatric comorbidity is rather rule than exception. This highlights the fact that HPA axis alterations are not specific for psychiatric disorders according to the Diagnostic and Statistical Manual of Mental Disorders. Instead, a functional phenotype predicting vulnerability for stress-related disorders, combining HPA axis parameters with individual factors (e.g. sex, age, sex hormone levels and genetic variation), as well as other mediators of allostasis (e.g. GABA), may be necessary to improve diagnostics and therapeutics.

THE GABA SYSTEM

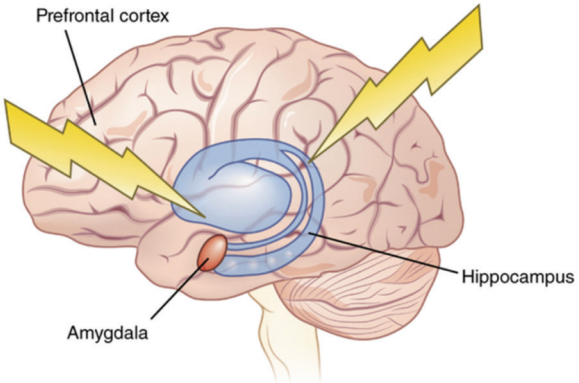
GABA system functionality

GABA is the most important inhibitory neurotransmitter and is present at synapses of one third of all neurons in the human brain (Petty, 1994). It exerts its action via two receptors: the metabotropic $GABA_B$ receptor and the ionotropic $GABA_A$ receptor. Most research has focused on the $GABA_A$ receptor, which is a pentamer of subunits, some of which are mostly extrasynaptic (e.g. the δ subunit), whereas others are generally synaptic (e.g. the γ2 subunit) (Skilbeck et al., 2010). The shaping role of GABA in brain network dynamics makes it essential for a plethora of functions, including memory and attention, sensory processing and stress reactivity (Mody and Pearce, 2004; Möhler, 2007; Vinkers et al., 2010).

We here focus on the role of GABA in stress reactivity, which influences and is influenced by the HPA axis. Brain structures that are key in shaping stress reactivity (see Figure 1.2) target the CRH secreting cells in the paraventricular nucleus (PVN) of the hypothalamus via relay areas (e.g. the bed nucleus of the stria terminalis and the peri-PVN area) (Herman et al., 2003). As such, the hippocampus and the prelimbic prefrontal cortex inhibit CRH secretion via glutamate-GABA projections, whereas the amygdala and the infralimbic prefrontal cortex stimulate CRH secretion via GABA-GABA projections (Ulrich-Lai and Herman, 2009). Conversely, neuroactive steroids (e.g. cortisol, progesterone derivatives and brain-produced steroids) which rise after stress, rapidly inhibit CRH secretion via positive allosteric modulation of the $GABA_A$ receptor (Gunn et al., 2015), and by causing a

retrograde release of nitric oxide via a G protein signaling pathway, which in turn stimulates GABAergic inhibitory control (Di et al., 2009).
Animal research has demonstrated that GABAergic neurotransmission generally decreases within several minutes following acute stress (Gunn et al., 2015), and is restored after 30 minutes, when neuroactive steroid concentrations peak (Barbaccia et al., 2001). These dynamics, however, are sex- and brain region-specific and vary with stress duration and type (Acosta and Rubio, 1994; Borsini et al., 1988; De Groote and Linthorst, 2007; Otero Losada, 1988; Skilbeck et al., 2010). There is only parsimonious human literature examining the role of GABA in stress reactivity and its association with HPA axis functionality. Following acute stress, lower prefrontal GABA levels were observed in subjects anticipating an electrical shock compared with controls (Hasler et al., 2010). With regard to the importance of the GABA system for HPA axis functionality, Uhart et al. (Uhart et al., 2004) showed that genetic variation in a subunit of the $GABA_A$ receptor influenced cortisol stress reactivity. Extensive research in humans, focusing on relevant brain areas and types of stress are needed to shed light upon the role of GABA in acute stress and its interaction with the HPA axis.

Figure 1.2. Brain structures influencing the PVN (partly) through GABAergic projections.
Reprinted by permission from Macmillan Publishers Ltd: Nat. Neurosci. (McEwen et al., 2015)



Measurement of GABA system functionality

During the last two decades of the 20th century, human GABA measurement was predominantly carried out in peripheral sources, such as plasma or cerebrospinal fluid (Gerner and Hare, 1981; Petty et al., 1995, 1992; Petty and Coffman, 1984). Around the turn of the century, proton magnetic resonance spectroscopy (^{1}H-MRS), a technique that allows *in vivo* brain GABA measurement, increasingly gained popularity (Sanacora et al., 1999) and is still widely used (Brix et al., 2015; Rowland et al., 2015). Moreover, positron emission tomography (PET) has proven a very valuable tool for the measurement of *in vivo* brain $GABA_A$ receptor binding (Geuze et al., 2008; Klumpers et al., 2010). Interestingly, a recent PET study (Klumpers et al., 2010) has demonstrated the value of peripheral GABA levels to inform about central GABAergic neurotransmission, as $GABA_A$ receptor binding in several brain regions was correlated to plasma GABA levels.

GABA system functionality in relation to traumatic stress

Throughout life the GABA system undergoes dynamic developmental changes. In the early postnatal period a shift takes place from a predominance in α2 to α1 $GABA_A$ receptor subunits, possibly partially explaining the depolarizing effects of GABA in the pre- and neonatal period as opposed to the hyperpolarizing effects later in life (Hornung and Fritschy, 1996). During puberty $GABA_A$ receptor subunit composition is again altered, now as a result of changing neurosteroid concentrations (Skilbeck et al., 2010). Traumatic stress during these periods may influence long-term programming of GABA system functionality, which could alter the vulnerability for developing psychiatric disorders (Skilbeck et al., 2010).

Studies in rats have demonstrated that prenatal stress alters $GABA_A$ receptor subunit composition in the amygdala (e.g. decreased γ2 subunits) (Barros et al., 2006; Laloux et al., 2012) and reduces GABAergic inhibition in the Cornu Ammonis 1 (CA1) subregion of the hippocampus, (Grigoryan and Segal, 2013).

Similar effects of stress have been described in the early postnatal period, with a decrease in γ2 subunits in the amygdala (Caldji et al., 2000). Moreover, some evidence suggests that early life stress causes a disruption in the shift from α2 to α1 receptors, with more α2 subunits in the adult animals that had been subjected to early life stress (Skilbeck et al., 2010).

The paradoxical excitatory effect of allopregnanolone on GABAergic tone in CA1 during puberty, as was observed in mice (Shen et al., 2007), may indicate higher vulnerability to traumatic stress in this period. However, it is unknown whether and how traumatic stress in puberty enduringly alters GABA system functionality.

In adulthood, chronic stress in rats leads to reduced GABA levels in the hippocampus (Gronli et al., 2007) and the prefrontal cortex (Otero Losada, 1988), as well as reduced $GABA_A$ receptor binding in the prefrontal cortex (Gruen et al., 1995). In humans, increased extrasynaptic $GABA_A$ receptor sensitivity has been described after chronic stress, possibly as a result of lower neurosteroid levels (Bäckström et al., 2013).

These studies demonstrate that traumatic stress may lead to region-dependent changes in GABA system functionality across the lifespan, but the timing, type, duration and magnitude of stress will probably determine the risk for subsequent development of psychiatric disorders.

GABA system functionality in relation to psychiatric disorders

Alterations in GABA system functionality have been most extensively documented in MDD (Luscher et al., 2011), as is the case for HPA axis dysregulation. In addition, convincing evidence exists for GABAergic involvement in PTSD (Geuze et al., 2008; Vaiva et al., 2006), anxiety disorders (Kalueff and Nutt, 2007), schizophrenia (Gonzalez-Burgos et al., 2015) and bipolar disorder (Brambilla et al., 2003). Decreased GABA system functionality has been proposed as a vulnerability factor for the development of both MDD (Luscher et al., 2011; Petty et al., 1995) and PTSD (Vaiva et al., 2006). However, the stability of this trait is questionable given the normal GABA levels in individuals with remitted MDD (Hasler et al., 2005). Moreover, the evidence is fairly equivocal, with most studies showing lower, but some studies showing higher or similar *in vivo* brain GABA levels in patients with MDD compared with healthy individuals (Abdallah et al., 2014; Epperson et al., 2006; Sanacora et al., 2004). This possibly reflects the heterogeneity of the disorder, as well as publication bias.

Of note, animal studies have demonstrated that GABA system dysfunctionality can be at the basis of behavioral changes, possibly via modification of HPA axis functionality (Liu et al., 2007). Alternatively, HPA axis aberrations may lead to GABA system dysfunctionality (Orchinik et al., 2001) and this might mediate the risk for developing psychiatric disorders. Again, integrating information about the GABA system, the HPA axis and other mediators of allostasis seems crucial for a better understanding of pathophysiological processes that may lead to the development of stress-related psychiatric disorders. Given the variety of psychotropic medication targeting the $GABA_A$ receptor (Rudolph and Mohler, 2006) and the HPA axis (DeBattista et al., 2006; Otte et al., 2010), a better understanding of these systems may be an ideal starting point leading to improved therapies for stress-related disorders.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is to further extend preclinical evidence that established the impact of traumatic stress on the HPA axis and the GABA system to the human condition, and to investigate the potential roles of these two systems in the development of psychopathology following traumatic stress. In addition, it explores the association of these two systems in relation to acute stress. Finally, it extends beyond these systems to investigate the contribution of common genetic risk variants on the development of stress-related psychopathology.

PART 1 focuses on the HPA axis in relation to traumatic stress and psychopathology. In chapter 2 we conducted a meta-analysis to evaluate HPA axis reactivity following acute psychosocial stress across psychiatric disorders. In chapter 3 we examined longitudinal methylation changes in exon 1_F of the glucocorticoid receptor gene, a proxy for negative feedback on the HPA axis, in relation to exposure to potentially traumatic events during military deployment and to the development of psychopathology after deployment.

PART 2 examines the GABA system in relation to psychopathology. In chapter 4 we performed a meta-analysis to summarize the literature on brain GABA levels (measured using ^{1}H-MRS) across psychiatric disorders. In chapter 5, we longitudinally examined plasma GABA levels around military deployment in relation to the development of psychopathology.

PART 3 (chapter 6) focuses on the association of the GABA system and the HPA axis in response to acute psychosocial stress in healthy individuals.

PART 4 (chapter 7) explores the value of the interaction between polygenic risk scores for PTSD and MDD with trauma exposure in relation to psychopathology after deployment, as well as GABA and HPA axis parameters.

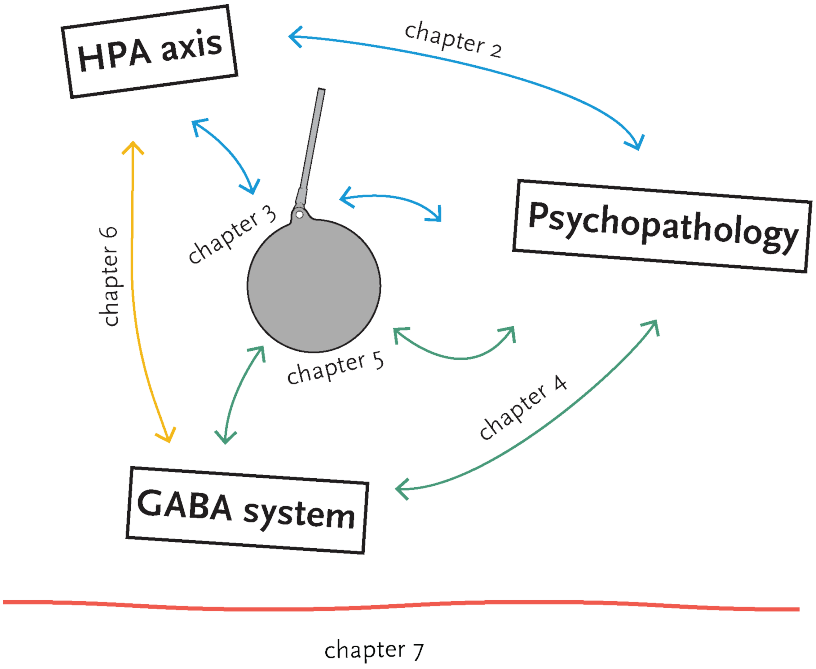
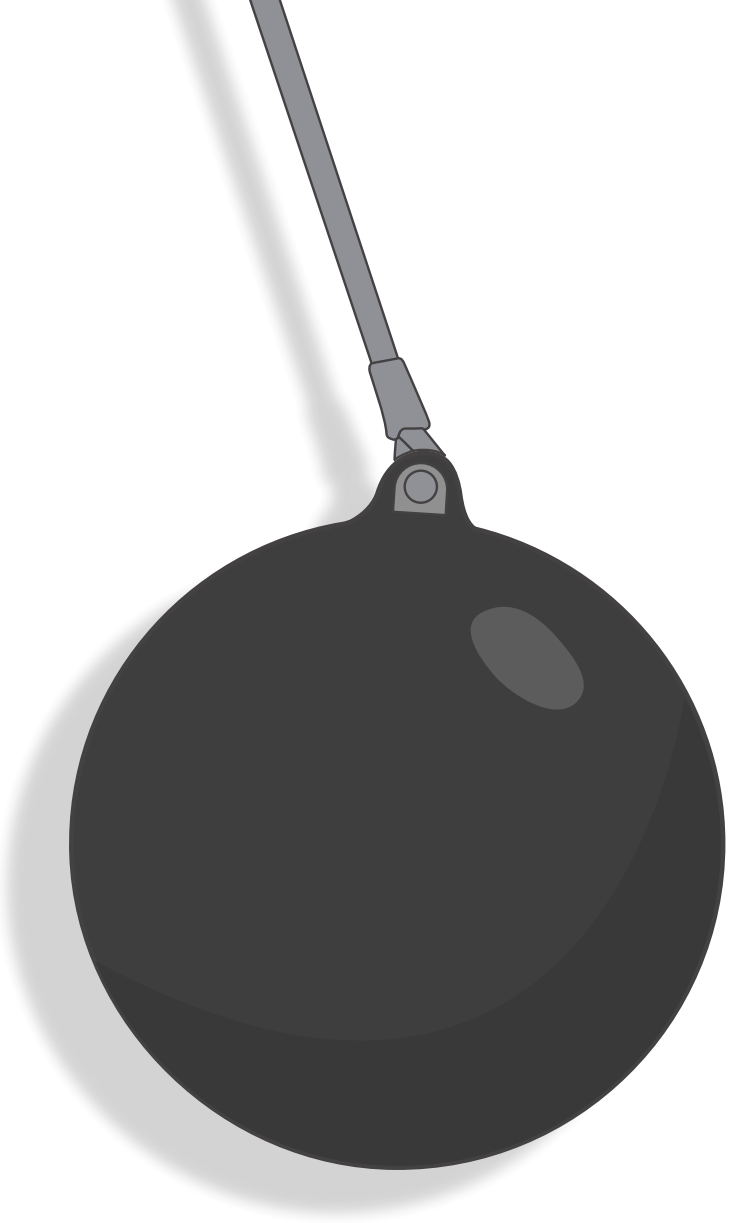


Figure 1.3. Schematic overview of how the chapters in this thesis relate to the main themes.

PART 1

The HPA axis



CHAPTER 2

Cortisol stress reactivity across psychiatric disorders: A systematic review and meta-analysis

Jelle V. Zorn#, Remmelt R. Schür#, Marco P. Boks, René S. Kahn, Marian Joëls, Christiaan H. Vinkers

# Authors contributed equally to the manuscript

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ABSTRACT

The hypothalamus-pituitary-adrenal (HPA) axis and its end product cortisol are essential for an adequate response to stress. Considering the role of stress as a risk factor for psychiatric disorders, it is not surprising that cortisol stress reactivity has frequently been investigated in patients versus healthy individuals. However, the large heterogeneity in measures of the cortisol stress response has hampered a systematic evaluation of the evidence. We here report of a systematic literature review and meta-analysis on cortisol reactivity to psychosocial stress across psychiatric disorders. Original data from authors were obtained to construct standardized cortisol outcomes (the areas under the curve with respect to increase (AUCi) and ground (AUCg)) and to examine the influence of sex and symptomatic state on cortisol stress reactivity. Fourteen studies on major depressive disorder (MDD) (n = 1129), 9 on anxiety disorders (n = 732, including social anxiety disorder (SAD), posttraumatic stress disorder, panic disorder and mixed samples of anxiety disorders) and 4 on schizophrenia (n = 180) were included that used the Trier Social Stress Test or an equivalent psychosocial stress task. Sex-dependent changes in stress reactivity were apparent in MDD and anxiety disorders. Specifically, women with current MDD or an anxiety disorder exhibited a blunted cortisol stress response, whereas men with current MDD or SAD showed an increased cortisol response to psychosocial stress. In individuals with remitted MDD, altered cortisol stress reactivity was less pronounced in women and absent in men. For schizophrenia, cortisol stress reactivity was blunted in both men and women, but the number of studies was limited and showed evidence for publication bias. These findings illustrate that sharing individual data to disentangle the effects of sex, symptom levels and other factors is essential for further understanding of the alterations in cortisol stress reactivity across psychiatric disorders.

1. INTRODUCTION

In order to successfully deal with stress, an adaptive and efficient response of the organism is essential. The hypothalamus-pituitary-adrenal (HPA) axis and its end product cortisol play a crucial role in the response to stress. A dynamic cortisol response, marked by a rapid rise and decline in cortisol levels following stress, is thought to be adaptive and to facilitate adequate coping with perceived threats in the environment. Conversely, changes in cortisol stress reactivity may increase susceptibility to the negative effects of stress. Prolonged, excessive or insufficient activation of the HPA axis may lead to changes in the brain and may subsequently result in the development of psychiatric disorders (McEwen, 2004). Indeed, exposure to repetitive stress is a major risk factor for many psychiatric disorders, including schizophrenia (Holtzman et al., 2013), bipolar disorder (Agnew-Blais and Danese, 2016), major depressive disorder (Kendler et al., 1999), and anxiety disorders (Moreno-Peral et al., 2014). Generally, HPA axis reactivity is assumed to play a crucial role in this relationship between stress and psychopathology (Heim et al., 2008; Holtzman et al., 2013).

Over the past two decades, an increasing number of studies have employed psychosocial stress tests to examine whether psychiatric disorders are associated with changes in HPA axis and cortisol stress reactivity. These laboratory stress tests probe an individual's response to acute social stress and may serve as a proxy for the response to stressful situations in real life. Even though these studies have the potential to inform about HPA axis functionality across psychiatric disorders, the interpretation of the current evidence is hampered by two factors. First, standardized cortisol outcomes based on all available cortisol data (e.g. areas under the curve (Pruessner et al., 2003)), are often not reported in original articles. This problem is reflected in a recent meta-analysis which focused on cortisol levels during the anticipatory and peak phase of the stress response (Ciufolini et al., 2014). Second, the current evidence cannot be reliably interpreted without taking parameters into account which influence stress-induced cortisol levels. Among these parameters, sex and age are known to affect cortisol stress reactivity (Kirschbaum et al., 1999; Kudielka et al., 2004). In support, sex hormones change cortisol stress reactivity (Juster et al., 2016; Stephens et al., 2016). Also, it is currently unknown whether changes in cortisol stress reactivity are consistent across psychiatric disorders and whether these effects are irreversible or dependent on symptomatic state. Finally, several studies suggest an association between medication use and HPA axis activity (Cohrs et al., 2006; Houtepen et al., 2015; Manthey et al., 2011). Detailed information on these relevant parameters is often lacking in the original studies and their influence has not been systematically reviewed.

We here present a meta-analysis of the scientific literature on cortisol reactivity in response to acute psychosocial stress across psychiatric disorders. To this end, we obtained standardized cortisol data from the original studies, as well as data on sex, age, current or remitted symptoms, and medication use. We carried out stratified analyses to investigate whether cortisol stress reactivity across psychiatric disorders depends on sex and current symptomatic state.

2. METHODS

2.1. Literature search and study selection

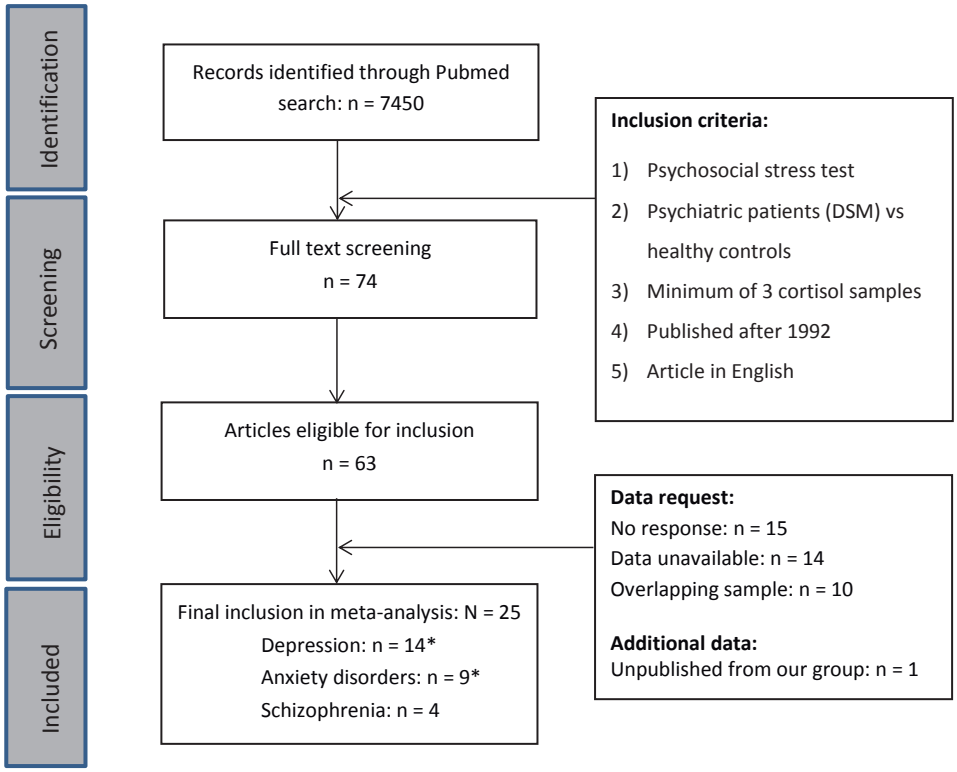
We searched Pubmed on September 1st 2016 for studies investigating cortisol reactivity in response to a laboratory psychosocial stressor in individuals with any psychiatric disorder (DSM axis I) compared with healthy controls (for search terms see Supplementary Table S1). Reference lists of selected articles were screened for additional articles. A minimum of three independent studies was needed for any psychiatric disorder to be included in the meta-analysis. To be selected for inclusion, studies had to: 1) include a laboratory psychosocial stress challenge containing explicit social evaluation (either direct (in front of a public) or indirect (video camera/mirror wall); studies with anticipation of social evaluation were excluded as it refers to anticipation of, rather than response to an acute stressor), 2) compare patients with a psychiatric disorder (according to DSM axis I) with healthy individuals, 3) measure cortisol over at least three consecutive time points (including a sample at baseline and at least 30 minutes after onset of the social stressor to include the peak of the cortisol stress response), 4) be published from 1993 onwards when the Trier Social Stress Test (TSST) was introduced (Kirschbaum et al., 1993), and 5) be written in English.

The literature search resulted in 63 unique studies. Previously unpublished data from our group from a cohort of schizophrenia patients were also included in the meta-analysis, yielding a total of 64 studies. In case of multiple publications based on the same patient cohort, the study with the largest sample size was included after deliberation with the study's authors. Authors of all studies were contacted with a data request, yielding complete cortisol stress data of 25 studies. Two studies included both an MDD and an anxiety disorder group (Dietz et al., 2013; Young et al., 2004), yielding a total of 14 studies on MDD (n = 1129 subjects), 9 studies on anxiety disorders (n = 732) and 4 studies on schizophrenia (n = 180). Thirty-nine studies could not be included due to unavailability of data (n = 14), no response after approaching at least two authors (n = 15), and sample overlap between studies (n = 10) (see Figure 1 for the PRISMA diagram and Supplementary Table S2, for a list of excluded studies).

Individual cortisol levels per time point were used to calculate the area under the curve with respect to increase (AUCi) and ground (AUCg) as measures of stress reactivity, since the use of both measures is recommended (Pruessner et al., 2003). Authors also provided average cortisol values for each time point. AUCs were first calculated for each individual and then averaged across groups. A maximum of one missing cortisol value (at one time point) was replaced by the average of the surrounding data points. If the first, last, or more than one sample was missing, data were discarded.

2.2. Statistical analyses

To evaluate effect sizes across studies, we calculated the standardized mean differences (SMD) in AUCi and AUCg between patients and controls with subsequent stratified analyses for sex. For current and remitted symptom levels, stratified analyses were only carried out for MDD since no data were available in individuals with remitted anxiety disorders or remitted schizophrenia. Separate secondary analyses were carried out for social anxiety disorder (SAD), which was the most frequently studied anxiety disorder.



*Two of the included studies comprised both a depression and anxiety disorder(s) group: Dietz et al. 2013, Young et al. 2004.

Figure 1. PRISMA diagram of the literature search.

Comprehensive Meta-Analysis (Biostat) was used for all analyses (Borenstein et al., 2005). Assuming methodological and clinical heterogeneity, as well as a common among-study variance component across subgroups, we used random effects models to calculate both subgroup results and results over subgroups. We focused on the AUCi to explore evidence for heterogeneity, and publication bias, as it is most sensitive to stress-induced changes. Cochrane's Q-test and the I^2 statistic were used to evaluate heterogeneity (Higgins et al., 2003). Publication bias was assessed using Egger's test and by constructing funnel plots (Egger et al., 1997). Finally, baseline cortisol levels of patients and controls, stratified for sex, were compared using two-sample t-tests.

2.3. Visualization of average cortisol levels (including baseline cortisol levels)

Timing of cortisol measurements varied considerably across studies. To display the cortisol stress response across studies, weighted means and standardized errors of the means of cortisol levels were calculated over seven time bins (-10-+4, 5-14, 15-24, 25-34, 35-44, 45-59, 60-80 min) based on an even sample distribution across different time bins. If more than one cortisol value was present in a certain time bin, data were averaged (17 studies). There was a large variability in absolute cortisol levels due to heterogeneity in analytic methods and tissue type. To enable the direct comparison of fluctuating cortisol levels over time across studies, averaged pre-stress (baseline) cortisol levels per study were set to 100% and consecutive cortisol levels were plotted relative to the initial 100%, thereby informing about the AUCi. To compare baseline cortisol levels between patients and healthy controls, averaged baseline (pre-stress) cortisol levels for patients were calculated relative to those of controls (100%) and subsequently weighed for sample size per study.

2.4. Exploration of the influence of age and medication use on effect size

To assess the influence of age and medication use on effect size, we focused on the AUCi. Mixed-effects meta-regressions were performed to explore the effect of age over all included psychiatric disorders (MDD, anxiety, and schizophrenia). Two included studies comprised multiple patient groups (Young et al., 2004: cMDD, anxiety disorders; Dietz et al., 2013: cMDD, rMDD, anxiety disorders) and only a single control group. In both cases, only the patient group with the largest sample size was included in the analyses. For medication use, the mean percentage of patients using psychotropic medication for MDD or an anxiety disorder was used. Studies in which mean percentage medication use was below 5% were excluded from the analysis. For schizophrenia, medication use was known for each individual patient. Antipsychotic drug doses were transformed to chlorpromazine (CPZ) equivalents (Woods, 2003) and a standard linear regression was carried out to assess the ability of CPZ equivalents to predict cortisol stress reactivity (AUCi), including only individuals with complete dose information.

3. RESULTS

3.1. Study characteristics

3.1.1. General

Study characteristics are shown in Table 1. Additional information on method of diagnostic assessment, medication use, contraception use and type of psychosocial stress intervention is included in the Supplementary Information (Supplementary Table S3). In addition, the graphs of cortisol levels over time from the original studies are included in the Supplementary Information (Supplementary Figure S1).

3.1.2. Major Depressive Disorder (MDD)

For MDD, a total of 14 studies were included. Eight studies investigated currently depressed patients (Klimes-Dougan et al., 2014; Mazurka et al., 2016; Morris et al., 2014; Pierrehumbert et al., 2009; Stewart et al., 2013; Taylor et al., 2009; Weinstein et al., 2010;

Young et al., 2004), 5 studies examined remitted individuals (Ahrens et al., 2008; Bagley et al., 2011; Höhne et al., 2014; Lange et al., 2013; Morris and Rao, 2014), and one study included both depressed and remitted individuals, using the same control group (Dietz et al., 2013). A total of 308 currently depressed patients (92 males/216 females) compared with 392 healthy controls (157 males/235 females), as well as 219 remitted individuals (97 males/122 females) compared with 351 healthy controls (173 males/178 females) were included. The mean age of subjects in the studies was 30.5 years (range: 15.7-62.3). All studies used the TSST or a modified version, except for two studies. The study of Ahrens et al. (Ahrens et al., 2008) used the Groningen acute social stress test (GAST), whereas Weinstein and colleagues (Weinstein et al., 2010), had subjects perform an anger recall and mental arithmetic task in front of an evaluating panel. All studies were conducted in the afternoon. Ten studies measured cortisol in saliva, whereas 4 studies used blood samples (see Table 1). Five studies required patients to be free of psychotropic medication, while the percentage of medication use in MDD patients in the other 9 studies ranged from 14 to 68%. Eight studies provided no information on contraceptive use, in 3 studies female individuals used no contraceptives and in 3 studies contraceptives were used (in 64% of the sample in one study and in an unknown percentage in the two other) (see Supplementary Table S3). Finally, with regard to comorbidity, 6 studies provided no information, one study reported its absence and 4 studies included patients with comorbid psychiatric disorders (in 9 to 67% of the sample and unknown in one study) (see Table 1).

3.1.3. Anxiety disorders

For anxiety disorders, a total of 9 studies were included. Five studies examined SAD (Klumbies et al., 2014; Krämer et al., 2012; Roelofs et al., 2009; Van West et al., 2008; Yoon and Joormann, 2012), one of which also included patients with posttraumatic stress disorder (PTSD) (Roelofs et al., 2009). Four other studies examined patients with PTSD (Zaba et al., 2015), panic disorder (Petrowski et al., 2013), and various types of anxiety disorders (Dietz et al., 2013; Young et al., 2004). A total of 311 patients with an anxiety disorder (129 males/182 females) and 421 healthy controls (204 males/217 females) were included. The mean age of subjects in the studies was 25.8 years (range: 9.5-40.8). All but one study used the TSST or a modified version. In the study by Yoon and colleagues (Yoon and Joormann, 2012), subjects were told that their speech would be recorded and evaluated by a committee (no direct evaluation). This was the only study that failed to show a significant stress effect on cortisol levels in healthy controls (see Supplementary Figure S1). All studies were conducted in the afternoon. Seven studies used saliva samples, and two studies used blood samples (see Table 1). Five studies included only medication-free patients, whereas the percentage of medication use in the other 4 studies ranged from 20 to 72%. Three studies provided no information on contraceptives, in two studies subjects were free of contraceptives, in 3 studies contraceptive use was known (range: 25-47%) and in one study the percentage was unknown (see Supplementary Table S3). Finally, in one study subjects had no comorbid psychiatric disorders, in 5 studies comorbidity was present but in an unknown fraction of the sample, and in 3 studies comorbidity ranged from 37 to 58% (see Table 1).

	Population	Sample (Pt/C)	Sex (% F)	Age, mean (SD) (Pt/C)	Sample type	Time of testing	Comorbidity (%)
Depression							
Young 2004[1]	cMDD	81 (33/48)	63	25.7 (7.1)[8]/26.0 (7.1)	Blood	Afternoon	55
Taylor 2009[2]	cMDD	65 (46/19)	60	62.3 (6.4)/62.5 (6.0)	Saliva	Afternoon	Unknown
Ahrens 2008	rMDD	39(19/20)	100	51.0 (1.7)/54.2 (1.6)	Blood	Afternoon	Unknown
Pierrehumbert 2009	cMDD	41 (14/27)	100	33.0 (7.1)[9]	Saliva	Afternoon	Unknown
Weinstein 2010	cMDD	28 (14/14)	50	41.7 (9.6)/39.3 (5.6)	Blood	Afternoon	Unknown
Bagley 2011	rMDD	57 (22/35)	40	34.2 (1.7)/31.8 (1.2)	Saliva	Afternoon	No
Dietz 2013[3]	cMDD/rMDD	213 (cMDD 55/ rMDD 17/C 141)	49	17.2 (3.5)[8,10]	Saliva	Afternoon	Yes, % unclear[11]
Lange 2013	rMDD	147 (70/77)	51	38.1 (11.8)/38.6 (11.1)	Saliva	Afternoon	No
Stewart 2013	cMDD	64 (31/33)	73	16.0 (1.6)/14.6 (2.0)	Saliva	Afternoon	55
Klimes-Dougan 2014	cMDD	75 (49/26)	77	15.7 (1.7)/16.3 (2.1)	Saliva	Afternoon	67
Morris and Wang 2014	rMDD	53 (30/23)	62	23.2 (3.9)/22.6 (3.8)	Saliva	Afternoon	Unknown
Morris 2014[4]	cMDD	50 (24/26)	62	18.3 (4.7)/20.9 (3.2)	Saliva	Afternoon	21
Höhne 2014	rMDD	116 (61/55)	48	34.7 (3.3)/34.0 (3.6)	Blood	Afternoon	Unknown
Mazurka 2015	cMDD	100(42/58)	72	16.6(2.1)/15.8 (2.5)	Saliva	Afternoon	9-11%
Anxiety							
Young 2004[1]	M anx[6]	63 (15/48)	62	25.4 (8.5)/26.0 (7.1)	Blood	Afternoon	Yes, % unclear
Van West 2008	SAD	50 (25/25)	32	9.5 (0.8)/8.9 (1.5)	Saliva	Afternoon	56
Roelofs 2009	SAD/ PTSD	57(SAD 18/PTSD 17/C 22)	58	SAD 32.3 (3.2)/PTSD 35.8 (3.3)/C 39.9 (2.9)	Saliva	Afternoon	Yes, % unclear[12]
Yoon 2012	SAD	62 (40/22)	50	32.7 (11.4)[8]/37.8 (10.8)	Saliva	Afternoon	58[12]
Krämer 2012	SAD	77 (37/40)	49	10.1 (1.4)/10.0 (1.1)	Saliva	Afternoon	37
Petrowski 2013	PD	64 (32/32)	62	32.9 (11.2)/31.2 (11.1)	Saliva	Afternoon	No
Dietz 2013[4]	M anx[7]	177 (36/141)	53	17.2 (3.5)[8,10]	Saliva	Afternoon	Yes, % unclear[11,12]
Klumbies 2014	SAD	141 (68/73)	48	29.7 (9.6)/30.2 (10.0)	Saliva	Afternoon	Yes, % unclear[12]
Zaba 2015	PTSD	41 (23/18)	100	40.8 (11.5)/39.0 (11.6)	Blood	Afternoon	Yes, % unclear[12]
Schizophrenia							
Jansen 1998	Scz	19 (10/9)	0	27.1 (7.0)/26.9 (5.8)	Saliva	All day	Unknown
Jansen 2000	Scz	39 (18/21)	38	27.7 (4.3)/27.0 (5.4)	Saliva	All day	Unknown
Brenner 2011	Scz	59 (30/29)	24	30.5 (7.3)/29.3 (8.1)	Saliva	Afternoon	Unknown
Houtepen[5]	Scz	63 (15/48)	49	40.1(13.9)/43.5(15.6)	Saliva	Afternoon	Unknown

Table 1. Study Characteristics

All reported sample sizes are based on data received from authors. If a study assessed both blood and saliva, we used saliva data. Pt = patients; C = controls; F = female; SD = standard deviation; c/rMDD = current/remitted major depressive disorder; M anx = mixed anxiety; SAD = social anxiety disorder; PTSD = posttraumatic stress disorder; PD = panic disorder; Scz = schizophrenia.

(1) The original study contained 4 groups (only MDD, comorbid MDD, only anxiety, controls). We combined data from the only and comorbid MDD group. All patients are unique, whereas controls reported under MDD and anxiety are identical.
(2) High cardiovascular disease risk.
(3) Patients vs controls was not the primary interest of the original article (bereaved vs non-bereaved youth). The authors created an MDD (major depressive disorder, dysthymia, and depressive disorder not otherwise specified), anxiety and healthy control group on request. All patients are unique, whereas controls reported for MDD and anxiety are identical.
(4) Participants underwent the TSST twice, during a major depressive episode and again during remission. As the authors reported evidence of habituation effects, only data of the first TSST were included in the meta-analysis.
(5) Unpublished data from our institution.
(6) Panic, generalized, or social anxiety disorder.
(7) Type unknown.
(8) Weighted mean and pooled standard deviation of two separate groups (Young: pure/comorbid MDD; Dietz: bereaved vs non-bereaved youth; Yoon: pure/comorbid SAD).
(9) Patients vs controls was not the primary interest of the original article (abused vs non-abused). The authors created an MDD and healthy control group on request.
(10) Overall age.
(11) ADHD, alcohol/substance abuse and or dependence, behavioral disorders (oppositional defiant disorder and/or conduct disorder), PTSD, bipolar disorder and psychosis.

3.1.4. Schizophrenia

For schizophrenia, 4 studies (including unpublished data by Houtepen et al.) investigated the cortisol response to psychosocial stress (Brenner et al., 2011; Jansen et al., 2000, 1998). A total of 73 patients (54 males/19 females) and 107 healthy controls (66 males/41 females) were included. The mean age of participants in the studies was 31.3 years (range: 27.1-40.1). Two studies were conducted in the afternoon and two over the course of the day. All studies used saliva samples (see Table 1). Medication use in the 4 studies ranged from 80-100%. In one study subjects were free of contraceptives, in another study subjects used contraceptives but the fraction was unknown, one study provided no information on contraceptive use, and one study included only men (Supplementary Table 3). None of the studies provided information on comorbidity (Table 1).

3.2. Cortisol stress response across psychiatric disorders

3.2.1. Major Depressive Disorder (MDD)

Overall, patients with MDD showed a significantly lower AUCi (SMD = -0.18, 95% confidence interval (CI): -0.336 to -0.028, p = 0.02) but a similar AUCg (SMD = -0.077, 95% CI: -0.214 to 0.061, p = 0.28) compared with healthy controls (n = 1249, 515 patients). Results of stratified analyses were not significant for currently depressed (n = 680, 296 patients; AUCi: SMD = -0.180, 95% CI: -0.382 to 0.023, p = 0.08; AUCg: SMD = -0.048, 95% CI: -0.0229 to 0.133, p = 0.60) and remitted individuals (n = 569, 219 patients; AUCi: SMD = -0.184, 95% CI: -0.422 to 0.053, p = 0.13; AUCg: SMD = -0.115 95% CI: -0.327 to 0.096, p = 0.28).

Stratified analyses for sex showed clear differences between men and women. Overall, female patients with MDD exhibited a significantly lower AUCi (SMD = -0.342, 95% CI: -0.538 to -0.145, p < 0.01) (Figure 2) and AUCg (SMD = -0.254, 95% CI: -0.410 to -0.099, p < 0.01) (see Supplementary Figure S2) compared with healthy controls (n = 736, 329 patients). Subgroup analyses based on current symptomatic state demonstrated that the effect was significant in depressed women with MDD for both the AUCi (SMD = -0.384, 95% CI: -0.637 to -0.131, p < 0.01) and the AUCg (SMD = -0.239, 95% CI: -0.437 to -0.040, p = 0.02) (n = 437, 207 patients). In remitted women, the AUCg was significantly lower compared with controls (SMD = -0.280, 95% CI: -0.532 to -0.028, p = 0.03), but the AUCi was not (SMD = -0.276, 95% CI: -0.590 to 0.038, p = 0.09) (n = 299, 122 remitted individuals). The lower AUCi for females was supported by the visualization of the cortisol stress response over time (Figure 5A), with less reactivity after stress in depressed female patients compared with healthy controls. Moreover, in agreement with the statistical results, remitted females showed no clear differences compared with controls (Figure 5C).

In contrast, currently depressed men with MDD showed an elevated AUCg compared with controls (SMD = 0.399, 95% CI: 0.061 to 0.738, p = 0.02; n = 243, 89 patients) (see Supplementary Figure S3), but not a significantly different AUCi (SMD = 0.210, 95% CI: -0.136 to 0.557, p = 0.23) (Figure 3). No significant differences were found in remitted men (n = 270, 97 remitted individuals) for both the AUCi (SMD = -0.046, 95% CI: -0.420 to 0.327 p = 0.81) and the AUCg (SMD = 0.100, 95% CI: -0.252 to 0.452, p = 0.58), nor in the overall group, independent of symptomatic state (n = 513, 186 patients; AUCi: SMD = 0.092, 95% CI: -0.162 to 0.346, p = 0.48; AUCg: SMD = 0.254, 95% CI: -0.039 to 0.547, p = 0.09). Cortisol reactivity after stress appeared consistently higher for men with MDD

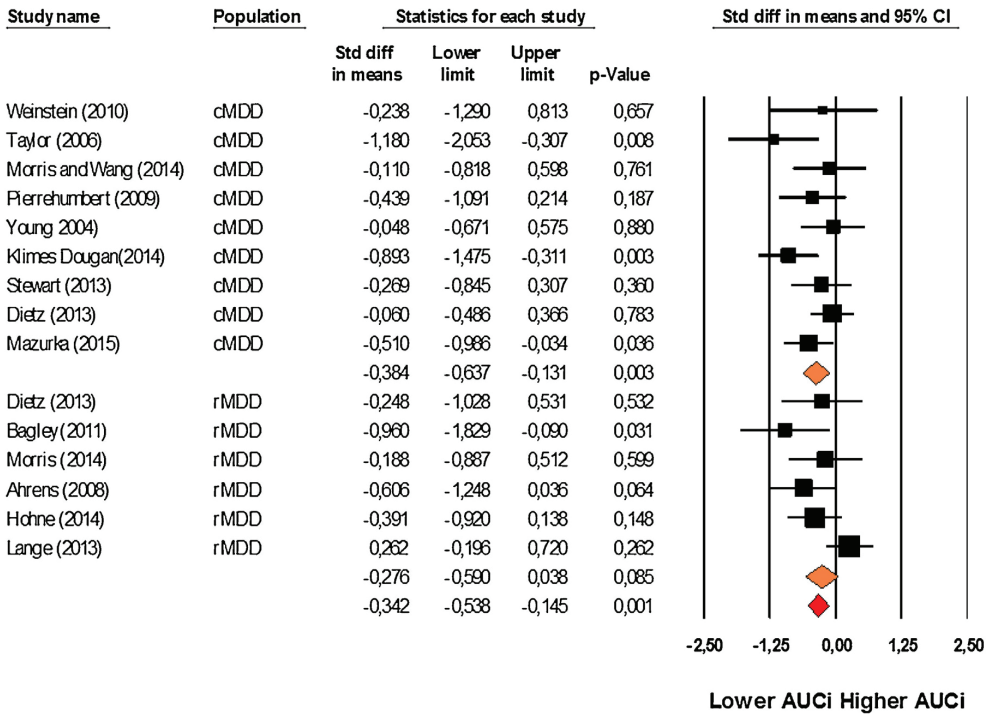


Figure 2. Forest plot of the cortisol AUCi in response to a psychosocial laboratory stressor in women with major depressive disorder. Diamond shaped orange symbols represent current MDD (upper symbol) and remitted MDD (lower symbol). Diamond shaped red symbol represents a summary of all MDD studies. Size of the black squares is proportionate to the sample size used. cMDD = current major depressive disorder; rMDD = remitted major depressive disorder. The same control group was used for the analyses of cMDD and rMDD of Dietz et al.

compared with controls, whereas it seemed similar to controls in remitted individuals (see Figures 5B and 5D). Nevertheless, these observations were not statistically tested and could not be directly deduced from the AUCi.

3.2.2. Anxiety disorders

Overall, no statistically significant difference in cortisol following psychosocial stress was found between patients with an anxiety disorder and healthy controls (n = 723, 308 patients; AUCi: SMD = -0.101, 95% CI: -0.285 to 0.082, p = 0.28; AUCg: SMD = -0.031, 95% CI: -0.345 to 0.406, p = 0.87). Stratified analyses based on sex showed a significantly lower AUCi in female patients with an anxiety disorder compared with healthy controls (SMD = -0.288, 95% CI: -0.564 to -0.012, p = 0.04) (Figure 4), but a similar AUCg (SMD = 0.010, 95% CI: -0.440 to 0.459, p = 0.97) (see Supplementary Figure S4) (n = 393, 180 patients). No significant differences were found in men for both outcomes (n = 330, 128 patients; AUCi: SMD = 0.077, 95% CI: -0.163 to 0.316, p = 0.53; AUCg: SMD = 0.182, 95% CI: -0.235 to 0.599, p = 0.39). The lower AUCi for females was supported by the course of the cortisol stress response (see Figure 5E), with less reactive cortisol levels after stress in female

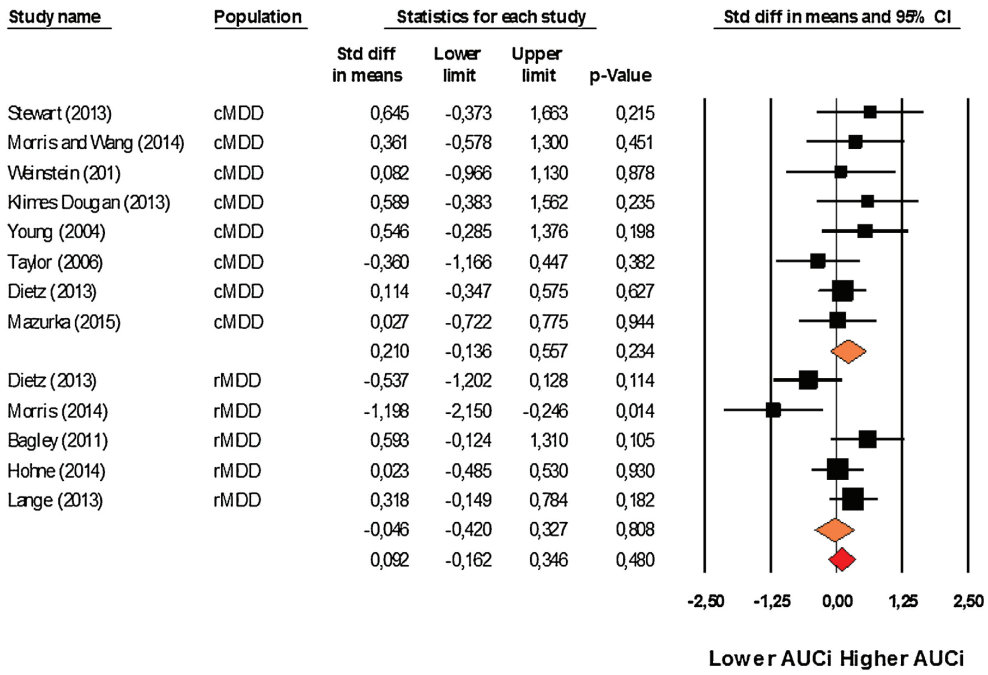


Figure 3. Forest plot of the cortisol AUCi in response to a psychosocial laboratory stressor in men with major depressive disorder. Diamond shaped orange symbols represent current MDD (upper symbol) and remitted MDD (lower symbol). Diamond shaped red symbol represents a summary of all MDD studies. Size of the black squares is proportionate to the sample size used. cMDD = current major depressive disorder; rMDD = remitted major depressive disorder. The same control group was used for the analyses of cMDD and rMDD of Dietz et al.

patients with an anxiety disorder compared with healthy controls. For male patients the opposite pattern was apparent, albeit with marginally higher cortisol stress reactivity than controls (Figure 5F). Secondary analyses in patients with SAD alone revealed a higher AUCg in male patients compared with controls (n = 195, 95 patients; SMD = 0.50, 95% CI: 0.055 to 0.948, p = 0.03), but a similar AUCi (SMD = 0.077, 95% CI: -0.227 to 0.381, p = 0.62) (see Supplementary Figures S5A and S5B). No significant differences were present in women (n = 175, 93 patients; AUCi: SMD = -0.187, 95% CI: -0.601 to 0.226, p = 0.38; AUCg: SMD = 0.282, 95% CI: -0.300 to 0.865, p = 0.34).

3.2.3. Schizophrenia

There was a significant overall difference in the cortisol stress response between schizophrenia patients and healthy controls (n = 180, 73 patients; AUCi: SMD = -0.594, 95% CI: -1.150 to -0.037, p = 0.04; AUCg: SMD = -0.543, 95% CI: -0.968 to -0.118, p = 0.01). Subgroup analyses revealed a significantly lower AUCi (SMD = -0.840, 95% CI: -1.591 to -0.088, p = 0.03) (Figure 4) and AUCg (SMD = -0.641, 95% CI: -1.018 to -0.263, p < 0.01) (see Supplementary Figure S4) in male patients compared with controls (n = 120, 54 patients). Female patients only showed a significantly lower AUCg compared with controls

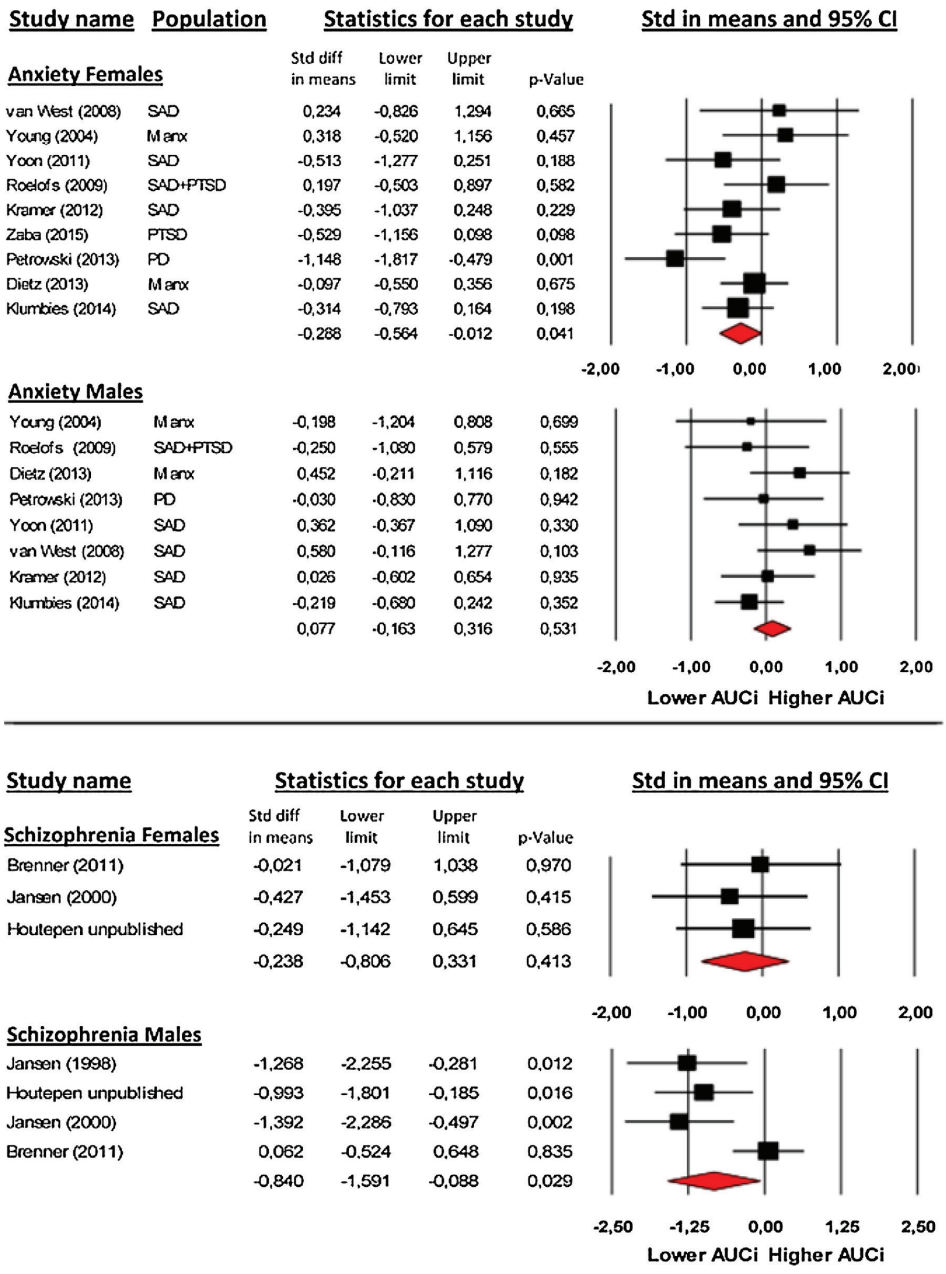


Figure 4. Forest plots of the cortisol AUCi in response to a psychosocial laboratory stressor in anxiety disorders and schizophrenia. Diamond shaped red symbols represent summaries of all studies per disorder, stratified for sex. Size of the black squares is proportionate to the sample size used. SAD = social anxiety disorder; PTSD = posttraumatic stress disorder; PD = panic disorder, M anx = mixed anxiety.

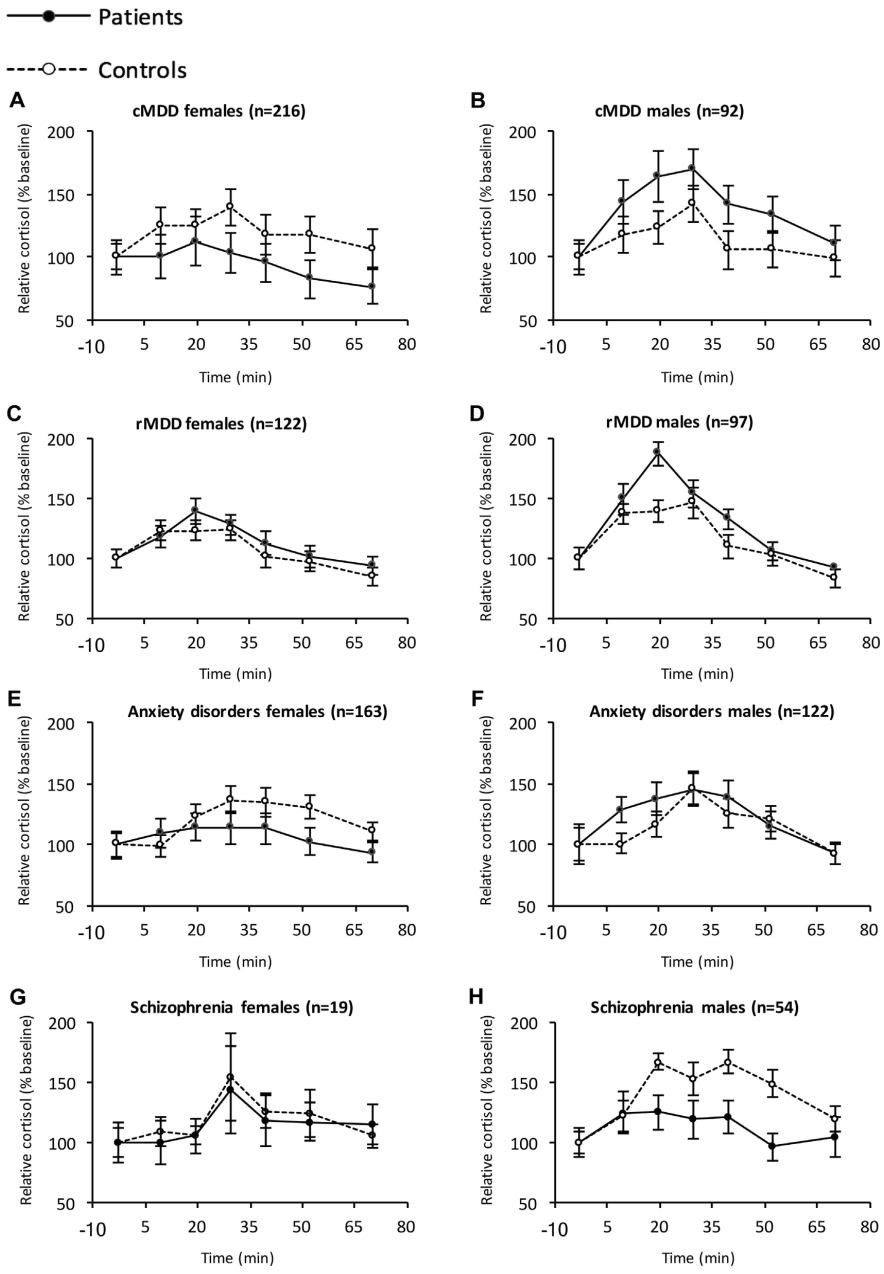


Figure 5. Graphs of the averaged cortisol response across different subgroups. Weighted means ± SD are shown for all subgroups. Averaged pre-stress (baseline) cortisol levels per study were set to 100% and consecutive cortisol levels were plotted relative to the initial 100%, thereby informing about the AUCi. cMDD = current major depressive disorder; rMDD = remitted major depressive disorder.

(n = 60, 19 patients; AUCi: SMD = -0.238, 95% CI: -0.806 to 0.331, p = 0.41; AUCg: SMD = -0.752, 95% CI: -0.146 to -0.042, p = 0.04). The AUCi findings were supported by the course of the cortisol stress response (see Figure 5H and 5G).

3.2.4. Age and cortisol stress reactivity

Meta-regressions stratified for sex were performed to assess the effect of age on effect size (SMD) over all included psychiatric disorders (MDD, anxiety disorders and schizophrenia). For women, 24 studies with 488 patients and 501 healthy controls, and for men 22 studies with 355 patients and 419 healthy controls were included in the analyses. No significant relationship between age and effect size was found for women (coefficient: -0.005, 95% CI: -0.016 to 0.006, p = 0.41), or men (coefficient: -0.005, 95% CI: -0.018 to 0.008, p = 0.44) (see Supplementary Figures S6A and S6B).

3.2.5. Medication use and cortisol stress reactivity

Twelve studies (9 MDD; 3 anxiety disorders) with 429 patients and 487 healthy controls were included in this meta-regression analysis (medication use ranging from 14-72%). No significant relationship between effect size and percentage medication use was found (coefficient: -0.002, 95% CI: -0.010 to 0.006, p = 0.60) (see Supplementary Figure S7). For schizophrenia, the relationship between antipsychotic drug dose (CPZ equivalents) and cortisol stress reactivity was explored using individual patient data. Complete dose information was available for 54 patients (15 females, 39 males). CPZ equivalents were not significantly associated with cortisol stress reactivity ($F(1,54) = 0.12$, p = 0.39) (see Supplementary Figure S8).

3.2.6. Baseline cortisol levels across studies

Baseline cortisol levels were similar for female patients compared with female controls (cMDD: n = 449, t = 0.85, p = 0.40; rMDD: n = 300, t = 0.86, p = 0.39; anxiety disorders: n = 421, t = 1.01, p = 0.31; schizophrenia: n = 60, t = 0.44, p = 0.66) and for male patients compared with male controls (cMDD: n = 247, t = 0.64, p = 0.52; rMDD: n = 270, t = 0.06, p = 0.95; anxiety disorders: n = 333, t = 0.49, p = 0.62; schizophrenia: n = 119, t = 0.42, p =0.67) (Supplementary Figure S9).

3.2.7. Publication bias

Funnel plots were created for studies on cMDD, rMDD, anxiety disorders, and schizophrenia, including separate publication bias analyses for male and female analyses (Supplementary Figure S10). No apparent publication bias was present, except for the comparison of male schizophrenia patients with healthy controls (Egger's test two-tailed p = 0.03).

3.2.8. Heterogeneity

Heterogeneity was tested for cMDD, rMDD, anxiety disorders, and schizophrenia, also stratified based on sex. No significant heterogeneity was detected for studies investigating MDD (p = 0.13, I^2 = 30%), including analyses restricted to females (p = 0.13, I^2 = 30%) and males (p = 0.11, I^2 = 34%). No significant heterogeneity was detected in studies investigating anxiety disorders (p = 0.23, I^2 = 24%), including stratified sex analyses

(females: p = 0.12, I^2 = 38%; males: p = 0.50, I^2 = 0%). For schizophrenia, significant heterogeneity was detected for the overall analyses (p = 0.04, I^2 = 65%) and for males (p = 0.02, I^2 = 71%) but not for females (p = 0.86, I^2 = 0%).

4. DISCUSSION

In this meta-analysis we investigated the cortisol response to psychosocial stress across psychiatric disorders. We found sex-specific changes in cortisol stress reactivity for MDD and anxiety disorders. Specifically, we found that women with current MDD or an anxiety disorder exhibited a blunted cortisol stress response compared with healthy controls, whereas men with current MDD or SAD showed an elevated cortisol response. For schizophrenia, the cortisol response to psychosocial stress was blunted in both male and female patients, but the limited sample size and possible publication bias preclude firm conclusions. In contrast to a previous meta-analysis (Ciufolini et al., 2014), we used standardized measures for the cortisol response (AUCi and AUCg), taking all cortisol measurements into account. Moreover, we visualized the stress response over time and stratified our analyses based on sex and symptomatic state.

4.1. Influence of sex and sex hormones

Our results highlight that sex is an important factor when studying cortisol stress reactivity across psychiatric disorders. An increasing body of evidence indicates substantial sex differences in the cortisol response to stress in healthy individuals and these effects are more evident in saliva (only free cortisol) than in blood (total cortisol) (Foley and Kirschbaum, 2010). Specifically, women in the luteal phase of the menstrual cycle have a similar cortisol response as men, while women in the follicular phase, menopause (with low estrogen and progesterone levels) and those using oral contraceptives (which reduce free cortisol (Kirschbaum et al., 1999; Kudielka et al., 2004)) show blunting of the cortisol response (Kirschbaum et al., 1999). This finding was further substantiated in two recent studies (Juster et al., 2016; Stephens et al., 2016). Stephens and colleagues (Stephens et al., 2016) demonstrated a greater TSST cortisol response in men than in women (all women in the follicular phase). Furthermore, higher testosterone levels were associated with lower cortisol responses in men and higher progesterone levels had the same effect in women. In contrast, Juster and colleagues (Juster et al., 2016) demonstrated a negative association between TSST cortisol reactivity and progesterone in men, and between TSST cortisol reactivity and estradiol or testosterone in women. Moreover, despite the rise of sex hormones in response to acute stress (Lennartsson et al., 2012), baseline levels of testosterone, estradiol and progesterone could partially explain sex differences in cortisol stress reactivity (Juster et al., 2016). Although the neurobiological mechanisms underlying sex-dependent HPA axis activity remain largely elusive (Handa and Weiser, 2014), the present meta-analysis underlines the importance of accounting for sex when investigating cortisol reactivity to psychosocial stress in psychiatric patients.

4.2. The cortisol stress response as a resilience marker

Our data suggest a dynamic cortisol stress response in relation to psychiatric illness: the response was altered in patients with current MDD or an anxiety disorder and more comparable to healthy controls in individuals with remitted MDD. It is of note that recent evidence supports a model in which chronicity of depressive symptoms and recurrent depressive episodes change the cortisol response to stress more permanently (Booij et al., 2013). Booij and colleagues (Booij et al., 2013) found a higher cortisol response to stress in individuals with recent depressive symptoms, as opposed to a blunted response in individuals with chronic symptoms compared with controls. In addition, the association between depressive episodes and major stressful life events weakens with each episode (Morris et al., 2010), suggesting increased vulnerability to minor stressors over time. This evidence, further substantiated by our findings, points towards a model of limited flexibility which potentially reflects the extent to which individuals are resilient. Longitudinal evidence in individuals with recurrent depressive episodes is needed to shed light upon the usefulness of cortisol reactivity to stress as a marker of such resilience. In contrast to the proposed progressive blunting in MDD, hypocortisolemia (Van Zuiden et al., 2011) and a blunted cortisol response to psychosocial stress (Wintermann et al., 2016) have been suggested to precede PTSD and panic disorder, respectively. These possible vulnerability factors may be due to genetic variation (Uhart et al., 2004) or result from childhood trauma (Lupien et al., 2009).

In light of the above, it is important that baseline cortisol levels did not differ in patients compared with healthy controls, supporting our hypothesis that cortisol reactivity to stress better reflects, or informs about, psychopathological processes than baseline cortisol levels.

4.3. Strengths and limitations

To the best of our knowledge, this is the first meta-analysis comparing cortisol stress reactivity across psychiatric disorders with standardized cortisol outcomes. Stratified analyses based on sex and current symptoms add to the interpretation of the data that are visualized by mean cortisol level increase over time. Similar to a previous meta-analysis (Ciufolini et al., 2014), we focused on studies using a psychosocial stress test, as these tests elicit a physiological response involving suprahypothalamic structures (Schmidt-Reinwald et al., 1999), such as the limbic system, thereby ensuring high face validity. Moreover, we only included patients with a psychiatric disorder according to DSM criteria to minimize symptom heterogeneity and facilitate interpretation of findings.

This meta-analysis also has several limitations. First, there may have been limited statistical power in some of the analyses. Both visualization (Figure 5) and forest plots suggest a higher AUCi in depressed men compared with controls, but this finding did not reach statistical significance (in contrast with the significant difference in AUCg). Unfortunately, statistical differences per time point could not be analyzed using repeated measures analysis of variance, as individual level data was not available. Second, other aspects of the cortisol stress response that were not investigated in the present meta-analysis, such as steepness of increase and speed of recovery, may hold additional information about HPA axis functionality. Third, limited numbers of individuals with schizophrenia and the presence of possible publication bias, as indicated by Egger's test, compromise conclusions about cortisol stress reactivity in male schizophrenia patients compared with

healthy controls. Fourth, it should be noted that the studies on anxiety disorders included in our meta-analyses formed a heterogeneous sample that included SAD, PTSD, panic disorder and mixed anxiety disorders. The small number of studies for each disorder did not allow us to investigate them separately, except for SAD. Therefore, we cannot exclude that the cortisol response to psychosocial stress depends on the specific type of anxiety disorder. Fifth, the results of this meta-analysis pertain to the cortisol response to psychosocial stress and it is of note that other types of stressors may yield different cortisol responses. In support, Elzinga et al. (Elzinga et al., 2003) found elevated instead of blunted cortisol responses in women with PTSD after reading personalized trauma scripts. Finally, notwithstanding our intended comprehensive approach, missing data of parameters at the individual level hampered a thorough exploration of their influence in this meta-analysis. In particular, we did not know the age and specific medication use (in MDD and anxiety disorders) for each subject, and could therefore only investigate the mean or fraction (in case of medication use) of these parameters, despite evidence for their effect on the cortisol response to psychosocial stress (Houtepen et al., 2015; Kudielka et al., 2004). Information about other variables associated with the cortisol response was not available and could therefore not be explored, such as comorbidity (Pinna et al., 2014), depression severity, duration and the previous number of depressive episodes (Booij et al., 2013), childhood maltreatment (Harkness et al., 2011; Heim et al., 2000; Houtepen et al., 2016), and sex hormone levels (Juster et al., 2016; Stephens et al., 2016). Foley and Kirschbaum (Foley and Kirschbaum, 2010) present an extended list of variables influencing the cortisol response to the TSST, all relevant to take into account in future studies. In the absence of these variables, we cannot exclude that the sex-dependent differences between patients and controls found in the current meta-analysis are (partly) attributable to some of these potentially confounding factors.

4.4. Future directions

The large variation in cortisol stress reactivity may be attributable to many factors and is illustrated by the effects of sex and symptomatic state in the present meta-analysis. There is a lot to gain by sharing individual data on stress reactivity, including information about sex, contraceptives, symptom levels, age, disease duration and number of episodes, comorbidity, childhood trauma, circulating sex hormones and details on medication use. If stress reactivity is studied across psychiatric disorders, it may prove to be a functional phenotype which, corresponding to the Research Domain Criteria (RDoC) initiative of the National Institute of Mental Health, extends across classical diagnostic boundaries. A recent example is the Neuropattern tool (Hellhammer et al., 2012), which combines stress-induced HPA axis activity with parasympathetic and sympathetic information.

4.5. Conclusions

Overall, this meta-analysis found that women with current MDD or an anxiety disorder exhibit a blunted cortisol response to psychosocial stress compared with healthy controls. In contrast, male patients with current MDD or SAD showed an elevated cortisol response to psychosocial stress, while no firm conclusions could be drawn for schizophrenia. The results of the present study indicate that sharing individual data to disentangle the effects of sex, symptom levels and other factors is essential for further understanding of the alterations in cortisol stress reactivity across psychiatric disorders. A cross-disorder functional phenotype approach to HPA axis dysfunction in relation to psychiatric symptoms may prove a fruitful way to more accurate diagnostics and novel treatments.

SUPPLEMENTARY INFORMATION

Title/abstract (OR)		AND	Title/abstract (OR)
MDD	Phobics		Trier
Depression	PTSD		Social stress
Depressed	Schizophrenia		Acute stress
Depressive	Schizophrenic		TSST
Anxiety	Psychosis		Cold pressor
Anxious	Psychotic		MAST
Agoraphobia	Psychotics		Public speaking task
Disorder			Stress AND cortisol
SAD			Stress AND HPA axis
Panic			Stress AND HPA axis
Phobia			
Phobic			

Supplementary Table S1. Search terms.

DATA UNAVAILABLE

MDD

- Chopra et al., 2009. Sex differences in hormonal responses to a social stressor in chronic major depression. Psychoneuroendocrinology. 34. 1235-41.
- Furlan et al., 2005. The role of stress-induced cortisol in the relationship between depression and decreased bone mineral density. Biol Psychiatry. 57. 911–917.
- Rao et al.,2008. Effects of Early and Recent Adverse Experiences on Adrenal Response to Psychosocial Stress in Depressed Adolescents. Biol Psychiatry. 64. 521–526.
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ANXIETY DISORDERS

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- Dorn et al., 2003. Psychological comorbidity and stress reactivity in children and adolescents with recurrent abdominal pain and anxiety disorders. J Am Acad Child Adolesc Psychiatry. 42. 66–75.
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- Gerra et al., 2000. Neuroendocrine responses to psychological stress in adolescents with anxiety disorder. Neuropsychobiology. 42. 82–92.
- Graver, C., White, P., 2007. Neuropsychological effects of stress on social phobia with and without comorbid depression. Behav Res Ther. 45. 1193–1206.
- Martel et al., 1999. Salivary cortisol levels in socially phobic adolescent girls. Depress Anxiety. 10. 25-7.
- Petrowski et al., 2010. Striking pattern of cortisol non-responsiveness to psychosocial stress in patients with panic disorder with concurrent normal cortisol awakening responses. Psychoneuroendocrinology. 35. 414–421.
- Simeon, D et al., 2007. Hypothalamic-Pituitary-Adrenal Axis Function in Dissociative Disorders, Post-Traumatic Stress Disorder, and Healthy Volunteers. Biol Psychiatry. 61. 966–973.
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SCHIZOPHRENIA

- Van Venrooij et al., 2012. Impaired Neuroendocrine and Immune Response to Acute Stress in Medication-Naive Patients With a First Episode of Psychosis. Schizophr Bull. 38. 272–279.

NO RESPONSE

MDD

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- Dienes, K. A., Hazel, N. A., Hammen, C. L., 2013. Cortisol secretion in depressed, and at-risk adults. Psychoneuroendocrinology. 38. 927–940.
- Heim, C., Newport, D.J., Heit, S., Graham, Y.P., Wilcox, M., Bonsall, R., Miller, A.H., Nemeroff, C.B., 2000. Pituitary-adrenal and autonomic responses to stress in women after sexual and physical abuse in childhood. JAMA. 284. 592–597.
- Hellman, N., Morris, M.C., Rao, U., Garber, J., 2015. Depression history as a moderator of relations between cortisol and shame responses to social-evaluative threat in young adults. Biol Psychol. 109. 159-65.
- Jabbi et al., 2007. Catechol-o-methyltransferase polymorphism and susceptibility to major depressive disorder modulates psychological stress response. Psychiatr Genet. 17. 183–193.
- Melhem et al., 2016. Blunted HPA Axis Activity in Suicide Attempters Compared to those at High Risk for Suicidal Behavior. Neuropsychopharmacology. 41. 1447-56.
- Miller et al., 2005. Clinical Depression and Regulation of the Inflammatory Response During Acute Stress. Psychosom Med. 67. 679–687.
- Rao, U., Morris, M. C., 2015. Cortisol Responses to Psychosocial Stress: The Role of Childhood Maltreatment and Depression. Int J Public Ment Health Neurosci. 2. 37-42.
- Slattery et al., 2013. Neurocognitive function and state cognitive stress appraisal predict cortisol reactivity to an acute psychosocial stressor in adolescents. Psychoneuroendocrinology 38, 1318–1327.

ANXIETY DISORDERS

- Ahs et al., 2006. Hypothalamic Blood Flow Correlates Positively With Stress-Induced Cortisol Levels in Subjects With Social Anxiety Disorder: Psychosom Med. 68, 859–862.
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- Melhem et al., 2016. Blunted HPA Axis Activity in Suicide Attempters Compared to those at High Risk for Suicidal Behavior. Neuropsychopharmacology. 41. 1447-56.
- Slattery et al., 2013. Neurocognitive function and state cognitive stress appraisal predict cortisol reactivity to an acute psychosocial stressor in adolescents. Psychoneuroendocrinology 38, 1318–1327.
- Wintermann, G.B., Kirschbaum, C., Petrowski, K., 2016. Predisposition or side effect of the duration: the reactivity of the HPA axis under psychosocial stress in panic disorder. Int J Psychophysiol. 107. 9-15.

SAMPLE OVERLAP

MDD

- Harkness et al., 2011. Cortisol reactivity to social stress in adolescents: Role of depression severity and child maltreatment. Psychoneuroendocrinology. 36. 173–18.
- Heim et al., 2002. The role of early adverse experience and adulthood stress in the prediction of neuroendocrine stress reactivity in women: A multiple regression analysis. Depress Anxiety. 15. 117–125.
- Höhne, N., Poidinger, M., Merz, F., Pfister, H., Brückl, T., Zimmermann, P., Uhr, M., Holsboer, F., Ising, M., 2014. Increased HPA axis response to psychosocial stress in remitted depression: the influence of coping style. Biol Psychol. 103. 267–275.
- Morris, M.C., Kouros, C.D., Hellman, N., Rao, U., Garber J., 2014. Two prospective studies of changes in stress generation across depressive episodes in adolescents and emerging adults. Dev Psychopathol. 26. 1385-400.
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ANXIETY DISORDERS

- Elzinga et al., 2010. The role of childhood abuse in HPA axis reactivity in Social Anxiety Disorder: A pilot study. Biol Psychol. 83. 1–6.
- Hoyer et al., 2013. Depersonalization/ derealization during acute social stress in social phobia. J Anxiety Disord. 27. 178–18.
- Schmitz et al., 2010. Post-event Processing in Children with Social Phobia. J Abnorm Child Psychol. 38, 911–919.

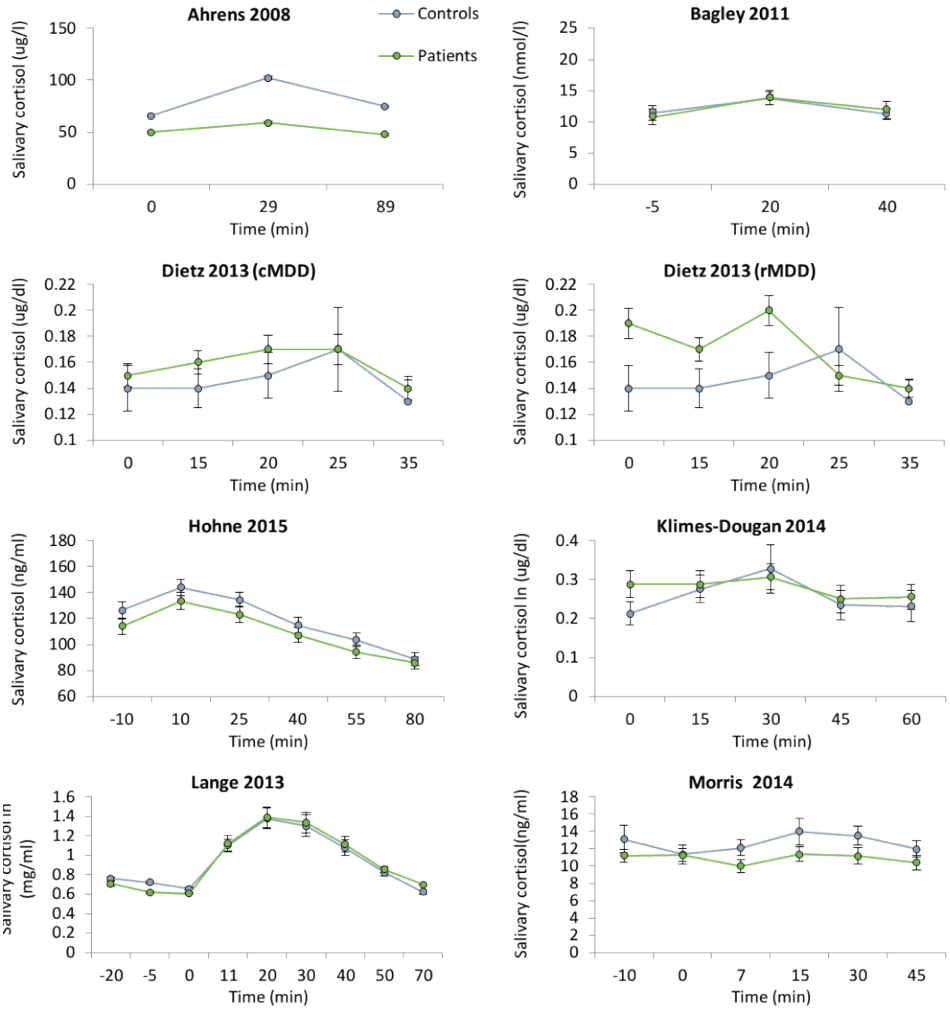
Supplementary Table S2. List of excluded studies.

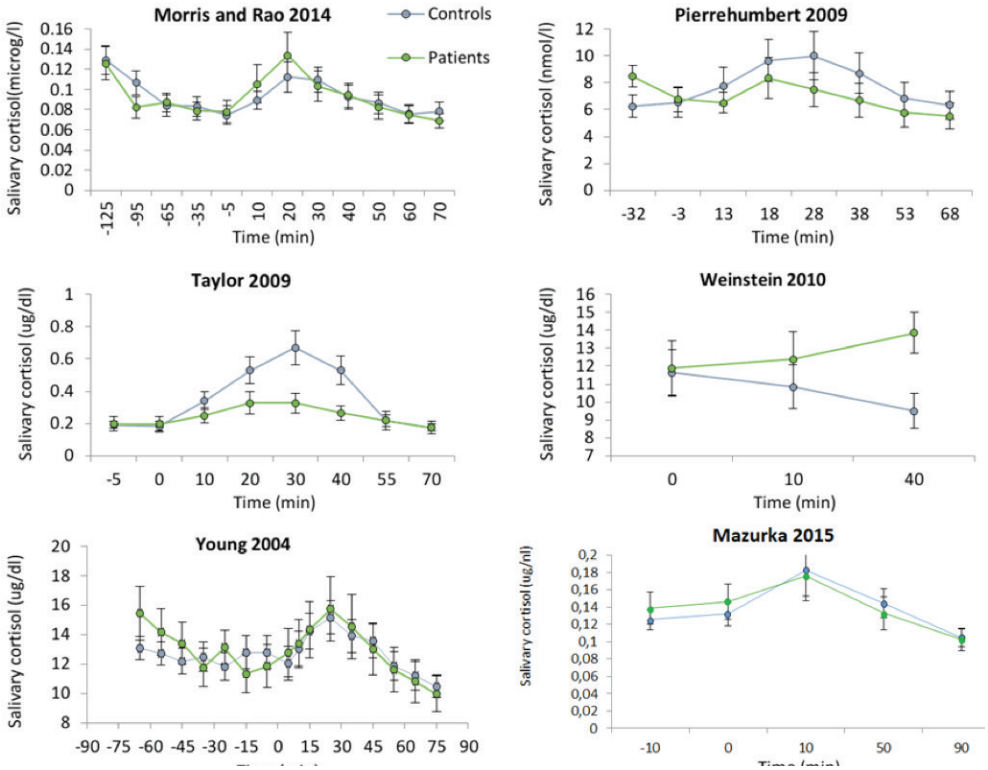
	Diagnosis assessment	Psychotropic medication (%)	Contraceptive use (%)	Psychosocial stress intervention
Depression				
Young 2004	DSM-IV SCID	No	No	TSST
Taylor 2009	DISH	No[2]	Yes, % unclear	TSST modified
Ahrens 2008	DSM-IV SCID	68	Unknown	GAST
Pierrehumbert 2009	MINI	No	No[6]	TSST
Weinstein 2010	DSM-IV SCID	14	Unknown	Alternative[7]
Bagley 2011	DSM-IV SCID	14	No	TSST modified
Dietz 2013[1]	DSM-IV SCID	32[3]	Unknown	TSST modified
Lange 2013	DSM-IV by psychiatrist	56	Unknown	TSST modified
Stewart 2013	DSM-IV KSADS	29	Unknown	TSST
Klimes-Dougan 2014	DSM-IV KSADS	31	Unknown	TSST modified
Morris 2014	DSM-IV SCID	40[4]	62	TSST modified[8]
Morris and Wang 2014	DSM-IV SCID, K-SADS	No	Unknown	TSST
Höhne 2015	DSM-IV M-CIDI	No	Unknown	TSST
Mazurka 2015	DSM-IV KSADS	29	Yes, % unclear	TSST
Anxiety				
Young 2004	DSM-IV SCID	No	No	TSST
Van West 2008	ADIS-IV-C/P	No	No	TSST modified
Roelofs 2009	DSM-IV, SCID	69	25	TSST
Yoon 2012	DSM-IV, SCID	20	Yes, % unclear	Alternative[9]
Krämer 2012	DSM-IV, Kinder-DIPS	No[1]	Unknown	TSST-C
Petrowski 2013	DSM-IV, SCID	No	45	TSST
Dietz 2013	DSM-IV SCID	32[3]	Unknown	TSST modified
Klumbies 2014	DSM-IV, M-CIDI	No[5]	47	TSST
Zaba 2015	DSM-IV, M-CIDI	72	Unknown	TSST
Schizophrenia				
Jansen 1998	DSM-IV, CASH	>=80	Unknown	Alternative[10]
Jansen 2000	DSM-IV, CASH	100	Unknown	Alternative[10]
Brenner 2011	Psychiatrist	Yes, % unclear	Yes, % unclear	TSST modified[11]
Houtepen	Psychiatrist	100	No	TSST

Supplementary Table S3. Study characteristics.
DSM = Diagnostic and Statistical Manual of Mental Disorders; SCID = Structured Clinical Interview for DSM Disorders; DISH = Depression Interview and Structured Hamilton; MINI = Mini International Neuropsychiatric Interview; KSADS = Child and Adolescent Schedule of Affective Disorders and Schizophrenia; M-CIDI = Munich version of the Composite International Diagnostic Interview; ADIS-IV-C/P = Anxiety Disorders Interview Schedule for DSM-IV: Child and Parent Versions; Kinder-DIPS = Diagnostic Interview for Mental Disorders for Children; CASH = Comprehensive Assessment of Symptoms and History; TSST(-C) = Trier Social Stress Test (Children); GAST = Groningen Acute Stress Test.

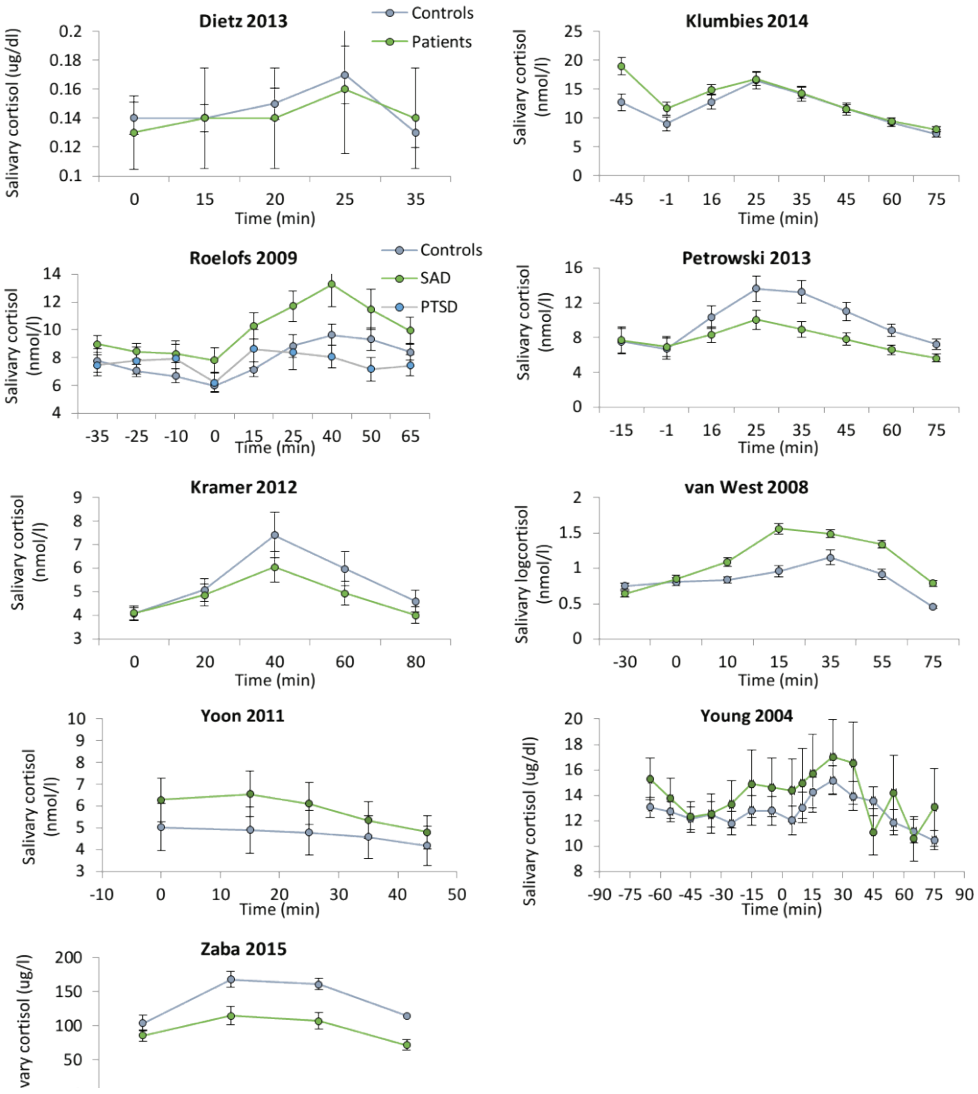
(1) Correspondence with author.
(2) Only antihypertensives and lipid-lowering medication.
(3) Calculated over whole sample. Controls might have been using psychotropic medication as well.
(4) 2 healthy controls used antidepressants.
(5) Only proton pump inhibitors, analgesics, nutritional supplements and homeopathics.
(6) Recommended women with oral contraceptives to stop taking pills during the entire cycle in which the TSST took place.
(7) Anger recall task and mental arithmetic in front of evaluating panel.
(8) Subjects were told that their speech and mental arithmetic would be recorded and then evaluated by a committee (no direct judgment).
(9) Subjects were told that their speech was recorded and then evaluated by a committee (no direct judgment). Subsequent unexpected working memory task.
(10) Speech in front of mirror-wall behind which the subject was led to believe there was a committee.
(11) Speech and mental arithmetic in front of mirror-wall behind which the subject was led to believe there was a committee.

MDD

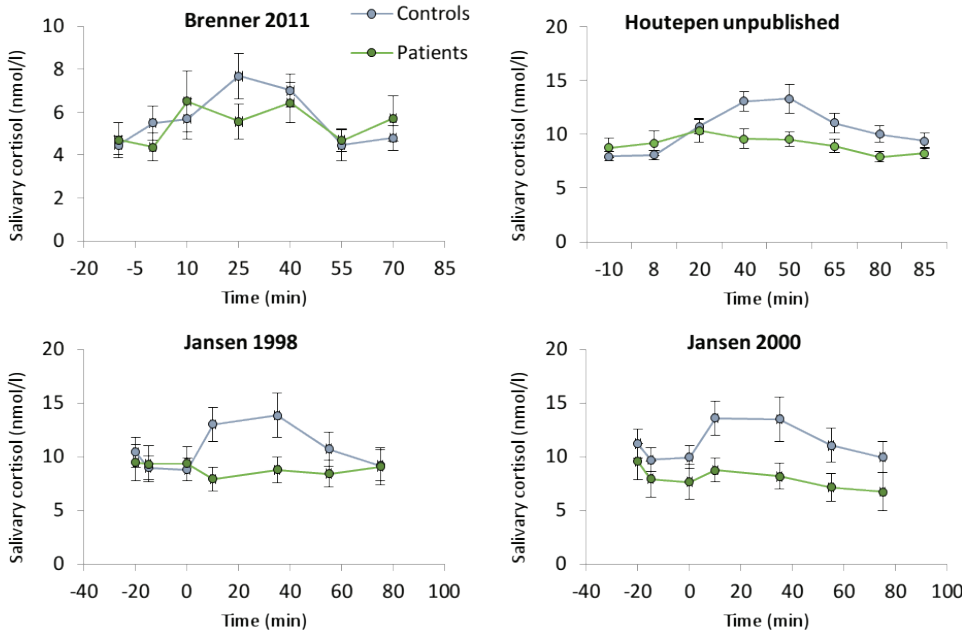

Ahrens 2008
Controls
Patients
Salivary cortisol (ug/l)
150
100
50
0
0
29
89
Time (min)
Bagley 2011
Salivary cortisol (nmol/l)
25
20
15
10
5
0
-5
20
40
Time (min)
Dietz 2013 (cMDD)
Salivary cortisol (ug/dl)
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0
15
20
25
35
Time (min)
Dietz 2013 (rMDD)
Salivary cortisol (ug/dl)
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0
15
20
25
35
Time (min)
Hohne 2015
Salivary cortisol (ng/ml)
180
160
140
120
100
80
60
-10
10
25
40
55
80
Time (min)
Klimes-Dougan 2014
Salivary cortisol ln (ug/dl)
0.4
0.3
0.2
0.1
0
0
15
30
45
60
Time (min)
Lange 2013
Salivary cortisol ln (mg/ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-20
-5
0
11
20
30
40
50
70
Time (min)
Morris 2014
Salivary cortisol(ng/ml)
18
16
14
12
10
8
6
4
2
0
-10
0
7
15
30
45
Time (min)


Morris and Rao 2014
Controls
Patients
Salivary cortisol(microg/l)
Time (min)
Pierrehumbert 2009
Salivary cortisol (nmol/l)
Time (min)
Taylor 2009
Salivary cortisol (ug/dl)
Time (min)
Weinstein 2010
Salivary cortisol (ug/dl)
Time (min)
Young 2004
Salivary cortisol (ug/dl)
Mazurka 2015
Salivary cortisol (ug/nl)

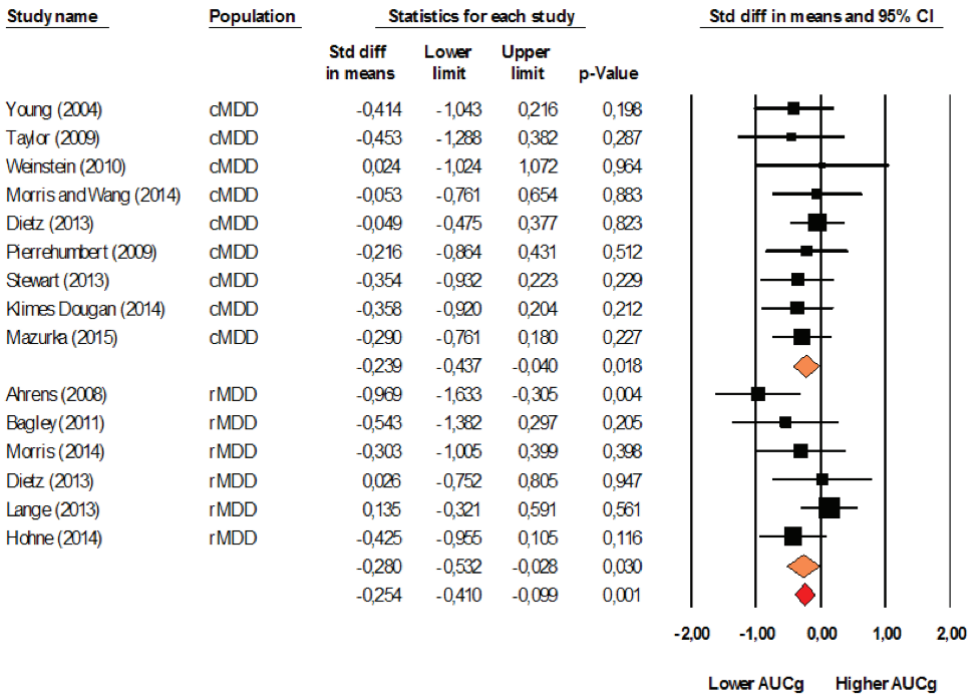
Anxiety disorders



Schizophrenia

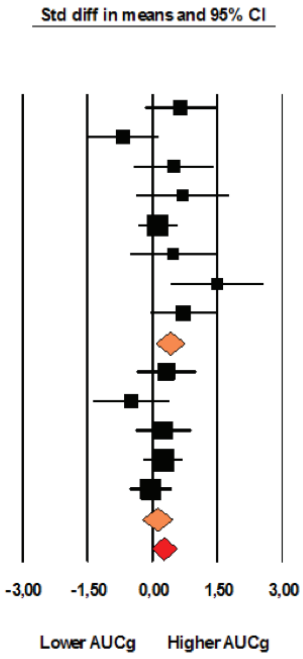


Supplementary Figure S1. Graphs of cortisol levels over time from individual articles.

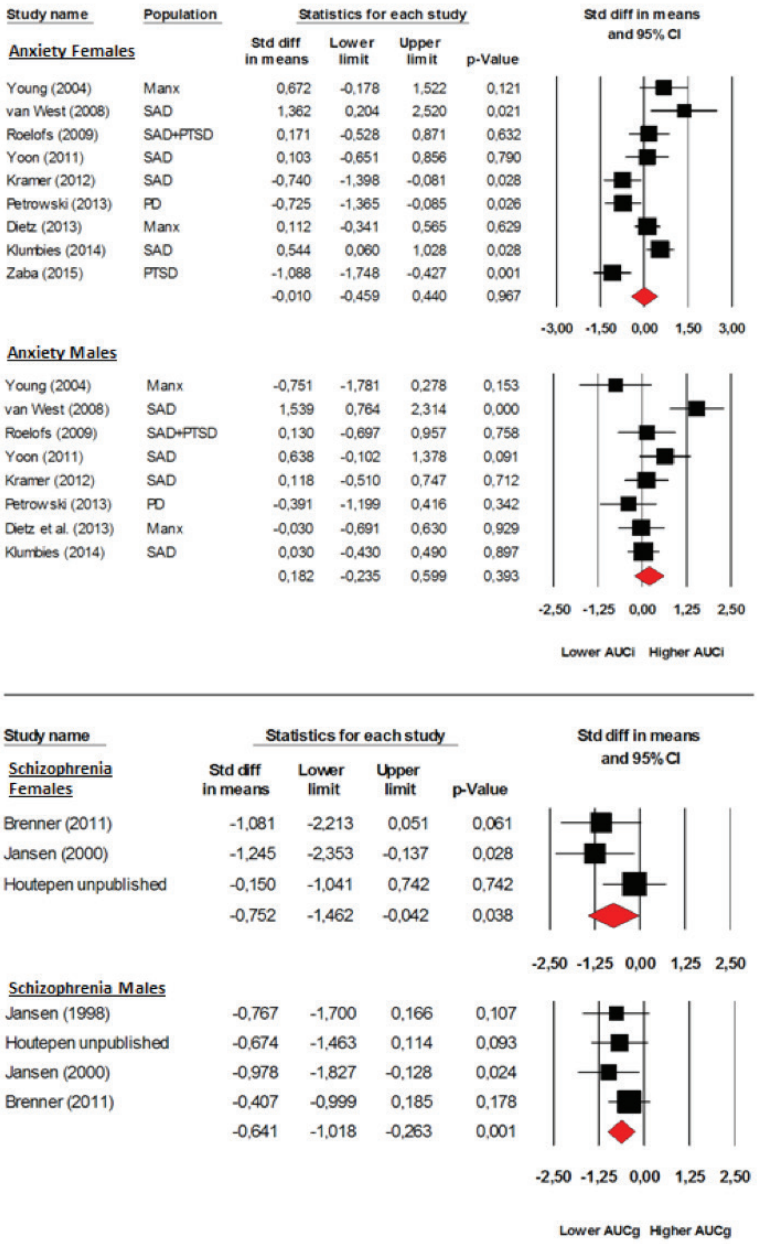


Supplementary Figure S2. Forest plot of the cortisol AUCg in response to a psychosocial laboratory stressor in women with major depressive disorder. Diamond shaped orange symbols represent current MDD (upper symbol) and remitted MDD (lower symbol). Diamond shaped red symbol represents a summary of all MDD studies. Size of the black squares is proportionate to the sample size used. cMDD = current Major Depressive Disorder; rMDD = remitted Major Depressive Disorder. The same control group was used for the analyses of cMDD and rMDD of Dietz et al

Study name	Population	Statistics for each study			
		Std diff in means	Lower limit	Upper limit	p-Value
Young (2004)	cMDD	0,650	-0,186	1,486	0,128
Taylor (2006)	cMDD	-0,677	-1,499	0,145	0,107
Morris and Wang (2014)	cMDD	0,493	-0,453	1,438	0,307
Weinstein (2010)	cMDD	0,691	-0,387	1,770	0,209
Dietz (2013)	cMDD	0,128	-0,333	0,589	0,587
Stewart (2013)	cMDD	0,483	-0,525	1,491	0,347
Klimes Dougan (2014)	cMDD	1,492	0,416	2,569	0,007
Mazurka (2015)	cMDD	0,712	-0,059	1,484	0,070
		0,399	0,061	0,738	0,021
Bagley (2011)	rMDD	0,324	-0,384	1,031	0,370
Morris (2014)	rMDD	-0,489	-1,378	0,401	0,282
Dietz (2013)	rMDD	0,255	-0,406	0,917	0,449
Lange (2013)	rMDD	0,243	-0,222	0,708	0,306
Hohne (2014)	rMDD	-0,035	-0,543	0,472	0,891
		0,100	-0,252	0,452	0,576
		0,254	-0,039	0,547	0,089



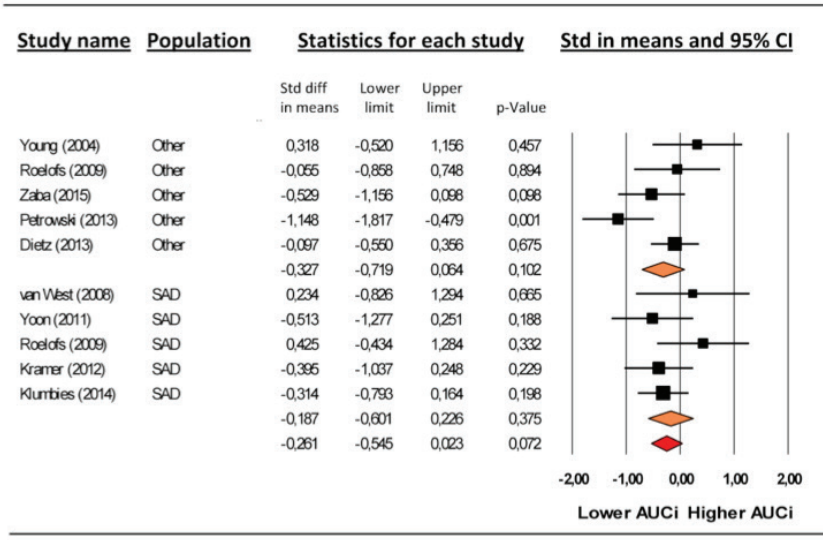
Supplementary Figure S3. Forest plot of the cortisol AUCg in response to a psychosocial laboratory stressor in men with major depressive disorder. Diamond shaped orange symbols represent current MDD (upper symbol) and remitted MDD (lower symbol). Diamond shaped red symbol represents a summary of all MDD studies. Size of the black squares is proportionate to the sample size used. cMDD = current Major Depressive Disorder; rMDD = remitted Major Depressive Disorder. The same control group was used for the analyses of cMDD and rMDD of Dietz et al.



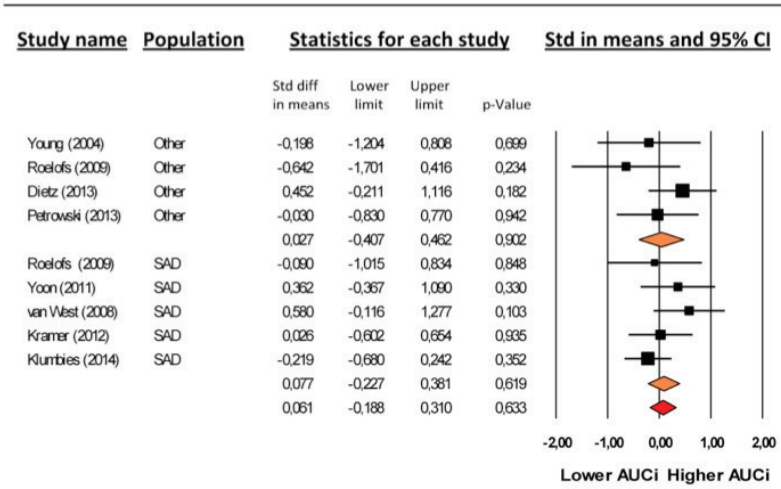
Supplementary Figure S4. Forest plots of the cortisol AUCg in response to a psychosocial laboratory stressor in anxiety disorders and schizophrenia. Diamond shaped red symbols represent summaries of all studies per disorder, stratified for sex. Size of the black squares is proportionate to the sample size used. SAD = social anxiety disorder; PTSD = posttraumatic stress disorder; PD = panic disorder, M anx = mixed anxiety.

A. AUCI

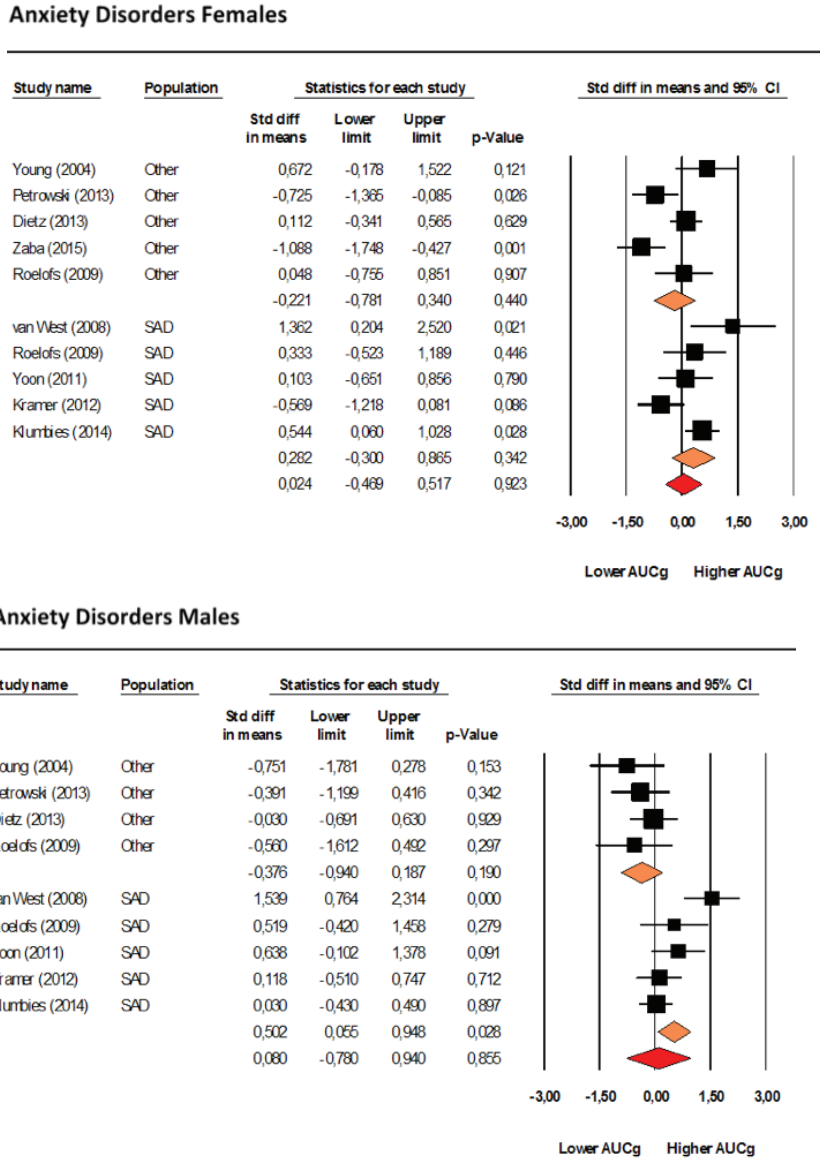
Anxiety Disorders Females



Anxiety Disorders Males

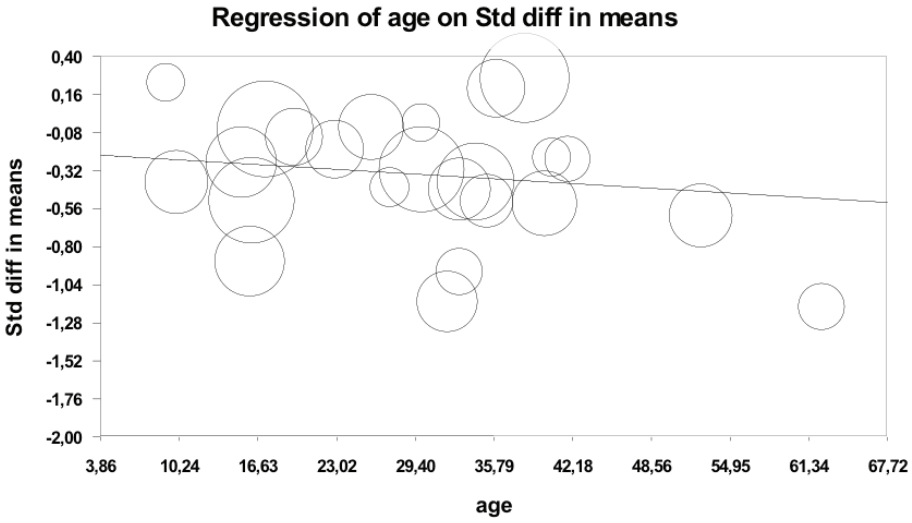


B. AUCG

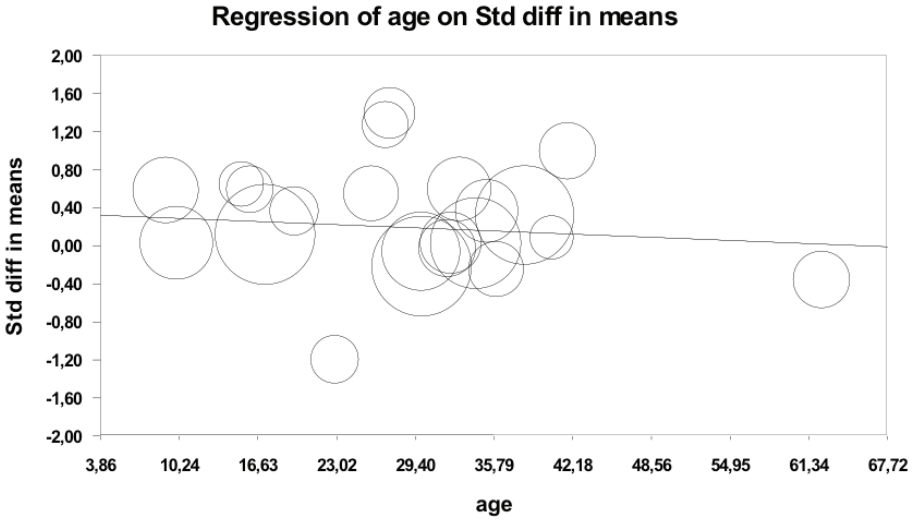


Supplementary Figure S5. Forest plot of the AUCi in response to a psychosocial laboratory stressor in patients with social anxiety disorder and other anxiety disorders. Diamond shaped orange symbols represent social anxiety disorder (lower symbol) and all other included anxiety disorders (upper symbol). Diamond shaped red symbol represents a summary of all studies on anxiety disorders. Size of the black squares is proportionate to the sample size used. SAD = social anxiety disorder; Other = all other included anxiety disorders.

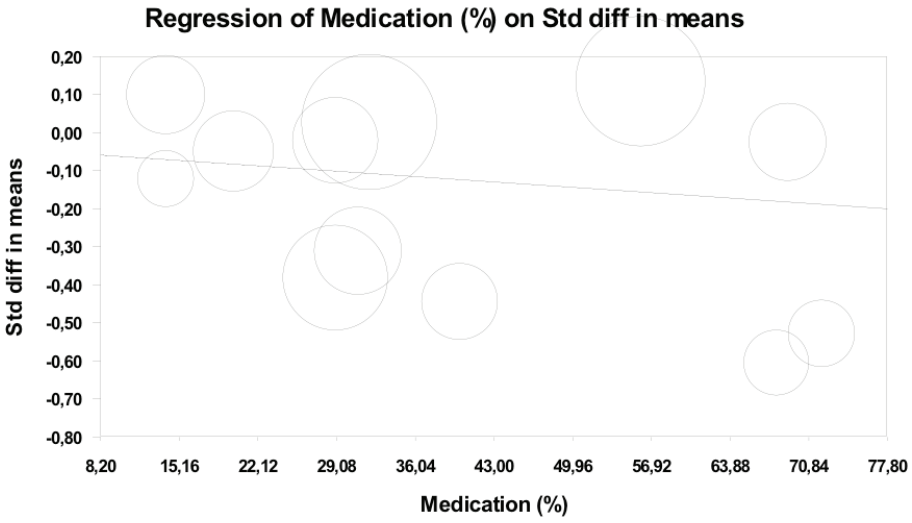
A. Women



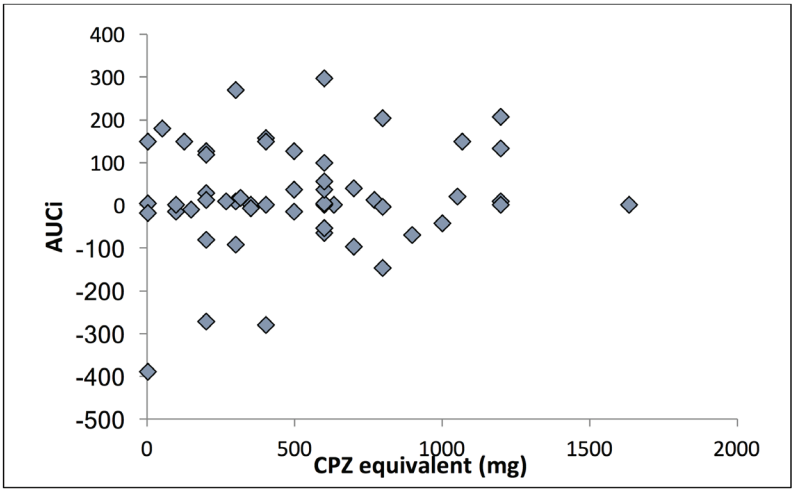
A. Men



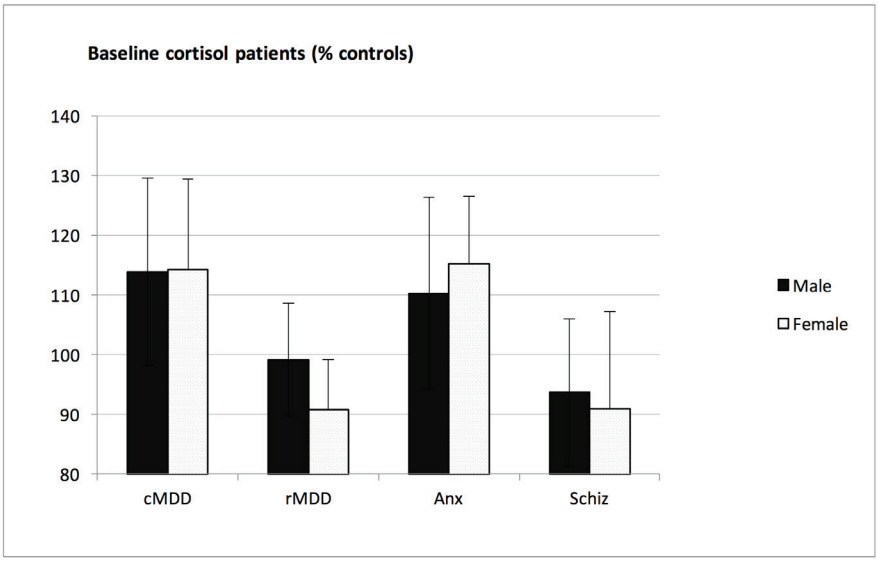
Supplementary Figure S6. Meta-regressions for the effect of age across all disorders. Meta-regressions were performed to assess the effect of age on effect size (SMD) for women (A) and men (B) separately. For women, a total of 24 studies with 488 patients and 501 healthy controls, and for men a total of 22 studies with 355 patients and 419 healthy controls were included in the analyses. No significant relationship between age and effect size was found for women (coefficient: -0.005, 95% CI: -0.016 to 0.006, p = 0.41), or men (coefficient: -0.005, 95% CI: -0.018 to 0.008, p = 0.44).



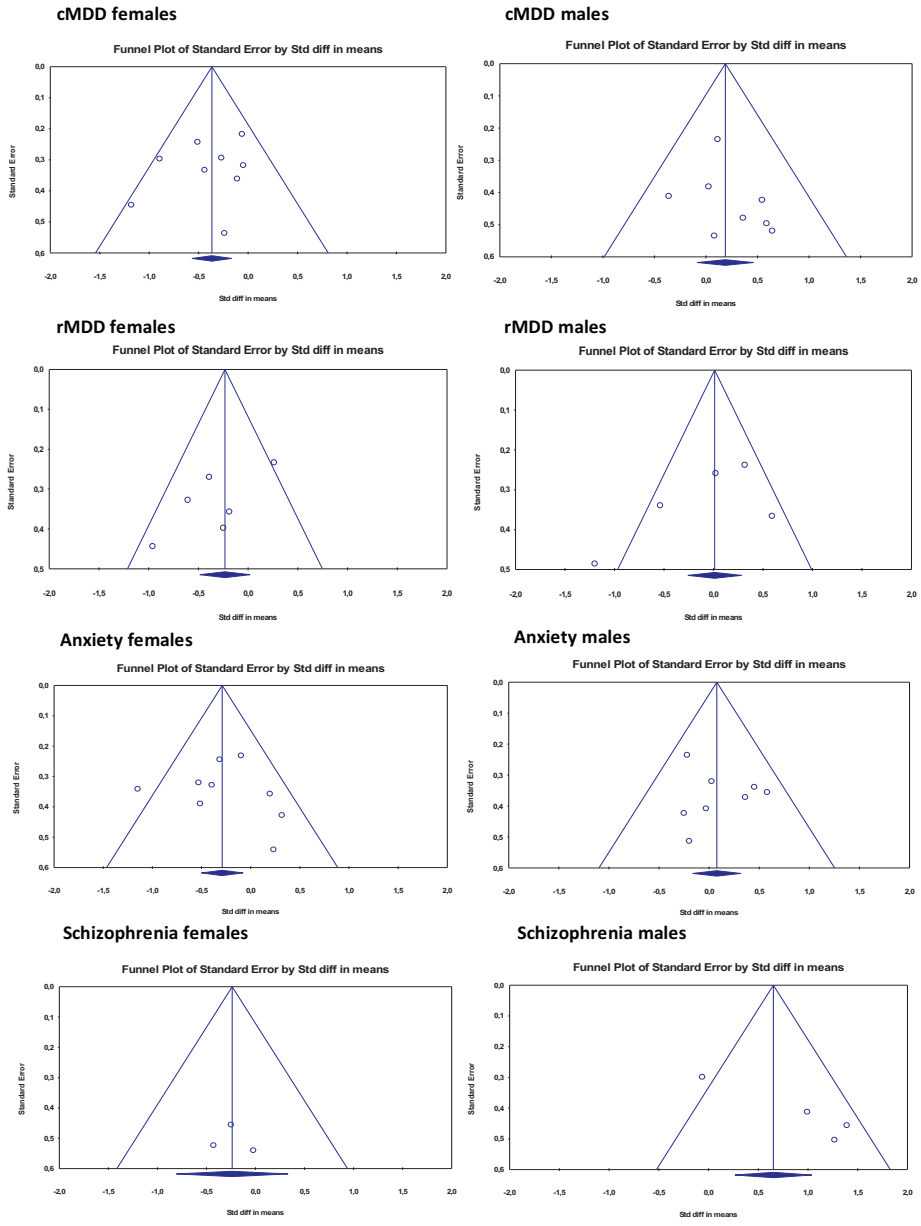
Supplementary Figure S7. Meta-regression for the effect of medication (major depressive disorder and anxiety disorders). A meta-regression for the effect of percentage medication use on effect size (SMD) from 12 studies with a total of 429 patients and 487 healthy controls was carried out. No significant relationship between effect size and percentage medication use was found (coefficient: -0.002, 95% CI: -0.010 to 0.005, p = 0.60).



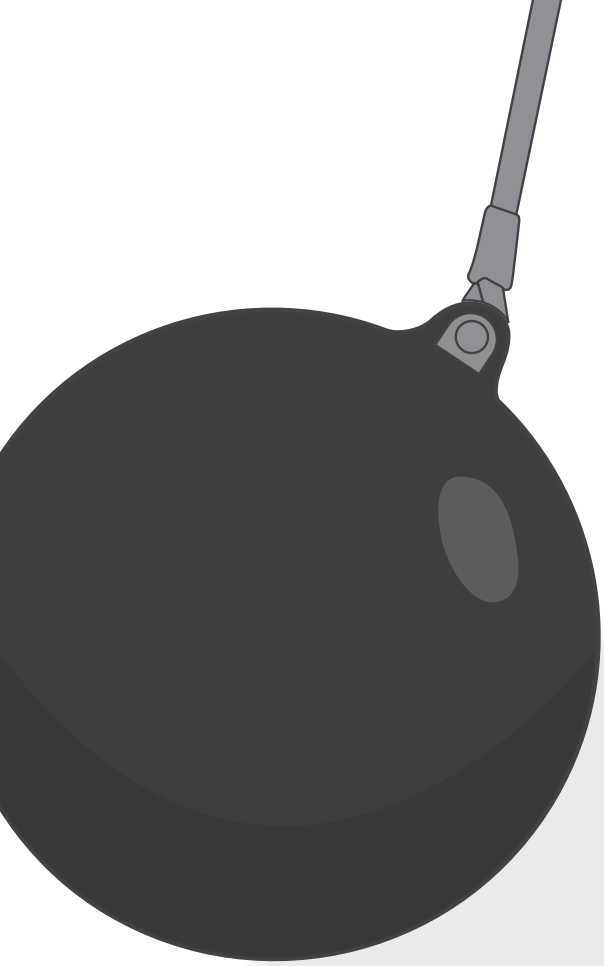
Supplementary Figure S8. Linear regression of chlorpromazine (CPZ) equivalents as determinant of the AUCi for schizophrenia patients. A simple linear regression was performed to investigate the relationship between CPZ equivalents and AUCi, using individual data (n = 54 subjects: 15 females, 39 males). CPZ equivalents were not significantly associated with cortisol stress reactivity: $F(1,54) = 0.12$, $p = 0.39$.



Supplementary Figure S9. Relative baseline cortisol level for each patient group. Baseline cortisol levels (mean ± SEM) for each patient group as the percentage of the corresponding baseline cortisol of the control group. Baseline cortisol levels were similar for female patients compared with female controls (cMDD: n = 449, t = 0.85, p = 0.40; rMDD: n = 300, t = 0.86, p = 0.39; anxiety disorders: n = 421, t = 1.01, p = 0.31; schizophrenia: n = 60, t = 0.44, p = 0.66) and for male patients compared with male controls (cMDD: n = 247, t = 0.64, p = 0.52; rMDD: n = 270, t = 0.06, p = 0.95; anxiety disorders: n = 333, t = 0.49, p = 0.62; schizophrenia: n = 119, t = 0.42, p =0.67).



Supplementary Figure S10. Funnel plots for the studies on major depressive disorder, anxiety disorders and schizophrenia showing the relation between the standardized difference in means and the standard error for each study. Egger's test was significant for studies investigating men with schizophrenia compared with controls (p = 0.03), but it was not significant for cMDD (p = 0.37), rMDD (p = 0.26) or anxiety disorders in men (p = 0.64), nor for schizophrenia (p = 0.85), cMDD (p = 0.43), rMDD (p = 0.10) or anxiety disorders (p = 0.74) in female patients compared with healthy controls.



CHAPTER 3

Longitudinal changes in glucocorticoid receptor exon 1_F methylation and psychopathology after military deployment

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ABSTRACT

Several cross-sectional studies have demonstrated the relevance of DNA methylation of the glucocorticoid receptor exon 1_F region (GR-1_F) for trauma-related psychopathology. We conducted a longitudinal study to examine GR-1_F methylation changes over time in relation to trauma exposure and the development of post-deployment psychopathology. GR-1_F methylation (52 loci) was quantified using pyrosequencing in whole blood of 92 military men one month before and six months after a four-month deployment period to Afghanistan. GR-1_F methylation overall (mean methylation and the number of methylated loci) and functional methylation (methylation at loci associated with GR exon 1_F expression) measures were examined. We first investigated the effect of exposure to potentially traumatic events during deployment on these measures. Subsequently, changes in GR-1_F methylation were related to changes in mental health problems (total Symptom Checklist 90 score) and PTSD symptoms (Self-Report Inventory for PTSD). Trauma exposure during deployment was associated with an increase in all methylation measures, but development of mental health problems six months after deployment was only significantly associated with increased functional methylation. Emergence of post-deployment PTSD symptoms was not related to increased functional methylation over time. Pre-deployment methylation levels did not predict post-deployment psychopathology. This is the first study to prospectively demonstrate trauma-related increases in GR-1_F methylation and it shows that only increases at specific functionally relevant sites predispose for post-deployment psychopathology.

1. INTRODUCTION

Exposure to traumatic stress is a major risk factor for a wide range of psychiatric disorders, including posttraumatic stress disorder (PTSD) and major depressive disorder (MDD) (Kendler et al., 1999). The hypothalamus-pituitary-adrenal (HPA) axis is crucial for an adequate response to a stressful environment. The glucocorticoid receptor (GR) determines negative feedback on the HPA axis, and impaired GR functionality has been proposed as a potential pathophysiological mechanism underlying both PTSD and MDD (Raison and Miller, 2003).

Over a decade ago, Weaver et al. (Weaver et al., 2004) demonstrated that DNA cytosine methylation of the GR exon 1$_7$ promoter in the hippocampus of rats remains elevated after early life stress and leads to decreased GR expression and increased adult hypothalamus-pituitary-adrenal (HPA) axis activity. This methylation effect was particularly pronounced at a nerve growth factor-inducible protein A (NGFI-A) transcription factor binding site. McGowan et al. (McGowan et al., 2009) translated these findings to humans, showing elevated methylation in the GR exon 1$_F$ region (GR-1$_F$) in the hippocampus of suicide victims with a childhood abuse history compared with nonabused suicide victims and controls. Following these seminal reports, the number of studies examining GR-1$_F$ methylation in relation to traumatic stress and stress-related psychiatric disorders has steadily increased for reviews see (Daskalakis and Yehuda, 2014; Turecki and Meaney, 2014; Vinkers et al., 2015). Of note, all of these studies examined peripheral tissues to assess GR-1$_F$ methylation and significant findings were not limited to NGFI-A binding sites. In humans, both pre- and postnatal stress have consistently been linked to increased GR-1$_F$ methylation in most (Daskalakis and Yehuda, 2014), but not all studies (Tyrka et al., 2016). In addition, decreased GR-1F methylation has been found in patients with major depressive disorder (MDD) (Na et al., 2014), whereas both increased (Perroud et al., 2014) and decreased (Yehuda et al., 2015) GR-1$_F$ methylation were reported in patients with posttraumatic stress disorder (PTSD) compared to controls.

These studies all employed a cross-sectional design which precludes conclusions about causality. As a result, it is still unknown whether GR-1$_F$ methylation changes as psychopathology emerges and/or whether it represents a pre-existing vulnerability factor predicting the development of psychopathology.

We therefore quantified GR-1$_F$ methylation levels in peripheral blood cells of Dutch military personnel before and after deployment to Afghanistan. In line with the accumulating evidence (for an overview see Daskalakis and Yehuda, 2014), we hypothesized that trauma exposure during deployment would increase GR-1$_F$ methylation. In addition, we wanted to explore the direction of GR-1$_F$ methylation change in relation to emerging psychopathology symptoms after deployment, as previous evidence is equivocal (Daskalakis and Yehuda, 2014). As reported in a previous study (Yehuda et al., 2015), we expected methylation changes associated with changes in GR exon 1$_F$ expression to be most informative of change in psychopathology. Furthermore, we investigated the predictive value of pre-deployment GR-1$_F$ methylation for the development of post-deployment psychopathology symptoms. Finally, we explored the association between GR-1$_F$ methylation and six functional haplotypes of the GR gene (*NR3C1*), which were previously associated with GR sensitivity and might be relevant for the development of psychopathology (Hardeveld et al., 2015).

2. Materials and Methods

2.1. Participants and assessments

The present study was part of a large longitudinal cohort of Dutch military personnel that were deployed to Afghanistan (n = 1,032), as part of the International Security Assistance Force, between 2005 and 2008 (for details on procedures see Reijnen et al., 2015; Van Zuiden et al., 2012). Among their duties were searching and clearing of buildings, combat patrols, transport across enemy territory and demining operations. The individuals were exposed to potentially traumatic events such as armed combat, combat casualties and enemy fire. Individuals were assessed (questionnaires were administered and blood samples were drawn) one month before, and one and six months after a four-month period of deployment. This study examined methylation measures prior to and six months after deployment, since psychopathology levels were highest six months after deployment (Reijnen et al., 2015). To maximize power (Boks et al., 2007), three subgroups of approximately equal size were selected from this cohort (n = 92) based on exposure to potentially traumatic events during deployment (high or low) and post-deployment psychopathology (high or low, only in group with high trauma exposure) (Boks et al., 2015). The study was approved by the Institutional Review Board of the University Medical Center Utrecht (UMCU) and both oral and written informed consent was obtained from all participants.

Mental health problems were assessed with the validated Dutch version of the Symptom Checklist-90 (SCL-90), which includes the total score of eight symptom dimensions: agoraphobia, anxiety, depression, somatization, cognitive performance deficits, mistrust and interpersonal sensitivity, hostility, and sleep difficulties (Arrindell et al., 2003). PTSD symptoms were measured using the Self-Report Inventory for PTSD (SRIP) (Hovens et al., 2002). Changes in SCL-90 and SRIP scores were significantly correlated ($\rho = 0.52$). A 19-item self-report deployment experiences checklist was used to assess traumatic stress exposure during deployment, such as direct combat stressors (Reijnen et al., 2015). For childhood trauma, the Early Trauma Inventory-Self Report was used which includes emotional, physical, and sexual abuse (Bremner et al., 2007).
For prediction of the development of psychopathology after deployment, individuals were divided into groups with low or high symptom levels based on previously defined cutoff scores of 124 for total SCL-90 and 38 for the SRIP (Arrindell et al., 2003; Van Zuiden et al., 2009; Van Zuiden et al., 2012). This SCL-90 cutoff score delineates individuals in the general population exceeding average symptoms (Arrindell and Ettema, 2003). The SRIP cutoff score (Van Zuiden et al., 2012) is in the range with the best sensitivity and specificity for PTSD classification according to the DSM-IV (in a general population) (Van Zelst et al., 2003).

2.2. Biological measures

Methylation was quantified in 52 CpGs in and adjacent to the 1_F region (see Figure 1, numbering in accordance with Daskalakis and Yehuda, 2014). The thirteen NGFI-A binding sites in this region (CpG numbers: 17, 18, 21-26, 35-37, 42, 43) have been suggested to be relevant for GR-1_F transcription and HPA axis activity (Weaver et al., 2004).

Whole blood EDTA samples were collected one month before and six months after deployment. Standard salting procedures were used to extract DNA. Subsequently, DNA concentration and integrity were determined using riboGreen (Thermo Fisher Scientific, Waltham, MA) and BioAnalyser (Agilent Technologies, Santa Clara, CA), respectively.
GR-1_F methylation data were available for 92 individuals. For methylation analysis (EpigenDx Inc, MA, USA (Brakensiek et al., 2007; England and Pettersson, 2005; T. Liu et al., 2007)), DNA was denatured using 3N NaOH followed by 30-minute incubation at 42°C. Bisulfite salt solution was added to the DNA and incubated for 14 hours at 50°C. Bisulfite treated DNA was purified using Zymogen DNA columns and eluted in 20 µl $T_1E_{0.2}$ (pH 8.0) and 1 µl wasused for each PCR. PCR was performed using 5 ng of genomic DNA and 0.2 µM of each primer. The final PCR product was purified using Sepharose beads (GE Healthcare Life Sciences, Pittsburgh, PA) and the Pyrosequencing Vacuum Prep Tool (Qiagen, Velncia, CA) as recommended by the manufacturer. Purified PCR product was sequenced using 0.2 UM pyrosequencing primer on the Pyrosequencing PSQ96 HS System (Qiagen, Valencia, CA) following the manufacturer's instructions. The percentage of methylation at each locus was determined by looking at the CpG site as an artificial C/T SNP using QCpG software (Qiagen, Valencia, CA), where % C equals % methylation as calculated by the equation below:
C% = RLU (C peak)/RLU (C peak + T peak)
All samples resulted in good pyrosequencing signals and good quality data (for assays, sensitivity, coefficients of variance, numbers of CpG sites and replicates, and chromosomal regions targeted by the primers, see Supplementary Table S1). As GR-1_F methylation levels are generally low (Yehuda et al., 2014, 2013), two measures of overall GR-1_F methylation were examined: mean methylation across all CpGs and the number of methylated loci (the number of CpGs with > 0% methylation). In addition, to optimally investigate the functional dynamics of methylation, we focused on those CpGs where we observed that longitudinal changes in methylation were significantly related to change in GR exon 1_F mRNA expression (for the latter see below), defined as functional methylation. In exploratory analyses, we examined DNA methylation at NGFI-A binding sites.

GR exon 1_F expression data were available for 75 individuals, as RNA quality was not sufficient in 17 individuals. Pre- and post-deployment RNA from peripheral blood mononuclear cells (PBMCs) was isolated with TRIzol reagent (Invitrigon, Carlsbad, CA) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). One µg of total RNA was converted into first-strand cDNA with oligo(dT) primers using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) as described by the manufacturer. qPCR assays were carried out in duplicate in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with specific primers (see Supplementary Table S3) and SensiMix SYBR Hi-ROX (Bioline Reagents Ltd). For normalization of mRNA expression, RefFinder (OMICtools, Sotteville-Les-Rouen, France) was used to find the most stable reference gene among *PPIB, YWHAZ, RPL13a* and *RPL37a*. The relative abundance of mRNAs was standardized with *RPL13a* mRNA as the invariant control.

In line with the finding that lower GR-1_F methylation is associated with dampening of the cortisol response through higher GR-mediated negative feedback (Weaver et al., 2004), we hypothesized GR-1_F methylation to be negatively associated to GR sensitivity. Data on GR binding capacity of PBMCs were available for 47 individuals, as these were acquired several years earlier from a largely overlapping cohort (Van Zuiden et al., 2009). As previously described (Van Zuiden et al., 2009), GR binding capacity was quantified using a modification of the whole cell single point binding assay described by Yehuda et al. (Yehuda et al., 1995). Using this protocol, specific binding at 100 nM ^{3}H-dexamethasone gave a reliable Bmax, which was determined with a classical binding assay with 3-200 nM ^{3}H-dexamethasone in the absence or presence of an excess of unlabeled dexamethasone (r^2 = 0.92) (Van Zuiden et al., 2009). In short, Ficoll-Paque (Pharmacia and Upjohn, Uppsala, Sweden) was used to isolate PBMCs from heparinized whole blood, and 10^7 cells were frozen in RPMI-1640 (Gibco, Grand Island, NY) in addition of 10% fetal calf serum (FCS) (Gibco, Grand Island, NY) and 20% DMSO. Samples of every single individual were analyzed simultaneously. Cells were thawed, and subsequently washed twice in RPMI-1640 and incubated for 30 min at 37°C. Again, cells were washed twice, after which they were resuspended in assay buffer (RPMI- 1640 with 10% FCS) and incubated in duplicate with 100 nM ^{3}H-dexamethasone (Amersham, Buckinghamshire, UK) in the absence or presence of excess unlabeled dexamethasone (Sigma–Aldrich, Steinheim, Germany). After 60 minutes incubation at 37°C, cells were washed twice in ice cold assay buffer followed by density centrifugation over Ficoll hypaque, separating cell-bound label from free label. Liquid scintillation analysis was used to analyze cell-bound radioactivity. In two simultaneously processed samples the number of cells was counted.

To examine the effect of genetic variation in the GR gene (*NR3C1*) on GR-1_F methylation, six SNPs linked to glucocorticoid sensitivity (rs10052957, rs10482605, rs6189, rs6195, rs41423247 and rs6198) (Hardeveld et al., 2015) were selected. SNPs were genotyped on the Illumina Human OmniExpress-24 Beadchip array, and genetic data were subjected to quality control (see Supplementary Table S4 for quality control information). SNPs rs10482605, rs6189 and rs41423247 were imputed using the Haplotype Reference consortium release 1.1. R^2 values and average call rates were > 0.99 for all three SNPs while allele frequencies did not deviate from Hardy-Weinberg Equilibrium (all p-values > 0.05). Haplotypes were constructed using SNPHAP (Clayton, 2004), yielding the following *NR3C1* haplotypes : CTGACA (40%), CTGAGA (22%), TTGAGA (15%), TCGACG (13%), CTGGCA (4%) and TCAACG (4%). *NR3C1* haplotypes and individual SNPs from 85 individuals were available.

2.3. Statistical analyses

Longitudinal changes in GR-1_F methylation around deployment were analyzed by applying mixed models for repeated measures (MMRM) using the nlme package in R (Pinheiro et al., 2015) The relation of changes in GR methylation with trauma exposure was investigated in a model without adjustment for symptom outcomes whereas the relation with symptom outcomes was investigated in a model with adjustment for trauma exposure during deployment (*methylation ~ methylation$_{pre}$ + trauma during deployment × time + change in mental health problems × time, random factor: subject ID*). We only used continuous

measures. PTSD symptom scores were log-transformed to improve distribution (Boks et al., 2015). In secondary analyses, *NR3C1* haplotypes/SNPs or childhood trauma was added to the model. To investigate the influence of GR-1_F methylation on GR-1_F expression and GR binding, the latter two measures were used as dependent variables (e.g. *expression ~ expression$_{pre}$ + trauma during deployment × time + change in methylation × time, random factor: subject ID*).

Linear regression models were used to examine the cross-sectional relations between pre-deployment GR-1_F methylation and childhood trauma or *NR3C1* haplotypes/SNPs.

Pre- and post-deployment psychopathology symptoms were compared using paired two-tailed t-tests. Methylation values of one individual were > 8 SDs above the mean methylation and were excluded from further analyses (resulting in n = 91). Random effects and residuals of the main MMRM were plotted and evaluated for heteroscedasticity and did not show further aberrations.

Considering the relatedness and relatively low number of tests, the significance threshold was set at $p < 0.05$ in primary analyses. However, as 12 *NR3C1* haplotypes/SNPs were investigated without prior hypotheses, the significance threshold in these analyses was set at $p < 0.0042$ (0.05/12).

For prediction, receiver operation curves (ROC) were constructed to predict a high level of post-deployment mental health problems or PTSD symptoms with pre-deployment GR-1_F methylation. Individuals with a high level of pre-deployment mental health problems (n = 8) or PTSD symptoms (n = 0) were excluded in the respective analyses. Areas under the curve (AUCs) and corresponding p-values were determined using the verification package in R (NCAR - Research Applications Laboratory, 2015). Sensitivity, specificity, positive and negative predictive values were calculated using the Epi package (Carstensen B, Plummer M, Laara E, 2013).

Potential confounding was investigated by analyzing the correlations of age, body-mass-index, military rank, education, previous number of deployments and white blood cell counts (including values of neutrophils, lymphocytes, monocytes, eosinophils and basophils) with methylation before and after deployment. Moreover, we analyzed the interaction of these covariates with time in our main models to investigate whether they affected the relation between our variables of interest (trauma, and change in psychopathology, GR exon 1_F expression and GR binding) and change in GR-1_F methylation.

To investigate the possible influence of alcohol use, medication and smoking, we excluded all individuals that changed medication between baseline and follow-up (n = 18, for types of medication see Supplementary Table S3), as well as individuals with a changed alcohol use (n =1) or smoking status (n = 6).

3. RESULTS

Sample characteristics, including information on age, childhood trauma, number of previous deployments, number of deployment-related trauma events, BMI, education, rank, mental health problems and PTSD symptoms are listed in Table 1.
Mean methylation per locus ranged from 0 to 2.60% (see Supplementary Table S2). Methylation changes per locus were highly correlated, especially for CpGs 31 to 52 (see Supplementary Figure S1).
Change in GR-1_F methylation was associated with change in GR exon 1_F expression at a nominal significance level at the following 17 CpGs: 1, 9, 11, 12, 23, 24, 28, 34, 39, 40, 45-51 (all p-values < 0.05, see Figure 1, as well as Supplementary Table S2 and Supplementary Figure S2). The mean methylation at these sites constituted our measure of functional methylation. Changes in our three main methylation measures (mean methylation, number of methylated sites and functional methylation) over time were significantly correlated with a minimal ρ of 0.89. Increases in all three methylation measures were associated with decreases in GR exon 1_F expression (mean methylation: $\beta = -0.54$, $p = 0.011$; number of methylated loci: $\beta = -0.039$, $p = 0.001$; functional methylation: $\beta = -0.526$, $p < 0.001$, see Table 2 and Figures 2A and 2B) and GR binding (mean methylation: $\beta = -463$, $p = 0.014$; number of methylated loci: $\beta = -29.9$, $p = 0.010$; functional methylation: $\beta = -317$, $p = 0.016$). In exploratory analyses, methylation change at NGFI-A binding sites was not associated with a change in GR exon 1_F expression ($\beta = -0.22$, $p = 0.36$) or GR binding ($\beta = -413$, $p = 0.06$) and was therefore not further analyzed.

Characteristics	Mean			SD			Range		
Age	27.5			9.3			18 - 54		
Childhood trauma	3.0			2.6			0 - 11		
Number of previous deployments	0.9			1.2			0 - 5		
Number of deployment-related trauma events	5.4			4.2			0 - 13		
BMI	24.4			2.7			18.1 – 35.8		
Education	Low			Moderate			High		
	42			42			6		
Rank	Private		Corporal	Non-commissioned officer			(Staff) officer		
	45		20	17			8		
	Pre-deployment			*Post-deployment*			t	df	p
	Mean	SD	Range	Mean	SD	Range			
Mental health problems (SCL-90)	103.7	17.1	90 - 209	110.8	23.1	90 - 204	2.97	87	0.004
PTSD symptoms (SRIP)	26.4	4.0	22 - 36	31.9	9.9	22 - 56	4.81	87	0.000006

Table 1. Sample characteristics (Total: n = 91, all male).
Education: low: some years of high school; moderate: finished high school; high: some years of college or university. Information about rank and education was missing for one subject.

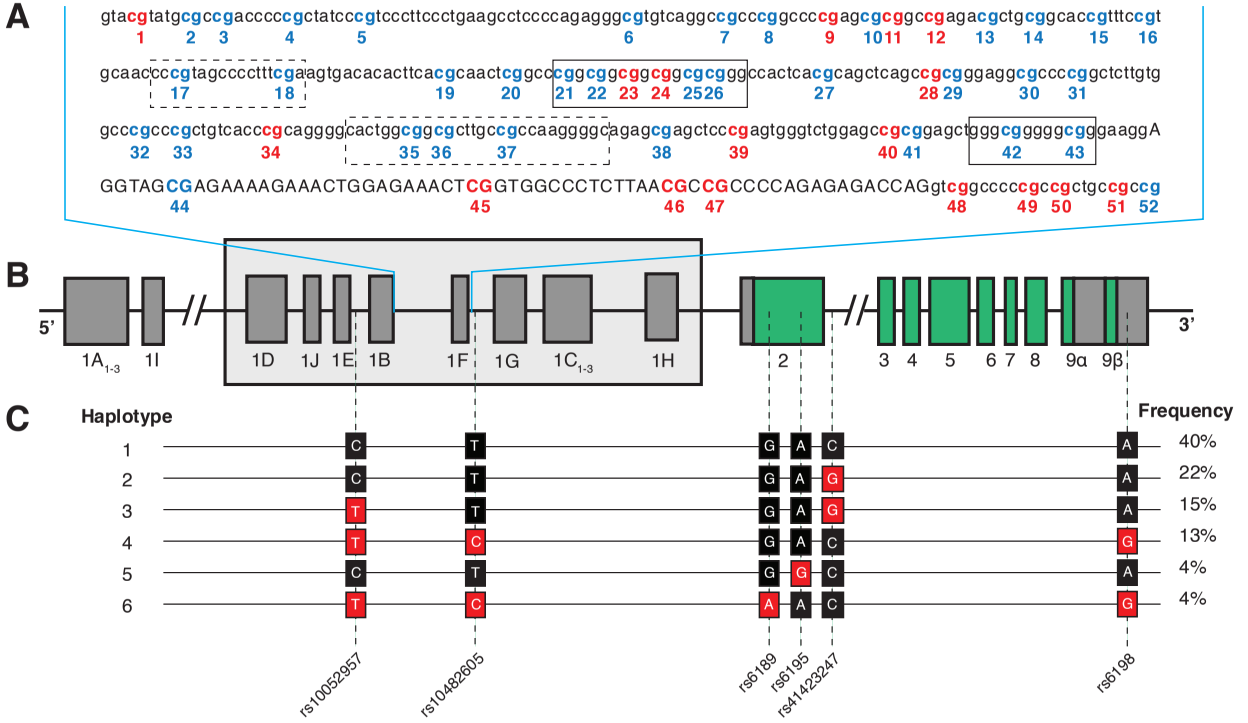


Figure 1. The glucocorticoid receptor exon 1_F region (GR-1_F) in NR3C1. **A:** All 52 5'—C—phosphate—G—3' sites (CpGs) where methylation was measured in the present study are numbered (red: CpGs where change in methylation was associated with GR exon 1_F expression change). Lower-case letters mark the promoter region, upper case letters the GR-1_F exon. Black-lined boxes represent canonical (solid) and non-canonical (dashed) NGFI-A binding sites, after McGowan et al. (McGowan et al., 2009) **B**: *NR3C1* (Chromosome 5: 142,657,496-142,815,077) from the 5' (left) to 3' (right) untranslated region. Boxes on the horizontal black line represent exons (grey: non-coding, green: coding sequence). The light grey box indicates a CpG island in the proximal promoter region (from 1D to 1H). **C**: Six common and functional haplotypes constituted by six SNPs (rs10052957, rs10482605, rs6189, rs6195, rs41423247 and rs6198) and their frequencies in the present study. Red letters represent the minor alleles. For a scaled representation of exons 2 through 9 in relation to the first exons, see (Sinclair et al., 2012).

3.1. GR-1$_F$ methylation and exposure to potentially traumatic events

Trauma exposure during deployment was significantly associated with an increase in all three methylation measures (mean methylation: β = 0.040, p = 0.003; number of methylated loci: β = 0.75, p = 0.002; functional methylation: β = 0.56, p = 0.002, see Table 2) and with an increase in methylation at 23 individual CpGs: 2, 4, 8, 9, 17, 19, 20, 22, 28, 31, 32, 34, 35, 39, 40, 45-52 (see Supplementary Table S2).

No significant associations were observed between childhood trauma and our main methylation measures (change in methylation over time: all p-values > 0.54; pre-deployment methylation: all p-values > 0.40).

3.2. GR-1$_F$ methylation and the development of psychopathology

Whereas the development of mental health problems was only at trend level significance associated with an increase in the number of methylated loci (β = 0.089, p = 0.050) (Figure 2C) and not with mean methylation (β = 0.004, p = 0.10), it was significantly associated with an increase in functional methylation (β = 0.010, p = 0.005) (see Table 2 and Figure 2D). Significant associations between change in mental health problems and methylation change existed at 14 CpGs (22, 31-35, 39-41, 44, 47, 48, 51, 52).

The development of PTSD symptoms was not associated with change in any methylation measure (all p-values > 0.12, see Table 2). At a nominal significance level, change in PTSD symptoms was associated with methylation change at 9 individual CpGs (10, 20, 25, 31, 35-37, 44, 51, see Supplementary Table S2).

	Trauma exposure (n = 91)			**Mental health problems (n = 88)**			**PTSD symptoms (n = 88)**			**GR exon 1$_F$ expression (n = 75)**			**GR binding (n = 47)**		
Methylation	*β*	*t*	*p*	*β*	*t*	*p*	*β*	*t*	*p*	*β*	*t*	*p*	*β*	*t*	*p*
Mean	0.040	3.02	*0.003*	0.004	1.65	0.10	0.233	1.05	0.30	-0.543	-2.63	*0.011*	-463	-2.56	*0.014*
Number of methylated loci	0.745	3.24	*0.002*	0.089	1.98	0.050	4.356	1.14	0.26	-0.039	-3.41	*0.001*	-29.9	-2.68	*0.010*
Functional	0.056	3.16	*0.002*	0.010	2.89	*0.005*	0.450	1.53	0.13	-0.526	-4.02	*< 0.001*	-317	-2.51	*0.016*

Table 2. Summaries of the main analyses. Associations of change in methylation (mean, number of methylated sites and functional) with trauma exposure and change in mental health problems, PTSD symptoms, GR exon 1$_F$ expression and GR binding.

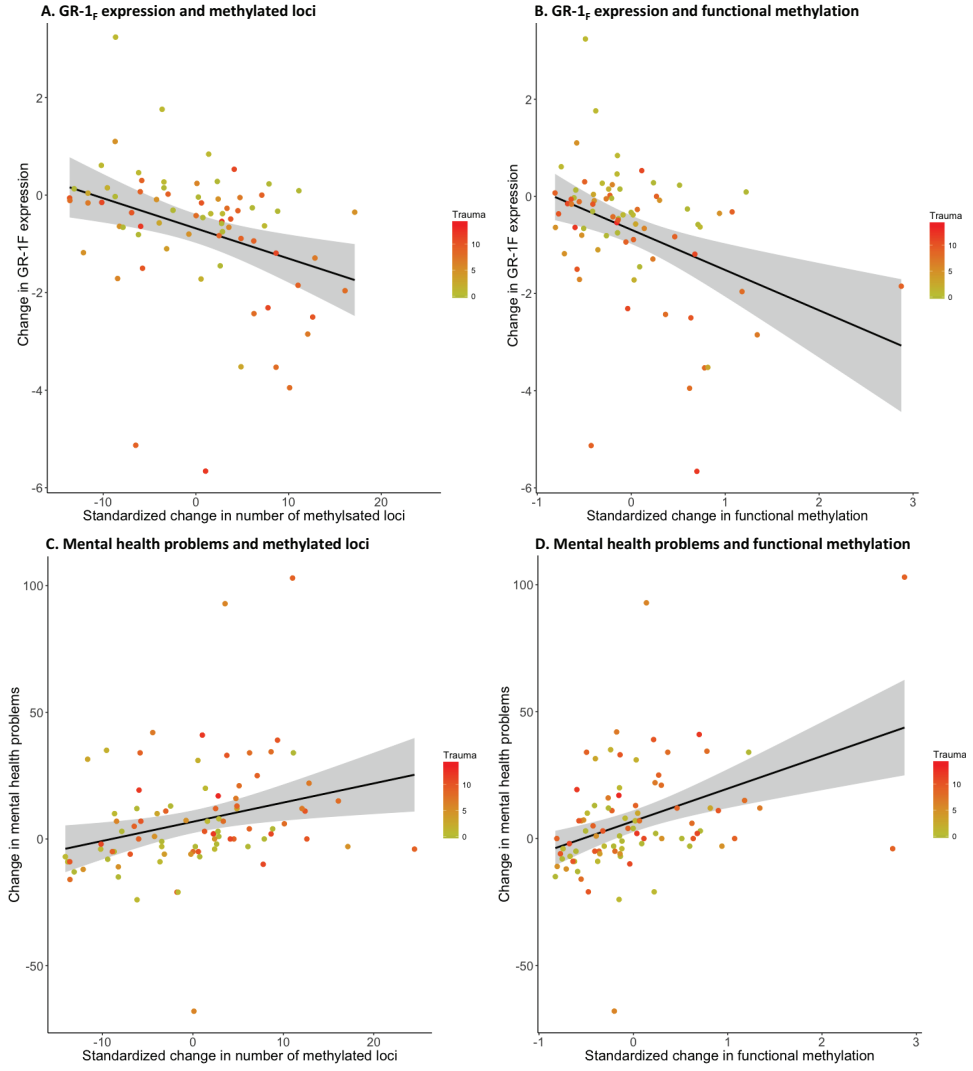


Figure 2. Change in mental health problems in relation to **A.** the number of methylated loci (n = 88, β = 0.089, p = 0.050) and **B.** functional methylation (n = 88, β = 0.010, p = 0.005), and change in GR exon 1_F expression in relation to **C.** the number of methylated loci (n = 75, β = -0.039, p = 0.001) and **D.** functional methylation (n = 75, β = -0.53, p < 0.001). Standardized change indicates that methylation levels after deployment were adjusted for pre-deployment values.

3.3. GR-1$_F$ methylation and genetic variation in NR3C1

None of the *NR3C1* haplotypes or individual SNPs was significantly associated with either pre-deployment or prospective changes in GR-1$_F$ methylation measures after correcting for multiple testing (data not shown). Nevertheless, rs10052957 showed an association with prospective methylation changes at a nominal significance level (TC carriers with CC as reference; mean methylation: $\beta = -0.25$, $p = 0.046$; number of methylated loci: $\beta = -4.21$, $p = 0.045$; functional methylation: $\beta = -0.38$, $p = 0.022$).

3.4. Confounder and sensitivity analyses

Confounding by age, body-mass-index (BMI), military rank, education, previous number of deployments and white blood cell counts (including values of neutrophils, lymphocytes, monocytes, eosinophils and basophils) was highly unlikely as these measures were not correlated with methylation before or after deployment (p-values > 0.05). In addition, cell-type composition was similar before and after deployment (Boks et al., 2015). Furthermore, including the interactions of these covariates with time did not affect the associations between change in GR-1$_F$ methylation with trauma exposure during deployment, change in mental health problems or PTSD symptoms, change in GR-1$_F$ expression or change in GR binding.

No individuals used antidepressants at any time point (see Supplementary Table S5). Exclusion of individuals with changed medication, smoking status or alcohol use status after deployment (total n = 23) strengthened most associations of GR-1$_F$ methylation in the longitudinal analyses (as indicated by the βs) except for the associations with GR binding, which did not remain significant (see Supplementary Table S6).

3.5. Prediction of post-deployment psychopathology by pre-deployment GR-1$_F$ methylation

Pre-deployment methylation did not significantly predict a high level of post-deployment mental health problems (mean methylation: AUC = 0.51, p = 0.47; number of methylated loci: AUC = 0.54, p = 0.32; functional methylation: AUC = 0.51, p = 0.47) or PTSD symptoms (mean methylation: AUC = 0.58, p = 0.11; number of methylated loci: AUC = 0.56, p = 0.18; functional methylation: AUC = 0.56, p = 0.17).

4. DISCUSSION

We examined longitudinal changes in whole blood GR-1_F methylation from one month prior to until six months after a four-month period of military deployment in relation to deployment-related trauma and the development of mental health problems and PTSD symptoms. We found that deployment-related trauma was associated with an increase in all GR-1_F methylation measures. However, only an increase in GR-1_F methylation at loci associated with GR exon 1_F expression (functional methylation) was significantly related to the development of post-deployment mental health problems. In contrast, the emergence of PTSD symptoms was not associated with an increase in functional methylation over time. Pre-deployment GR-1_F methylation did not predict a high level of psychopathology symptoms after deployment. Moreover, six functional *NR3C1* haplotypes and the six SNPs constituting these haplotypes were not associated with changes in GR-1_F methylation.

4.1. GR-1_F methylation as a vulnerability factor

This study provides the first longitudinal evidence in humans that methylation differences between trauma- and non-trauma-exposed individuals previously reported in the majority of cross-sectional studies (see for review Daskalakis and Yehuda, 2014) reflect a response to traumatic stress exposure. An increase in GR-1_F methylation at functionally relevant sites could result in impaired negative feedback on the HPA axis (Tyrka et al., 2012), leading to increased vulnerability to psychopathology. The here reported association between functional GR-1_F methylation increase and emerging mental health problems fits this hypothesis. In contrast, we did not find any associations between childhood trauma and GR-1_F methylation, whereas most, but not all (Tyrka et al., 2016), previous studies found a positive association (see for review Daskalakis and Yehuda, 2014). Of note, the fact that elevated GR-1_F methylation levels could constitute a vulnerability factor for the development of psychopathology was not supported by our prediction models and has not been investigated in prior studies. Moreover, the only two previous longitudinal studies on GR-1_F methylation do not indicate that psychotherapeutic interventions influence this hypothesized pathophysiological mechanism. Perroud et al. (Perroud et al., 2013) reported no change in mean GR-1_F methylation in 61 patients with borderline personality disorder after intensive dialectical behavior therapy. Yehuda et al. (Yehuda et al., 2013) showed that pre-treatment GR-1_F methylation levels (sum and number of methylated loci) significantly differed between responders (n = 8) and non-responders (n = 8) receiving psychotherapy for PTSD, but did not find a significant methylation change over time.

4.2. GR-1_F methylation in relation to psychopathology

In contrast to our study, it was previously observed that individuals with a high level of post-deployment PTSD symptoms had increased GR sensitivity (Van Zuiden et al., 2012) and GR binding (Van Zuiden et al., 2012, 2011) prior to deployment (while depressive symptom development was associated with decreased GR sensitivity (Van Zuiden et al., 2012)). Moreover, Yehuda et al. (Yehuda et al., 2015) found lower GR-1_F methylation and evidence for increased GR sensitivity in PTSD patients compared with trauma-exposed controls. These opposing directionalities of effects cannot be ascribed to a difference in pathophysiology between PTSD symptoms and mental health problems (measured with

the SCL-90), as Yehuda et al. (Yehuda et al., 2015) found negative associations of GR-1_F methylation with both outcomes, whereas we found positive associations (not significant for PTSD symptoms). Of note, both increased and decreased functionality of the GR have been linked to psychopathology, and Raison and Miller (Raison and Miller, 2003) previously suggested that these mechanisms are specific to PTSD and MDD, respectively (Raison and Miller, 2003). However, two recent studies contradict this hypothesis, showing decreased GR-1_F methylation in MDD (Na et al., 2014) and increased GR-1_F methylation in PTSD (Perroud et al., 2014). Moreover, PTSD and MDD often co-occur which complicates the interpretation of findings. This is illustrated by the significant correlation (ρ = 0.50) between change in depressive symptoms (subscale SCL-90) and change in PTSD symptoms in the present study.

4.3. Strengths

The main strength of the current study is its longitudinal design, with GR-1_F methylation, expression, GR binding and psychiatric outcomes assessed before and after military deployment. Evidence from longitudinal studies is paramount as it is much less influenced by genetic predispositions and other confounding factors that may influence both methylation levels and psychopathology than cross-sectional studies. In addition, the present study investigated the entire GR exon 1_F region, allowing examination of all CpGs in relation to GR exon 1_F expression. This approach highlights that associations between change in GR-1_F methylation and expression are site-specific and present at 17 out of 52 CpGs. 13 of these 17 CpGs have previously been associated with trauma and/or psychopathology (CpG numbers 1, 9, 23, 28, 34, 40 and 45-51, see this review (Daskalakis and Yehuda, 2014)). Our findings do not support an important role for NGFI-A binding sites in GR-1_F transcription. This is in line with recent evidence suggesting that several other transcription factors are important in the GR exon 1_F region (Witzmann et al., 2012). Other strengths are the generally healthy cohort at baseline, the absence of antidepressant use at any time point and the sensitivity analyses that exclude a confounding effect of change in any medication, smoking or alcohol use status. Finally, we explored the associations between *NR3C1* haplotypes and SNPs with GR-1_F methylation and did not find any significant associations with baseline GR-1_F methylation, which is in line with a recent study (Li-Tempel et al., 2016), or with methylation change. This finding highlights the fact that GR-1_F methylation is just one biological mechanism influencing GR sensitivity, while *NR3C1* haplotypes may be independently associated with GR functioning and psychopathology (Hardeveld et al., 2015).

4.4. Limitations

A possible limitation is the relevance of peripheral blood methylation to the brain. Several studies suggest that methylation differences across tissues are substantial (Davies et al., 2012; Hannon et al., 2015), even though consistent effects of various methylation quantitative trait loci (mQTLs) are found across tissues (Smith et al., 2014). However, the effect of trauma, mediated through stress hormones or cytokines, appears to affect the epigenome in a wide range of cell and tissue types (Turecki and Meaney, 2014). This is in line with findings of Tyrka and colleagues (Tyrka et al., 2012), who demonstrated a cross-sectional relation between trauma-related whole blood GR-1_F methylation with decreased

negative feedback of the HPA axis. Also, increased GR-1_F methylation in peripheral blood cells has consistently been linked to (early life) adversity (Daskalakis and Yehuda, 2014). The question of generalizability of methylation patterns across tissues also pertains to our measurement of GR exon 1_F expression and GR binding in PBMCs, instead of whole blood (Reinius et al., 2012). Another limitation is our focus on GR-1_F, while methylation in other parts of *NR3C1* may hold additional information about the relation between trauma and GR function (Labonte et al., 2012). In addition, only Dutch Caucasian males were investigated which limits the generalizability. This may be relevant since there is evidence for ethnic and sex-specific differences in cortisol stress responsivity in relation to DNA methylation (Houtepen et al., 2016). Furthermore, in addition to baseline GR exon 1_F expression data examined here, GR exon 1_F expression data following stress may provide additional information of its relation with GR-1_F methylation. Finally, we used self-report questionnaires to assess psychiatric outcomes, constituting a possible source of social desirability bias, and our questionnaire for exposure to potentially traumatic events does not take severity and impact into account.

4.5. Conclusions

In conclusion, this is the first longitudinal evidence linking changes in GR-1_F methylation to trauma exposure and the development of psychopathology symptoms. Our data indicate that trauma exposure increases GR-1_F methylation and that only increases at specific functionally relevant sites may predispose for post-deployment psychopathology. These results suggest an important role for epigenetic regulation of GR functioning after trauma exposure in the development of psychopathology.

ACKNOWLEDGEMENTS

We thank J.W. Deiman for creating Figure 1.

CONFLICT OF INTEREST

This study was funded by a grant from the Dutch Ministry of Defence. Methylation and gene expression analyses were funded by the VENI fellowship from the Netherlands Organisation for Scientific Research (NWO, grant number 451.13.001) to CHV.
Funders had no role in design and reporting of the study. All authors reported no biomedical financial interests or potential conflicts of interest.

Assay ID	Sensitivity (gDNA/bsDNA) in ng	CV	# CpGs	Pyrosequencing primers target chromosomal region Chr5:
ADS8063FS2	50/0.3125	0,00%	6	142783937-142783884
ADS1343FS2	5/5	0,99%	12	142783874-142783810
ADS1343FS3	20/10	1,40%	9	142783793-142783756
ADS1342FS	5/0.3125	2,02%	10	142783745-142783679
ADS749FS	10/2.5	1,74%	7	142783664-142783608
ADS2386FS	5/0.3125	0,72%	8	142783585-142783529

Supplementary Table S1. Summary of the sensitivity and reproducibility for all six pyrosequencing assays.

Section	CpG site	Chrom 5 GRCh37/hg19	Baseline mean methylation (%)	GR exon 1_F expression			Trauma exposure			Mental health problems			PTSD symptoms		
				β	*t*	*p*	*β*	*t*	*p*	*β*	*t*	*p*	*β*	*t*	*p*
Promoter 1F	**CpG_1**	142783937	0.026	-0.56676	-2.08	*0.041*	0.01336	1.52	0.133	-0.00099	-0.52	0.602	-0.12221	-0.81	0.419
	CpG_2	142783931	2.597	0.00113	0.02	0.981	0.13762	2.12	*0.037*	-0.00901	-0.65	0.517	0.80387	0.73	0.466
	CpG_3	142783928	0.327	0.03459	0.33	0.744	0.00826	0.36	0.718	0.00768	1.58	0.117	-0.55669	-1.43	0.156
	CpG_4	142783921	1.839	-0.01546	-0.34	0.734	0.15918	2.68	*0.009*	-0.01162	-0.94	0.351	0.48246	0.48	0.633
	CpG_5	142783913	0.704	-0.04790	-0.78	0.437	0.06423	1.40	0.166	0.00371	0.38	0.707	0.38090	0.48	0.629
	CpG_6	142783884	0.147	0.09273	0.91	0.365	0.02378	0.96	0.339	-0.00353	-0.71	0.482	-0.26617	-0.63	0.531
	CpG_7	142783874	0.176	0.12892	1.00	0.322	-0.03194	-1.67	0.099	0.00218	0.56	0.576	-0.23900	-0.73	0.468
	CpG_8	142783870	0.705	0.03229	0.55	0.585	0.08864	2.04	*0.044*	-0.00926	-1.07	0.288	-0.92221	-1.25	0.214
	CpG_9	142783864	0.818	-0.18695	-2.25	*0.027*	0.14292	4.00	*0.000*	0.00975	1.39	0.169	0.29411	0.48	0.629
	CpG_10	142783860	0.031	0.17728	0.86	0.391	0.01181	0.95	0.343	-0.00310	-1.24	0.218	-0.42783	-2.05	*0.043*
	CpG_11	142783858	0.056	-0.47742	-2.73	0.008	0.02183	1.68	0.096	-0.00111	-0.42	0.673	-0.30875	-1.40	0.166
	CpG_12	142783854	0.120	-0.64177	-2.55	*0.013*	0.01258	1.11	0.268	0.00044	0.19	0.846	-0.06827	-0.35	0.725
	CpG_13	142783849	0.070	-0.08949	-0.66	0.512	0.01003	0.55	0.581	-0.00628	-1.73	0.088	-0.23521	-0.76	0.450
	CpG_14	142783844	0.040	0.04110	0.29	0.771	0.03093	1.73	0.087	0.00032	0.09	0.930	-0.30139	-0.99	0.327
	CpG_15	142783838	0.070	-0.05040	-0.32	0.747	-0.01217	-0.75	0.454	-0.00136	-0.42	0.679	-0.41631	-1.51	0.135
	CpG_16	142783832	0.257	0.00908	0.10	0.920	0.01802	0.67	0.502	0.00215	0.40	0.693	-0.02881	-0.06	0.950
	CpG_17	142783822	0.264	0.09149	1.39	0.170	0.08256	2.07	*0.041*	-0.00773	-0.96	0.339	-0.31935	-0.47	0.641
	CpG_18	142783810	1.625	-0.06003	-1.66	0.100	0.03949	0.61	0.544	-0.00746	-0.58	0.566	-1.15168	-1.05	0.299
	CpG_19	142783793	0.392	-0.08201	-1.11	0.272	0.08411	2.33	*0.022*	-0.00479	-0.66	0.512	-0.23885	-0.39	0.698
	CpG_20	142783786	0.487	-0.08671	-1.02	0.310	0.07057	2.67	*0.009*	-0.00647	-1.22	0.227	-1.03294	-2.32	*0.023*
	CpG_21	142783781	0.212	-0.04317	-0.32	0.753	0.02111	0.66	0.510	-0.00269	-0.42	0.677	0.21349	0.39	0.697
	CpG_22	142783778	0.029	-0.11766	-0.90	0.369	0.04976	2.05	*0.043*	0.01075	2.26	*0.026*	0.51344	1.25	0.216
	CpG_23	142783775	0.070	-0.43771	-2.33	*0.023*	-0.02858	-1.00	0.321	-0.00535	-0.93	0.356	-0.68912	-1.42	0.159
	CpG_24	142783772	0.116	0.27892	3.05	*0.003*	-0.01811	-0.90	0.371	0.00102	0.25	0.802	-0.14259	-0.41	0.681
	CpG_25	142783769	0.087	-0.14097	-0.79	0.435	0.00807	0.76	0.448	0.00392	1.85	0.068	0.40906	2.29	*0.025*
	CpG_26	142783767	0.000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	CpG_27	142783756	0.000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	CpG_28	142783745	0.695	-0.23402	-2.31	*0.024*	0.05534	2.37	*0.020*	0.00606	1.31	0.193	0.62274	1.59	0.115
	CpG_29	142783743	0.529	0.18574	1.13	0.264	0.00678	0.44	0.663	0.00302	0.99	0.323	-0.21490	-0.82	0.416
	CpG_30	142783736	0.478	0.19760	1.06	0.293	0.01305	1.08	0.284	-0.00011	-0.05	0.963	-0.02984	-0.15	0.884
	CpG_31	142783731	1.395	-0.13154	-1.66	0.102	0.08068	2.35	*0.021*	0.01336	2.06	*0.042*	1.11631	2.03	*0.046*
	CpG_32	142783717	1.264	-0.04994	-0.68	0.498	0.10878	2.81	*0.006*	0.02323	3.19	*0.002*	0.81413	1.27	0.208
	CpG_33	142783713	1.024	-0.07980	-0.92	0.360	0.00413	0.13	0.897	-0.01401	-2.27	*0.026*	0.25625	0.48	0.631
	CpG_34	142783703	0.967	-0.22106	-2.50	*0.015*	0.09769	2.75	*0.007*	0.01707	2.48	*0.015*	0.96148	1.62	0.109
	CpG_35	142783688	1.179	-0.13436	-1.27	0.207	0.05950	2.24	*0.028*	0.01211	2.36	*0.021*	1.15815	2.68	*0.009*
	CpG_36	142783685	0.258	-0.08524	-0.44	0.661	0.00538	0.40	0.689	0.00233	0.87	0.387	0.48405	2.15	*0.034*
	CpG_37	142783679	1.257	-0.19383	-1.74	0.087	0.03801	1.54	0.127	0.00583	1.20	0.235	0.96238	2.38	*0.019*
	CpG_38	142783664	0.302	-0.31820	-1.67	0.099	0.00880	0.63	0.530	0.00106	0.38	0.702	0.26878	1.15	0.255
	CpG_39	142783656	1.322	-0.17340	-2.28	*0.025*	0.11354	3.41	*0.001*	0.01941	3.07	*0.003*	0.75107	1.34	0.183
	CpG_40	142783640	0.966	-0.22029	-2.81	*0.006*	0.06357	2.02	*0.047*	0.01910	3.23	*0.002*	0.69620	1.33	0.189
	CpG_41	142783638	0.201	0.02292	0.15	0.883	0.02290	1.33	0.186	0.00764	2.29	*0.024*	0.21759	0.74	0.461
	CpG_42	142783628	0.121	0.41824	1.23	0.223	0.00230	0.34	0.735	-0.00173	-1.30	0.196	-0.11652	-1.01	0.317
Exon 1F	CpG_43	142783622	0.025	-0.07359	-0.33	0.746	-0.01429	-1.18	0.240	-0.00037	-0.15	0.878	0.01488	0.07	0.943
	CpG_44	142783608	0.132	-0.20635	-1.23	0.221	0.02161	1.34	0.182	0.01087	3.62	*0.001*	0.68755	2.57	*0.012*
	CpG_45	142783585	1.285	-0.36176	-3.84	*0.000*	0.06171	2.49	*0.015*	0.00772	1.60	0.114	0.55153	1.34	0.185
	CpG_46	142783570	2.400	-0.18642	-2.68	*0.009*	0.09402	2.70	*0.008*	0.00882	1.31	0.194	0.50585	0.88	0.380
	CpG_47	142783567	0.982	-0.45279	-5.01	*0.000*	0.06402	2.68	*0.009*	0.01259	2.74	*0.008*	0.60559	1.51	0.135

5 Adjacent CpGs	**CpG_48**	142783548	1.256	-0.23842	-3.47	*0.001*	0.09347	2.72	*0.008*	0.02491	3.92	*0.000*	0.91766	1.59	0.115
	CpG_49	142783541	1.607	-0.18377	-2.43	*0.018*	0.09217	2.87	*0.005*	0.00876	1.40	0.165	0.68506	1.27	0.206
	CpG_50	142783538	0.819	-0.39752	-4.70	*0.000*	0.06140	2.20	*0.031*	0.01009	1.84	0.070	0.39477	0.83	0.409
	CpG_51	142783532	1.011	-0.43720	-4.41	*0.000*	0.07419	2.35	*0.022*	0.01533	2.67	*0.009*	1.11301	2.23	*0.029*
	CpG_52	142783529	0.796	-0.19658	-1.80	0.078	0.08114	2.49	*0.016*	0.02622	4.68	*0.000*	1.04481	1.97	0.054
	Mean		0.640	-0.54264	-2.63	*0.011*	0.04016	3.02	*0.003*	0.00431	1.65	0.104	0.23341	1.05	0.299

Supplementary Table S2. Mean methylation per locus prior to and after deployment, as well as the associations between change in methylation and change in GR exon 1_F expression, trauma exposure or change in mental health problems or PTSD symptoms. CpGs significantly associated with GR exon 1_F expression (functional methylation) are indicated in bold, p-values are uncorrected for multiple testing.

Gene Name	Reference	Primers pairs	
		Forward	Reverse
GR-1$_F$	Sinclair et al. 2012	CCGCCGCCACCCTTT	CAGGAGTTAATGATTCTTTGGAGTCCAT
PPIB		GTTTGAAGTTCTCATCGGGG	AAAACAGCAAATTCCATCGTG
RPL13a		CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA
RPL37a		CGACATGGCCAAACGTACCA	CAAGTGTACTTGGCGTGCTG
YWHAZ		ACTTGACATTGTGGACATCGGA	CAAAAGTTGGAAGGCCGGT

Supplementary Table S3. Human RT-qPCR primers pairs.

SNP	Position	Reference allele	Alternative allele	Alternative Allele freq	Average Call Rate	Genotyped/Imputed	Alternative allele frequency HRC reference panel
rs6198	5:142657621	T	C	0,17497	1	Genotyped	0,161903
rs41423247	5:142778575	G	C	0,37616	0,99831	Imputed	0,3585
rs6195	5:142779317	T	C	0,04253	0,99994	Genotyped	0,024284
rs6189	5:142780339	C	T	0,03848	0,99989	Imputed	0,0226363
rs10482605	5:142783521	A	G	0,17528	0,99903	Imputed	0,166846
rs10052957	5:142786701	G	A	0,33131	0,99994	Genotyped	0,316769

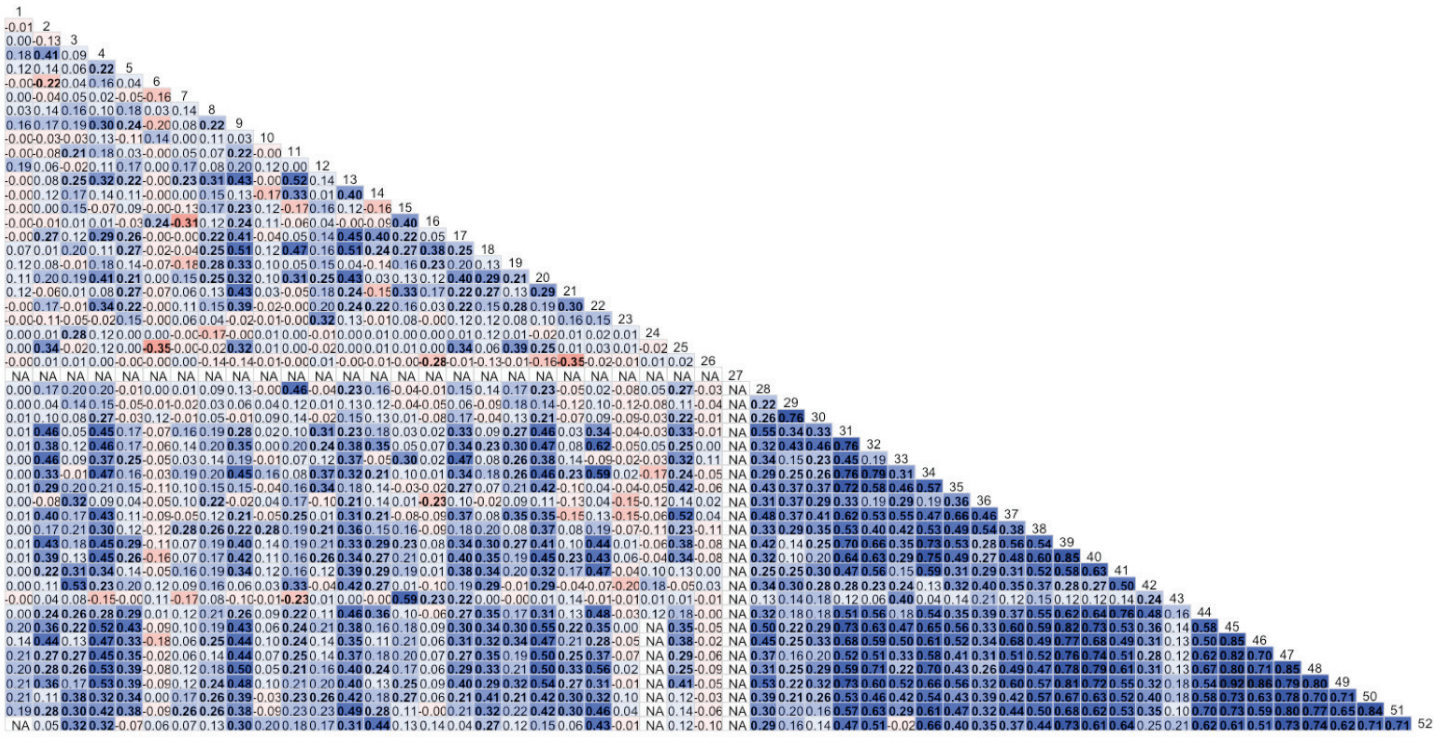
Supplementary Table S4. Overview genotyped and Haplotype Reference Consortium (HRC)-imputed SNPs.Data are based on all 963 genotyped individuals in the military cohort.

Subject	Medication T0	Medication T1
1	No medication	Sympatholytic drug
2	Albuterol	Simvastatin
3	No medication	Anti-tuberculosis drugs
4	Hydrochlorothiazide	Simvastatin
5	Enalapril	Chlortalidone
6	Seretide (salmeterol/fluticasone)	Desloratidine
7	No medication	Doxycycline
8	No medication	Antibiotics unspecified
9	Anti-diabetic medication	Antihypertensive
10	No medication	Isoniazid
11	No medication	Eye drops after laser eye surgery
12	No medication	Levocetirizine
13	No medication	Oxazepam
14	No medication	Perindopril
15	Unknown medication	No medication
16	Unknown medication	No medication
17	Unknown medication	No medication
18	Unknown medication	No medication

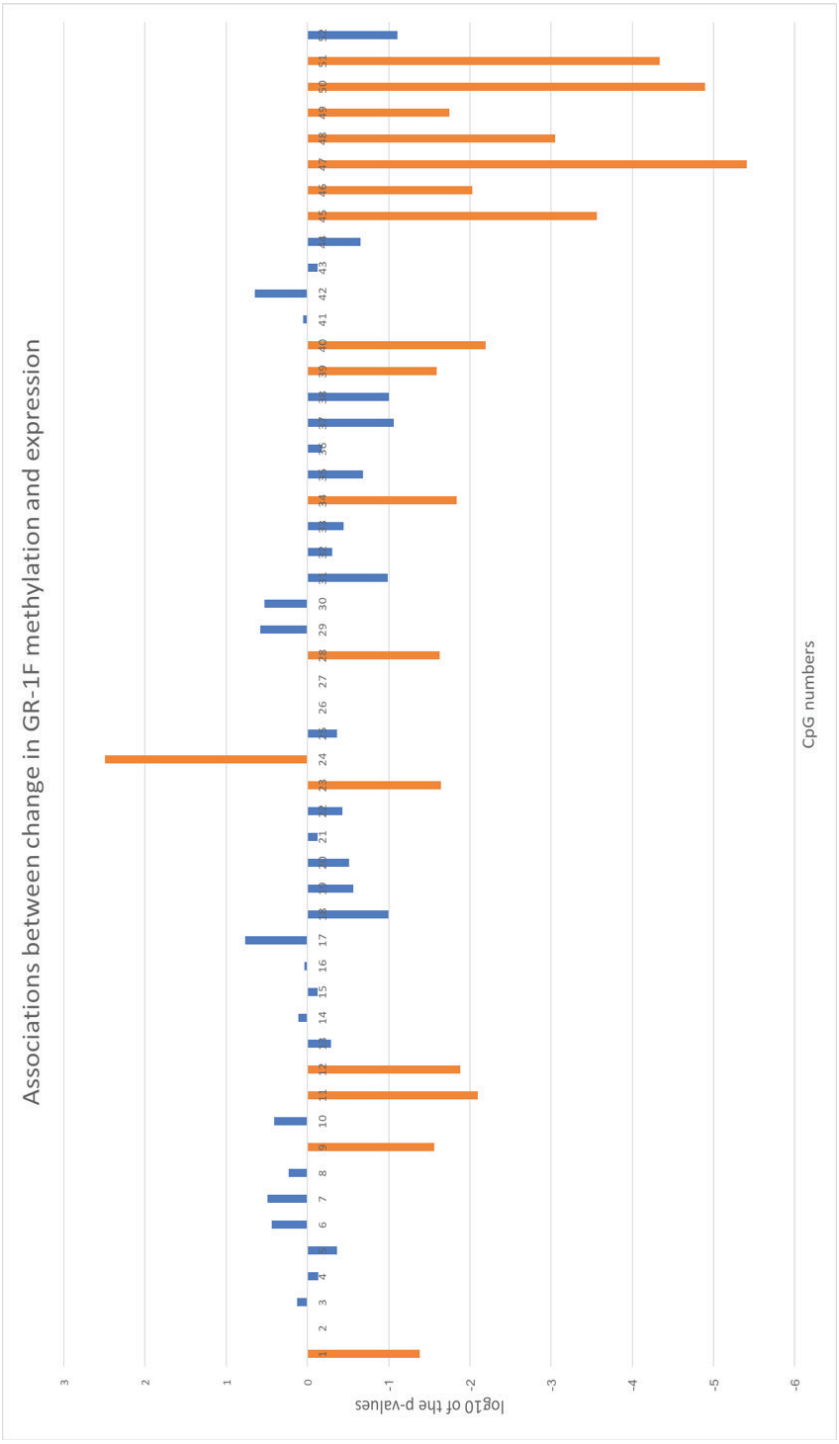
Supplementary Table S5. Medication of subjects who changed medication between T0 and T1. These subjects were excluded in sensitivity analyses.

	Trauma exposure (n = 68)			Mental health problems (n = 65)			PTSD symptoms (n = 65)			GR exon 1$_F$ expression (n = 55)			GR binding (n = 32)		
Methylation	*β*	*t*	*p*	*β*	*t*	*p*	*β*	*t*	*p*	*β*	*t*	*p*	*β*	*t*	*p*
Mean	0.049	2.91	*0.005*	0.005	1.58	0.12	0.37	1.29	0.20	-0.55	*-2.26*	*0.028*	-263	-1.37	0.18
Number of methylated loci	1.00	3.54	*< 0.001*	0.084	1.55	0.13	4.88	1.04	0.30	-0.04	-3.00	*0.004*	-23.3	-1.83	0.078
Functional	0.074	3.39	*0.001*	0.011	2.58	*0.012*	0.63	1.73	0.088	-0.54	-3.49	*0.001*	-180	-1.28	0.21

Supplementary Table S6. Summaries of the sensitivity analyses after excluding individuals who changed medication, smoking status or alcohol use status (total n = 23): associations of change in methylation (mean, number of methylated sites and functional) with trauma exposure and change in mental health problems, PTSD symptoms, GR exon 1$_F$ expression and GR binding.



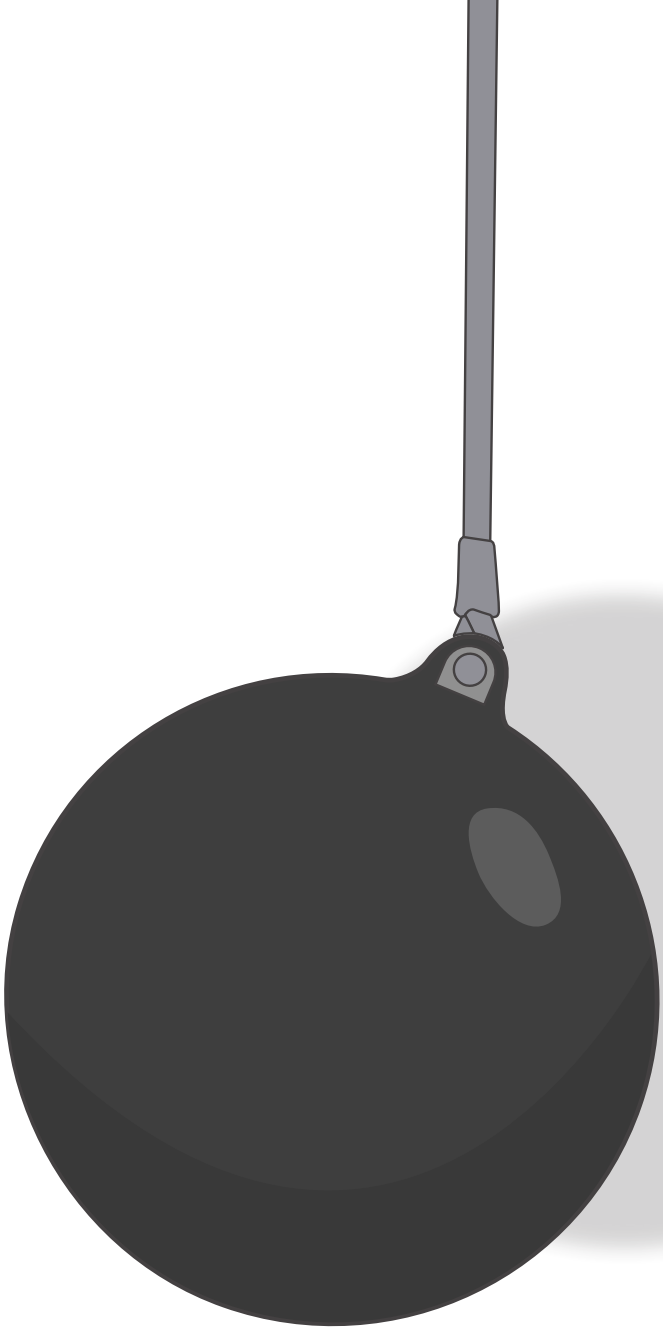
Supplementary Figure S1.
Correlogram of GR-1_F methylation change from pre- to post-deployment at all 52 CpGs. Blue and red colors indicate positive and negative correlations, respectively. Numbers in each cell represent Pearson's rhos. Bold numbers indicate significant correlations.



Supplementary Figure S2.
Log10 of the p-values of the association between longitudinal changes in GR-1$_F$ methylation and expression for each of the individual CpGs. Negative values represent negative associations. Red bars indicate significance ($p < 0.05$).

PART 2

The GABA system



CHAPTER 4

Brain GABA levels across psychiatric disorders: a systematic literature review and meta-analysis of ^{1}H-MRS studies

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ABSTRACT

The inhibitory gamma-aminobutyric acid (GABA) system is involved in the etiology of most psychiatric disorders, including schizophrenia, autism spectrum disorder (ASD) and major depressive disorder (MDD). It is therefore not surprising that proton magnetic resonance spectroscopy (^{1}H-MRS) is increasingly used to investigate *in vivo* brain GABA levels. However, integration of the evidence for altered *in vivo* GABA levels across psychiatric disorders is lacking. We therefore systematically searched the clinical ^{1}H-MRS literature and performed a meta-analysis. A total of 40 studies (n = 1591) in seven different psychiatric disorders were included in the meta-analysis: MDD (n = 437), schizophrenia (n = 517), ASD (n = 150), bipolar disorder (n = 129), panic disorder (n = 81), posttraumatic stress disorder (PTSD) (n = 104) and attention deficit/hyperactivity disorder (ADHD) (n = 173). Brain GABA levels were lower in ASD (standardized mean difference (SMD) = -0.74, p = 0.001) and in depressed MDD patients (SMD = -0.52, p = 0.005), but not in remitted MDD patients (SMD = -0.24, p = 0.310) compared to controls. In schizophrenia this finding did not reach statistical significance (SMD = -0.23, p = 0.089). No significant differences in GABA levels were found in bipolar disorder, panic disorder, PTSD, and ADHD compared to controls. In conclusion, this meta-analysis provides evidence for lower brain GABA levels in ASD and in depressed (but not remitted) MDD patients compared to healthy controls. Findings in schizophrenia are more equivocal. Even though future ^{1}H-MRS studies could greatly benefit from a longitudinal design and consensus on the preferred analytical approach, it is apparent that ^{1}H-MRS studies have great potential in advancing our understanding of the role of the GABA system in the pathogenesis of psychiatric disorders.

1. INTRODUCTION

There is ample evidence for involvement of the gamma-aminobutyric acid (GABA) system in psychiatric disorders such as schizophrenia (Gonzalez-Burgos et al., 2015; Lewis et al., 2005; Nakazawa et al., 2012), depression (Luscher et al., 2011), bipolar disorder (Brambilla et al., 2003), anxiety (Geuze et al., 2008; Kalueff and Nutt, 2007), autism (Marín, 2012), alcohol use disorder (Kumar et al., 2009) and attention deficit/hyperactivity disorder (ADHD) (Rivero et al., 2015). A role for GABA neurotransmission across a wide spectrum of psychiatric disorders is not surprising since GABA is present at approximately one third of all synapses in the central nervous system and shapes neural network dynamics via GABAergic interneurons (Möhler, 2007). As a result, GABA system functionality is pivotal for physiological processes that are often affected in psychiatric disorders, e.g. neural plasticity, stress reactivity, sensory processing, memory formation and attention (Mody and Pearce, 2004; Möhler, 2007; Vinkers et al., 2010).

A variety of approaches is applied to disentangle the role of the GABA system in the etiology of psychiatric disorders, e.g. involving (epi)genetics, post mortem studies and the measurement of GABA in plasma and cerebrospinal fluid see for example the review of (Luscher et al., 2011). Currently, the only methods to directly probe the GABA system in the living human brain are proton magnetic resonance spectroscopy (^{1}H-MRS), positron emission tomography (PET), and single photon emission computed tomography (SPECT). Of these methods, ^{1}H-MRS is the only one that does not require administration of radioactive tracers or drugs. Although GABA levels are relatively low in the human brain (±1 mmol/kg (Wijtenburg et al., 2015), compared to 5-15 mmol/kg for glutamate (Govindaraju et al., 2000) for example), recent advances in ^{1}H-MRS techniques and increased field strengths of MRI scanners have resulted in an improved GABA detection (Wijtenburg et al., 2015). In light of the major overlapping signal for GABA with glutamine and glutamate in standard MRS sequences due to its chemical structure, it is vital to acknowledge the importance of GABA-specific protocols reliably disentangling the GABA signal from the glutamate and glutamine signal. Moreover, editing techniques such as MEGA-PRESS or MEGA-sLASER (Andreychenko et al., 2012) allow for the quantification of brain GABA independent of overlapping spectral metabolites such as creatine (Mullins et al., 2014) and with reduced macromolecular contamination of the GABA signal (Arteaga De Castro et al., 2013).

These developments have resulted in a steady increase in ^{1}H-MRS studies examining GABA levels in psychiatric disorders ever since the first studies in 1999 (Behar et al., 1999; Sanacora et al., 1999). However, it is currently unknown whether brain GABA levels are consistently altered across a range of psychiatric disorders. We also do not know whether GABA levels in these disorders are state-dependent or represent a trait characteristic and whether brain GABA levels differ in developmental disorders (such as autism) from disorders with a stronger environmental component (such as MDD). In an attempt to clarify the potential relevance of brain GABA levels, we conducted a meta-analysis of the existing ^{1}H-MRS GABA studies across psychiatric disorders. Moreover, to enhance the interpretation of our results and the implications for future ^{1}H-MRS studies, we provide a critical discussion on the challenges of GABA quantification that are associated with the use of proton magnetic resonance spectroscopy.

2. METHODS

2.1. Search strategy and selection

We conducted Pubmed and Embase searches for relevant ^{1}H-MRS studies comparing brain GABA levels between patients with a psychiatric disorder and healthy controls (Supplementary Table 1, search performed August 21^{st,} 2015). Pre-specified inclusion criteria were: (1) human *in vivo* ^{1}H-MRS studies; (2) psychiatric patients compared to healthy controls; (3) use of an editing technique or J-resolved ^{1}H-MRS to measure GABA (to guarantee sufficient quality of distinct GABA signal); (4) original article; (5) article in English. Reference lists of retrieved articles were screened for additional relevant articles. Three studies per psychiatric disorder were minimally required for meta-analysis.

The initial search yielded 504 studies. All articles were screened on title and abstract. If uncertainty about aptness for inclusion remained, the full text article was read. This resulted in 49 relevant ^{1}H-MRS GABA studies (see Supplementary Figure S1 for a diagram according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis (Moher et al., 2009))). Screening of reference lists yielded two additional articles. The pre-specified minimum of three published studies was met for major depressive disorder (MDD) (n = 13) (Abdallah et al., 2014; Bhagwagar et al., 2008, 2007; Epperson et al., 2006; Gabbay et al., 2012; Hasler et al., 2007, 2005; Kugaya et al., 2003; Price et al., 2009; Sanacora et al., 1999, 2004; Shaw et al., 2013; Walter et al., 2009), schizophrenia (n = 10) (Goto et al., 2009; Kegeles et al., 2012; Kelemen et al., 2013; Marsman et al., 2014; Öngür et al., 2010; Rowland et al., 2015, 2013; Stan et al., 2015; Tayoshi et al., 2010; Yoon et al., 2010), autism spectrum disorder (ASD) (n = 5) (Brix et al., 2015; Cochran et al., 2015; Gaetz et al., 2014; Harada et al., 2011; Rojas et al., 2014), bipolar disorder (n = 4) (Bhagwagar et al., 2007; Brady et al., 2013; Kaufman et al., 2009; Wang et al., 2006), panic disorder (n = 3) (Goddard et al., 2001; Hasler et al., 2009; Long et al., 2013), posttraumatic stress disorder (PTSD) (n = 3) (Michels et al., 2014; Pennington et al., 2014; Rosso et al., 2014), and ADHD (n = 3) (Bollmann et al., 2015; Edden et al., 2012; Ende et al., 2015). Less than three ^{1}H-MRS studies were available for alcohol dependence (Behar et al., 1999; Mason et al., 2006), premenstrual dysphoric disorder (Epperson et al., 2002; Liu et al., 2015), primary insomnia (Plante et al., 2012; Winkelman et al., 2008), borderline personality disorder (Ende et al., 2015), cocaine dependence (Ke et al., 2004), obsessive-compulsive disorder (Simpson et al., 2012), nicotine dependence (Epperson et al., 2005), social anxiety disorder (Pollack et al., 2008) and Tourette syndrome (Tinaz et al., 2014).

2.2. Data extraction

The following study characteristics were extracted:

- Mean and standard deviations of GABA levels, selected brain region(s) and sample size. If means and/or standard deviations were not reported (Bhagwagar et al., 2008; Gaetz et al., 2014; Long et al., 2013; Rojas et al., 2014; Rowland et al., 2015; Yoon et al., 2010), freely available software was used (Window Ruler) to calculate these measures from the provided graphs. To ensure validity of this type of measurement, five studies were randomly chosen to calculate the correlation between factual and graphically acquired GABA levels, yielding a ρ of 0.9998 (Supplementary figure S2).
- Clinical characteristics (i.e. gender, age, diagnosis, instruments used for diagnosis,

use of psychotropic medication).
- Methodology details, including: magnetic field strength, voxel size, specific editing technique or J-resolved ^{1}H-MRS, GABA quantification using water or creatine as a reference, tissue composition correction and software used for metabolite quantification.

2.3. Statistical analysis

If data were available, patients with symptoms and remitted patients were separately compared to healthy individuals where appropriate. For the primary analyses, GABA levels across multiple brain areas in the same individuals were interpreted to be independent, assuming that they are not homogenously distributed or comparably altered across brain regions. This approach is analogous to Aoki et al. (Aoki et al., 2012) and has the advantage that more data can be taken into account. An important disadvantage is that there is no correction for the fact that GABA data from different brain regions in the same individual may not be independent. Therefore, in secondary analyses, we calculated the weighted average and standard deviation of GABA levels across multiple brain regions in the same individuals analogous to Luykx et al. (Luykx et al., 2012a). Moreover, we carried out analyses separately for frontal and occipital GABA levels in disorders for which sufficient studies were available (schizophrenia and MDD). Standardized mean differences (SMD) were calculated to compare effect sizes found in different studies. Heterogeneity was evaluated using Cochrane's Q-test and the I^2 statistic (Higgins et al., 2003). Funnel plots were constructed and Egger's test was used to establish possible publication bias (Egger et al., 1997). All analyses were carried out using the Comprehensive Meta-Analysis (Borenstein et al., 2005) software developed by Biostat. A random effects model was chosen since clinical and methodological heterogeneity was assumed to be present across studies. Moreover, we assumed a common/comparable among-study variance component across subgroups (based on region or disorder state) and combined subgroups using a random effects model.

3. RESULTS

3.1. Study characteristics

3.1.1. General

General study characteristics are shown in Table 1 for MDD, schizophrenia and ASD and in Table 2 for bipolar disorder, panic disorder, PTSD and ADHD. Additional information on diagnostic assessments and details of the applied ^{1}H-MRS methodology are included in the Supplementary Information (Supplementary Tables S2-3).

For MDD, nine studies investigated depressed patients and four studies examined remitted MDD patients (Table 1). For bipolar disorder, euthymic bipolar 1 disorder patients were generally included (Table 2).

Study	Diagnosis	Region(s)[2]	N (Pt/control)	Age (SD)	Female (%)	Meds (%, period)	Field Strength (T)
			MAJOR DEPRESSIVE DISORDER				
Kugaya 2003	MDD	OCC	11 (6/5)	34 (8)	0	0 (10 days)	2.1
Epperson 2006	MDD	OCC	23 (9/14)	31 (4)	100	0 (9 mo)[5]	2.1
Bhagwagar 2008	MDD-R	ACC	23 (12/11)[3]	38 (4)	52	0 (6 mo)	3
Walter 2009	MDD	ACC (R)	24 (11/13)	37 (NA)	67	0 (1 wk)	3
Hasler 2005	MDD-R	dm/daPFC vmPFC	31 (16/15)	41 (12)	77	0 (3 mo)[6]	3
Sanacora 1999	MDD	OCC	32 (14/18)	40 (10)	41	0 (2 wk)[7]	2.1
Bhagwagar 2007	MDD-R	OCC	33 (15/18)	40 (14)	57	0 (3 mo)	3
Shaw 2013	MDD-R	OCC PFC (L) Subcortical (L)	34 (18/16)	22 (2)	100	0 (n.s.)	3
Hasler 2007	MDD	dm/daPFC vmPFC	40 (20/20)	34 (12)	65	0 (1 mo)	3
Abdallah 2014	MDD	OCC	40 (23/17)	43 (12)	73	0 (4 wk)	4
Gabbay 2012	MDD	ACC	41 (20/21)	16 (2)	66	0 (3 mo)[6]	3
Price 2009	MDD	ACC OCC	57 (33/24)	40 (13)	48	0 (2 wk)	3
Sanacora 2004	MDD	OCC	71 (33/38)	39 (11)	48	0 (2 wk)[8]	2.1
			SCHIZOPHRENIA				
Rowland Old 2013[1]	SZ	ACC CSO	20 (10/10)	50 (4)	30	100	3
Rowland Young 2013[1]	SZ	ACC CSO	21 (11/10)	32 (7)	33	100	3
Yoon 2010	SZ	OCC	26 (13/13)	28 (9)	15	62	3
Marsman 2014	SZ	mPFC POC	32 (13/19) 34 (15/19)	28 (6)[4]	28[4]	100	7
Stan 2015	SZ	Hippocampus (L)	34 (18/16)	39 (10)	32	61	3
Goto 2009	SZ	FL Basal ganglia (L) POC	36 (18/18)	30 (11)	50	100	4
Ongur 2010	SZ or SZAD	POC ACC	40 (21/19)	38 (10)	35	100	3
Kelemen 2013	SZ	OCC	48 (28/20)	25 (8)	34	0 (naive)	3
Kegeles 2012	SZ or SZAD	dlPFC (L) mPFC	54 (32/22)	32 (10)	33	50 (2 wk)[9]	3
Tayoshi 2010	SZ	Basal ganglia (L) ACC	67 (38/29)	34 (10)	44	100	3

Rowland Old 2015[1]	SZ or SZAD	mPFC	68 (31/37)	50 (6)	35	90	3
Rowland Young 2015[1]	SZ or SZAD	mPFC	69 (29/40)	26 (5)	42	93	3
AUTISM SPECTRUM DISORDER							
Harada 2011	ASD	FL (L) Basal ganglia (L)	22 (12/10)	6 (3)	NA	NA[10]	3
Cochran 2015	5 autism, 6 Asperger's, 2 PDD-NOS	ACC	27 (13/14)	15 (2)	0	23	3
Gaetz 2014	ASD	OCC Temp (L) PMC (L)	18 (8/10) 24 (13/11) 32 (17/15)	12 (3)[4]	21[4]	24[4]	3
Rojas 2014	9 autism, 7 Asperger's, 1 PDD-NOS	Temp (L)	34 (17/17)	13 (5)	35	29	3
Brix 2015	ASD	ACC (L)	35 (14/21)	10 (2)	0	33	3

Table 1. Study Characteristics Major Depressive Disorder, Schizophrenia and Autism Spectrum Disorder
MDD(-R) = Major Depressive Disorder (in remission); SZ = Schizophrenia; SZAD = Schizoaffective Disorder; ASD = Autism spectrum disorder; PDD-NOS = Pervasive Developmental Disorder, not otherwise specified; OCC = Occipital cortex; ACC = Anterior cingulate cortex; R = Right; dm/daPFC = Dorsomedial/dorsal anterolateral prefrontal cortex (region partly overlaps with vmPFC in the same study); (vm)PFC = (Ventromedial) prefrontal cortex; CSO = Centrum semiovale; mPFC = Medial prefrontal cortex; POC = Parieto-occipital cortex; L = Left; FL = Frontal lobe; dlPFC = Dorsolateral prefrontal cortex; Temp = Temporal lobe; PMC = Primary motor cortex; Pt = Patients; HC = Healthy controls; SD = Standard deviation; NA = Not available; Meds = Psychoactive medication use; mo = months; wk = weeks n.s.=not specified; T = Tesla.

1. Rowland 2013 and Rowland 2015 are two studies, both distinguishing a young and an old sample. Conform the original articles we kept this distinction in our analyses;
2. Region is midline unless otherwise specified;
3. All participants from the study of 2007 by the same group;
4. Mean of total sample, differs per region;
5. 1 patient used lorazepam >2 weeks before imaging;
6. No antidepressants, other psychotropic medication not mentioned;
7. 1 patient used lorazepam, 1 patient used thioridazine hydrochloride, 1 control and 1 patient received hormone replacement therapy;
8. Diphenhydramine hydrochloride use was accepted for insomnia.
9. Separate analysis with medication-free patients.
10. 10 autistic patients and 9 normal controls were sedated with triclofos sodium 20 min before imaging.

Study	Diagnosis	Region(s)[1]	N (Pt/control)	Age (SD)	Female (%)	Meds (%, period)	Field Strength (T)
		BIPOLAR DISORDER					
Wang 2006	5 BD-I, 9 BD-II, 1 BD-NOS *(Eu: 8, D: 7)* 9 BD-I, 7 BD-II *(Eu: 10, D: 3, I/H: 3)*	mPFC OCC	21 (15/6)[2] 22 (16/6)[2]	37 (14) 34 (12)	48 64	40 75	3
Kaufman 2009	BD *(Eu: 10, D: 2, M: 1)*	Basal ganglia Whole brain	24 (13/11)	41 (13)	38	100	4
Brady 2013	BD-I *(Eu: all)*	ACC POC	28 (14/14)	35 (12)	36	86	4
Bhagwagar 2007	BD-I *(Eu: all)*	OCC	34 (16/18)	37 (14)	56	0 (3 mo)	3
		PANIC DISORDER					
Long 2013	PD	mPFC OCC	19 (11/8)	39 (12)	47	0 (4 wk)	3
Goddard 2001	PD	OCC	28 (14/14)	36 (8)	57	0 (1 wk)	2.1
Hasler 2009	PD	dm/daPFC vmPFC	34 (17/17)	35 (11)	69	0 (3 mo)	3
		PTSD					
Rosso 2014	PTSD	ACC Insula (R)	26 (13/13)	33 (12)	46	8	4
Michels 2014	PTSD	ACC dlPFC (L)	29 (12/17[3])	40 (13)	93	33	3
Pennington 2014	PTSD	Temp ACC POC (R)	40 (28/12)[4] 43 (31/12)[4] 49 (33/16)[4]	36 (11)[5]	0	0 (2 wk)	4
		ADHD					
Edden 2012	ADHD: 10 C, 3 IA	PMC (L)	32 (13/19)	10 (NA[6])	28	0 (1 day)	3
Bollmann Children 2015	ADHD	Basal ganglia (L)	35 (16/19)	11 (2)	43	0 (3 days)	3
Ende 2015	ADHD	ACC	52 (22/30)	29 (7)	100	0 (2 wk)	3
Bollmann Adults 2015	ADHD	Basal ganglia (L) dlPFC (L)	54 (16/38)	34 (10)	50	0 (3 days)	3

Table 2. Study Characteristics Bipolar Disorder, Panic Disorder, PTSD and ADHD
BD(-I/-II/-NOS) = Bipolar disorder type 1/type 2/not otherwise specified; Eu = Euthymic; D = Depressed; I/H = Irritable/hypomanic; PD = Panic Disorder; M = Manic; PTSD = Posttraumatic Stress Disorder; ADHD = Attention Deficit/Hyperactivity Disorder; C = Combined type; IA = Predominantly inattentive type; (v)mPFC = (ventro)Medial prefrontal cortex; OCC = Occipital cortex; ACC = Anterior cingulate cortex; POC = Parieto-occipital cortex; dm/daPFC = Dorsomedial/dorsal anterolateral prefrontal cortex (region partly overlaps with vmPFC in the same study); R = Right; dlPFC = Dorsolateral prefrontal cortex; L = Left; Temp = Temporal cortex; PMC = Primary motor cortex; Pt = Patients; HC = Healthy Controls; SD = Standard deviation; NA = not available; Meds = Psychoactive medication use; mo = months; wk = weeks; T = Tesla.

1. Region is midline unless otherwise specified;
2. No overlap between the two samples;
3. Healthy controls were trauma-exposed;
4. Also PTSD patients with comorbid alcohol abuse disorder;
5. Mean of total sample, differs per region;
6. Range 8.4-12.8 years old.

3.1.2. Medication use

All MDD and panic disorder studies required patients to be medication-free for at least one week (range: 1 week to 9 months). GABA data of unmedicated schizophrenia patients were only available in two studies (Kegeles et al., 2012; Kelemen et al., 2013) and no formal meta-analysis was carried out. Around 25% of ASD patients used medication. In one study, the majority of subjects were sedated with triclofos sodium prior to the ^{1}H-MRS measurements (Harada et al., 2011). The percentage of medicated bipolar disorder and PTSD patients per study ranged from zero (Bhagwagar et al., 2007; Pennington et al., 2014) to a hundred (Kaufman et al., 2009). All ADHD patients were off medication for at least one day.

3.1.3. Diagnostic criteria

Three out of 10 ^{1}H-MRS studies in schizophrenia also included patients with schizoaffective disorder (Table 1). Three out of 5 studies on ASD did not specify the Diagnostic and Statistical Manual of Mental Disorders (DSM) based diagnosis, while the other two included autism, Asperger's syndrome and pervasive developmental disorder not otherwise specified. The ADHD subtype (inattentive/hyperactive/combined) was only specified in one of the three ^{1}H-MRS studies.

3.1.4. ^{1}H-MRS methodology

Methodological ^{1}H-MRS parameters varied widely across studies (Supplementary Tables S2-3). In three studies from the same group, the two regions of interest partially overlapped but were treated as independent outcomes for this meta-analysis (Hasler et al., 2009, 2007, 2005). Voxel size ranged from 9 cm^3 to 75 cm^3 and MRI field strength varied from 2.1T to 7T. With regard to the editing technique, 21 studies used MEGA-PRESS and 18 studies used an in-house editing technique (J-editing, JPRESS, MEGA-sLASER or J-resolved MRS). Creatine was used as a reference compound in 26 studies, water (H_2O) in 13 studies and one study reported values for both (Brix et al., 2015). Eight studies adjusted GABA levels for voxel tissue composition (gray/white matter and cerebrospinal fluid), 18 studies explored differences in tissue composition between groups and included gray or white matter proportion as a covariate in case of a significant difference, while 14 studies did not correct for or did not mention tissue composition correction. Software used for the quantification of GABA and other metabolites was LCModel in 16 studies, other generally available software in 11 studies (including Gannet, (j)MRUI, SAGE, MPFIT, and ProFit) and customized in-house software in 12 studies.

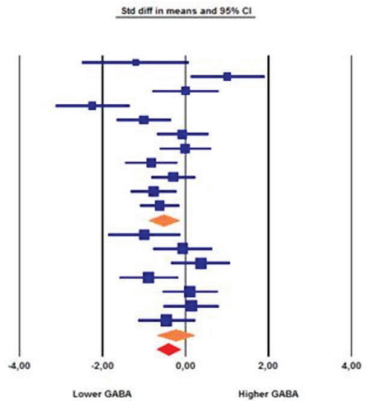
3.2. Brain GABA levels across psychiatric disorders

3.2.1. MDD

MDD patients exhibited significantly lower GABA levels compared to healthy controls (SMD = -0.41, 95% confidence interval (CI): -0.70 to -0.13, p = 0.005) (Figure 1A). Separate analyses of depressed and remitted MDD individuals demonstrated that this was the result of significantly lower GABA levels in depressed (SMD = -0.52, 95% CI:-0.89 to -0.16, p = 0.005), but not in remitted MDD patients (SMD = -0.24, 95% CI:-0.70 to 0.22, p = 0.31) (Figure 1A). Exclusion of the earliest ^{1}H-MRS study with the largest SMD (-2.24) (Sanacora et al., 1999) did not alter these results in depressed MDD patients (SMD = -0.39, 95% CI:-

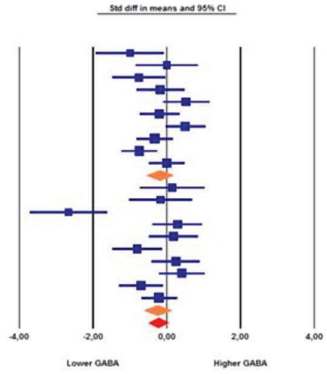
A Major Depressive Disorder

Study year	Region of interest	Outcome	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Kugaya 2003	OCC	MDD	-1,206	-2,495	0,084	-1,833	0,067
Epperson 2006	OCC	MDD	1,008	0,121	1,895	2,228	0,026
Walter 2009	ACC	MDD	0,000	-0,803	0,803	0,000	1,000
Sanacora 1999	OCC	MDD	-2,244	-3,132	-1,355	-4,948	0,000
Hasler 2007	dm/daPFC	MDD	-1,000	-1,657	-0,343	-2,981	0,003
Hasler 2007	vmPFC	MDD	-0,077	-0,697	0,543	-0,242	0,808
Abdallah 2014	OCC	MDD	0,000	-0,627	0,627	0,000	1,000
Gabbay 2012	ACC	MDD	-0,827	-1,465	-0,189	-2,541	0,011
Price 2009	ACC	MDD	-0,291	-0,820	0,237	-1,080	0,280
Price 2009	OCC	MDD	-0,766	-1,311	-0,222	-2,760	0,006
Sanacora 2004	OCC	MDD	-0,625	-1,103	-0,148	-2,566	0,010
			-0,520	-0,885	-0,156	-2,800	0,005
Bhagwagar 2008	ACC	MDD-R	-1,000	-1,868	-0,132	-2,259	0,024
Hasler 2005	dm/daPFC	MDD-R	-0,066	-0,771	0,638	-0,185	0,854
Hasler 2005	vmPFC	MDD-R	0,366	-0,344	1,077	1,011	0,312
Bhagwagar 2007	OCC	MDD-R	-0,886	-1,604	-0,169	-2,420	0,016
Shaw 2013	OCC	MDD-R	0,109	-0,565	0,783	0,318	0,751
Shaw 2013	PFC	MDD-R	0,135	-0,540	0,809	0,391	0,696
Shaw 2013	Subcortical	MDD-R	-0,456	-1,138	0,226	-1,311	0,190
			-0,238	-0,696	0,221	-1,015	0,310
			-0,411	-0,696	-0,126	-2,824	0,005



B Schizophrenia

Study year	Region of interest	Outcome	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Rowland Old 2013	ACC	Frontal region	-1,000	-1,930	-0,070	-2,108	0,035
Rowland Young 2013	ACC	Frontal region	0,000	-0,856	0,856	0,000	1,000
Marsman 2014	mPFC	Frontal region	-0,756	-1,465	-0,027	-2,031	0,042
Goto 2009	FL	Frontal region	-0,175	-0,829	0,480	-0,523	0,601
Ongur 2010	ACC	Frontal region	0,525	-0,106	1,156	1,631	0,103
Kegeles 2012	dlPFC	Frontal region	-0,201	-0,746	0,343	-0,725	0,468
Kegeles 2012	mPFC	Frontal region	0,508	-0,043	1,059	1,805	0,071
Tayoshi 2010	ACC	Frontal region	-0,326	-0,812	0,160	-1,314	0,189
Rowland Old 2015	mPFC	Frontal region	-0,744	-1,238	-0,251	-2,957	0,003
Rowland Young 2015	mPFC	Frontal region	0,000	-0,478	0,478	0,000	1,000
			-0,195	-0,555	0,164	-1,065	0,287
Rowland Old 2013	CSO	Non-frontal region	0,153	-0,724	1,031	0,342	0,732
Rowland Young 2013	CSO	Non-frontal region	-0,167	-1,025	0,691	-0,381	0,703
Yoon 2010	OCC	Non-frontal region	-2,668	-3,725	-1,611	-4,948	0,000
Marsman 2014	POC	Non-frontal region	0,288	-0,392	0,968	0,829	0,407
Stan 2015	Hippocampus	Non-frontal region	0,180	-0,495	0,855	0,523	0,601
Goto 2009	Basal ganglia	Non-frontal region	-0,801	-1,480	-0,122	-2,312	0,021
Goto 2009	POC	Non-frontal region	0,241	-0,415	0,896	0,719	0,472
Ongur 2010	POC	Non-frontal region	0,410	-0,217	1,037	1,281	0,200
Kelemen 2013	OCC	Non-frontal region	-0,700	-1,290	-0,109	-2,321	0,020
Tayoshi 2010	Basal ganglia	Non-frontal region	-0,212	-0,696	0,273	-0,857	0,392
			-0,256	-0,631	0,118	-1,343	0,179
			-0,225	-0,484	0,035	-1,698	0,089



C Autism Spectrum Disorder

Study year	Region of interest	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Harada 2011	Basal ganglia	0,000	-0,839	0,839	0,000	1,000
Harada 2011	FL	-1,672	-2,646	-0,698	-3,365	0,001
Cochran 2015	ACC	-0,667	-1,442	0,109	-1,685	0,092
Gaetz 2014	OCC	0,185	-0,746	1,117	0,390	0,697
Gaetz 2014	PMC	-0,880	-1,607	-0,153	-2,372	0,018
Gaetz 2014	Temp	-1,726	-2,665	-0,786	-3,599	0,000
Rojas 2014	Temp	-0,924	-1,631	-0,216	-2,560	0,010
Brix 2015	ACC	-0,394	-1,077	0,288	-1,133	0,257
		-0,739	-1,171	-0,307	-3,353	0,001

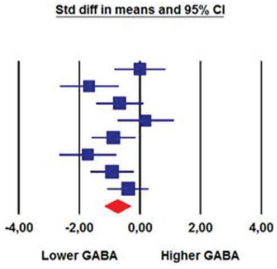
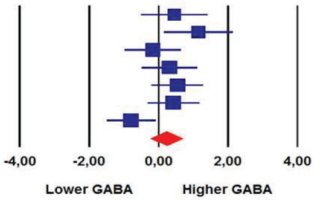


Figure 1. Forest plots of brain GABA levels in Major Depressive Disorder, Schizophrenia and Autism Spectrum Disorder. Diamond shaped orange symbols represent (from top to bottom) current MDD, remitted MDD, frontal regions in schizophrenia and non-frontal regions in schizophrenia. Size of the blue squares is proportionate to the sample size used. OCC = Occipital cortex; ACC = Anterior cingulate cortex; dm/daPFC = Dorsomedial dorsal anterolateral prefrontal (region partly overlaps with vmPFC in the same study); (vm/m)PFC = (Ventromedial/Medial) prefrontal cortex; MDD(-R) = Major Depressive Disorder (remitted). FL = Frontal lobe; dlPFC = Dorsolateral prefrontal cortex; CSO = Centrum semiovale; POC = Parieto-occipital cortex; Temp = Temporal lobe; PMC = Primary motor cortex.

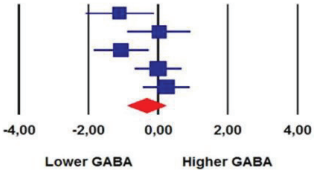
A Bipolar Disorder

Study year	Region of interest	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Wang 2006	mPFC	0,455	-0,502	1,412	0,932	0,351
Wang 2006	OCC	1,149	0,151	2,146	2,256	0,024
Kaufman 2009	Basal ganglia	-0,167	-0,972	0,637	-0,408	0,683
Kaufman 2009	Whole brain	0,314	-0,493	1,122	0,763	0,446
Brady 2013	ACC	0,541	-0,213	1,296	1,407	0,159
Brady 2013	POC	0,422	-0,327	1,171	1,105	0,269
Bhagwagar 2007	OCC	-0,792	-1,491	-0,092	-2,219	0,027
		0,231	-0,230	0,692	0,982	0,326



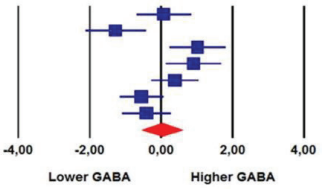
B Panic Disorder

Study year	Region of interest	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Long 2013	mPFC	-1,104	-2,080	-0,128	-2,216	0,027
Long 2013	OCC	0,025	-0,886	0,936	0,054	0,957
Goddard 2001	OCC	-1,068	-1,859	-0,276	-2,643	0,008
Hasler 2009	dm/daPFC	0,000	-0,672	0,672	0,000	1,000
Hasler 2009	mPFC	0,235	-0,440	0,909	0,682	0,495
		-0,341	-0,897	0,215	-1,201	0,230



C PTSD

Study year	Region of interest	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Rosso 2014	ACC	0,077	-0,692	0,846	0,195	0,845
Rosso 2014	Insula	-1,277	-2,121	-0,434	-2,968	0,003
Michels 2014	ACC	1,017	0,233	1,801	2,544	0,011
Michels 2014	dlPFC	0,902	0,127	1,677	2,282	0,022
Pennington 2014	ACC	0,384	-0,288	1,055	1,120	0,263
Pennington 2014	POC	-0,548	-1,155	0,058	-1,771	0,077
Pennington 2014	Temp	-0,409	-1,091	0,273	-1,175	0,240
		0,019	-0,555	0,594	0,066	0,948



D ADHD

Study year	Region of interest	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Edden 2012	PMC	-0,924	-1,665	-0,183	-2,444	0,015
Bollmann Children 2015	Basal ganglia	-0,401	-1,073	0,270	-1,171	0,242
Ende 2015	ACC	-0,741	-1,309	-0,172	-2,555	0,011
Bollmann Adults 2015	Basal ganglia	0,614	0,018	1,209	2,021	0,043
Bollmann Adults 2015	DLPFC	0,299	-0,288	0,886	0,999	0,318
		-0,215	-0,801	0,371	-0,720	0,472

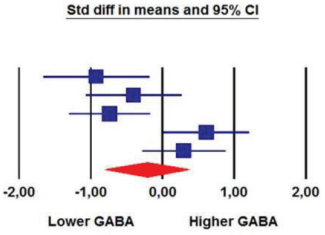


Figure 2. Forest plot showing brain GABA levels in Bipolar Disorder, Panic Disorder, PTSD and ADHD. Size of the blue squares is proportionate to the sample size used. (v)mPFC = (ventro)Medial prefrontal cortex; OCC = Occipital cortex; ACC = Anterior cingulate cortex; POC = Parieto-occipital cortex; dm/daPFC = Dorsomedial/dorsal anterolateral prefrontal cortex (region partly overlaps with vmPFC in the same study); dlPFC = Dorsolateral prefrontal cortex; Temp = Temporal cortex; PMC = Primary motor cortex.

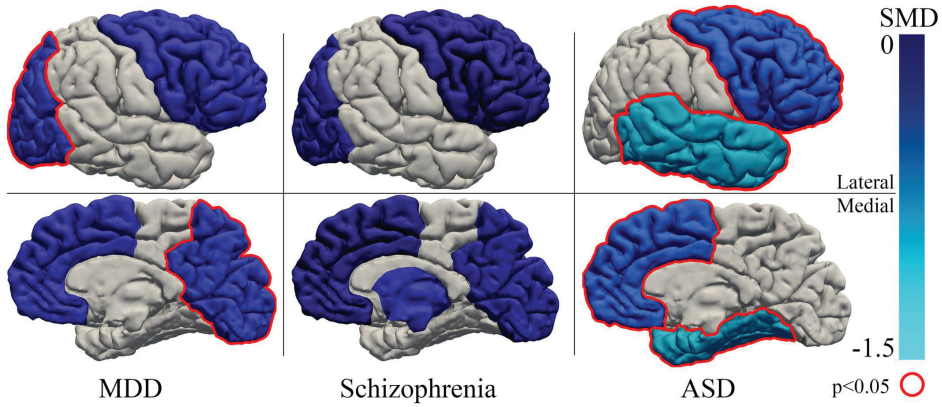


Figure 3. Schematic overview of SMDs in GABA per region of interest for MDD, schizophrenia and ASD (minimum of 2 studies per region). Frontal, temporal, parietal, occipital and basal ganglia are color coded and the brain is shown from a lateral and medial perspective. Dark blue: SMD close to 0, light blue: SMD close to -1.5, grey: not reported. Red outline: significant difference between patients and controls ($p < 0.05$). The following SMDs were found in this meta-analysis and used in this figure: MDD: occipital -0.597 ($p = 0.043$), frontal -0.445 ($p = 0.149$); schizophrenia: occipital -0.343 ($p = 0.232$), frontal -0.197 ($p = 0.313$), basal ganglia -0.483 ($p = 0.259$); ASD: frontal =-0.831 ($p = 0.001$), temporal -1.252 ($p = 0.001$).

0.71 to -0.06, p = 0.019). GABA differences between depressed MDD patients and controls were larger in occipital (SMD = -0.60, 95% CI:-1.18 to -0.02, p = 0.043) than in prefrontal regions (SMD = -0.45, 95% CI:-1.05 to 0.16, p = 0.149) (Figure 3 and Supplementary Figure S3). Averaging GABA levels across multiple brain regions from the same study yielded similar results for the total sample (SMD = -0.45, 95% CI:-0.79 to -0.10, p = 0.012), for depressed patients (SMD = -0.52, 95% CI:-0.93 to -0.11, p = 0.014) and for remitted MDD patients (SMD = -0.27, 95% CI:-0.92 to 0.38, p = 0.416) (Supplementary figure S4A).

3.2.2. Schizophrenia

No statistically significant differences in GABA levels were found between schizophrenia patients and healthy individuals, even though GABA levels tended to be lower in schizophrenia patients (SMD = -0.23, 95% CI:-0.48 to 0.04, p = 0.089) (Figure 1B). This trend level effect became statistically significant after averaging GABA levels across multiple brain regions from the same study (SMD = -0.29, 95% CI:-0.56 to -0.01, p = 0.039) (Supplementary figure S4B). Exclusion of one study with the largest SMD (-2.67) (Yoon et al., 2010) rendered these results non-significant (SMD = -0.18, 95% CI:-0.37 to 0.02, p = 0.078). A subanalysis in studies measuring GABA levels in frontal regions (medial (Kegeles et al., 2012; Marsman et al., 2014) and dorsolateral prefrontal cortex (Chen et al., 2014; Kegeles et al., 2012) and an unspecified region in the frontal lobe (Goto et al., 2009)) did not show significant differences (SMD = -0.20, 95% CI:-0.56 to 0.16, p = 0.287) (Figure 3).

3.2.3. ASD

Patients with ASD showed significantly lower GABA levels compared to healthy controls (SMD = -0.74, 95% CI:-1.17 to -0.31, p = 0.001) (Figure 1C). Averaging GABA levels across multiple brain regions from the same study yielded comparable results (SMD = -0.67, 95% CI:-0.94 to -0.39, p =2.4 × 10-6) (Supplementary Figure S4C). The largest SMD (-1.25) was found in the two studies examining the temporal lobe (see Figure 3 for a schematic overview of regional findings in ASD as well as in MDD and schizophrenia).

3.2.4. Bipolar disorder, panic disorder, PTSD, ADHD

No significant differences in GABA levels were found for bipolar disorder (SMD = 0.23, 95% CI:-0.23 to 0.69, p = 0.326), panic disorder (SMD = -0.34, 95% CI:-0.90 to 0.22, p = 0.230), PTSD (SMD = 0.02, 95% CI:-0.56 to 0.59, p = 0.948) and ADHD (SMD = -0.22, 95% CI:-0.80 to 0.37, p = 0.472) compared to controls (Figure 2). Averaging GABA levels across multiple brain regions from the same study did not change these non-significant differences in GABA levels (bipolar: SMD = 0.099, 95% CI:-0.48 to 0.68, p = 0.734; panic disorder: SMD = -0.46, 95% CI:-1.14 to 0.23, p = 0.192; PTSD: SMD = 0.05, 95% CI:-0.76 to 0.86, p = 0.909; ADHD: SMD = -0.41, 95% CI:-1.00 to 0.18, p = 0.176) (Supplementary Figure S5).

3.2.5. Age

SMD size did not significantly depend on age in the meta-analyses of at least five studies per diagnosis (MDD, schizophrenia and ASD; data not shown), even though the two studies examining GABA levels in a relatively older (approximately 50 years old) and a relatively younger sample (approximately 30 years old) (Rowland et al., 2015, 2013) only found significantly lower brain GABA levels in older schizophrenia patients compared to controls, even after adjusting for duration of the disorder (Rowland et al., 2015).

3.2.6. Publication bias

Funnel plots and Egger's tests showed no apparent publication bias and SMDs were more or less symmetrically distributed around the mean with greater dispersion of SMDs in studies that had higher standard errors (Supplementary Figures S6-7).

3.2.7. Heterogeneity

Significant heterogeneity was found for studies on MDD ($p < 0.001$, $I^2 = 68\%$), on current MDD ($p < 0.001$, $I^2 = 74\%$), but not on remitted MDD ($p = 0.07$, $I^2 = 49\%$). Exclusion of the study with the largest SMD (Sanacora et al., 1999) reduced heterogeneity but it remained significant ($p = 0.003$; $I^2 = 55\%$). A comparably large heterogeneity was found for schizophrenia studies ($p < 0.001$, $I^2 = 67\%$). Again, exclusion of the study with the largest SMD reduced heterogeneity, although it remained significant ($p = 0.006$, $I^2 = 51\%$) (Yoon et al., 2010). Significant heterogeneity was found also for ASD ($p = 0.026$, $I^2 = 56\%$), bipolar disorder ($p = 0.036$, $I^2 = 55\%$), panic disorder ($p = 0.043$, $I^2 = 59\%$); PTSD ($p < 0.001$, $I^2 = 77\%$) and ADHD ($p = 0.002$, $I^2 = 77\%$). Heterogeneity remained significant after averaging GABA levels across multiple brain regions from the same study (data not shown), except for ASD ($p = 0.775$, $I^2 = 0\%$). Collectively, these findings indicate that heterogeneity may have influenced the results for all psychiatric disorders in this meta-analysis.

4. DISCUSSION

The present study investigated whether brain GABA levels measured with ^{1}H-MRS are consistently altered across psychiatric disorders. Compared to healthy individuals, GABA levels were lower in depressed but not in remitted MDD patients. In addition, GABA levels were significantly lower in ASD patients compared to controls. For schizophrenia, the results were more equivocal: GABA levels were only significantly lower after averaging GABA levels across multiple brain regions from the same study. No significant differences in brain GABA levels were found in bipolar disorder, panic disorder, PTSD, and ADHD.

4.1. GABA in MDD

Our finding that brain GABA levels are lower in depressed MDD patient is in line with several studies showing that GABA deficits play a role in the etiology of MDD (Kalueff and Nutt, 2007; Luscher et al., 2011). Compared to healthy individuals, there is evidence for lower GABA levels in plasma (Petty et al., 1995, 1992; Petty and Sherman, 1984) and cerebrospinal fluid (Gerner et al., 1984; Kasa et al., 1982), as well as a loss of GABAergic interneurons (Rajkowska et al., 2007) in MDD patients. We found that low brain GABA levels in MDD were state-dependent, as there was no difference between remitted MDD patients and controls. Supporting state-dependent GABA changes in MDD, longitudinal ^{1}H-MRS studies have shown normalization of brain GABA levels in MDD patients after electroconvulsive (Sanacora et al., 2003), cognitive behavioral therapy (Sanacora et al., 2006) and treatment with selective serotonin reuptake inhibitors (Sanacora et al., 2002). Of note, some studies suggest that GABA levels in melancholic (Sanacora et al., 2004) and in treatment-resistant MDD patients (Price et al., 2009) are lower compared to atypical or non-treatment resistant MDD, respectively. Due to a lack of individual data, we could not distinguish between MDD subtypes in this meta-analysis. Nevertheless, these findings underscore the potential utility of *in vivo* GABA levels for a diagnostic subdivision of MDD patients.

4.2. GABA in ASD

The finding that GABA levels were consistently lower in ASD fits a growing body of evidence that point to increased excitatory and reduced inhibitory neurotransmission in ASD (Hussman, 2001). Both SPECT and PET studies have demonstrated a decrease in $GABA_A$ receptors in the frontal cortex (Mori et al., 2012) and of $GABA_A$ receptor $\alpha 5$ subunits in the nucleus accumbens and the amygdala in ASD patients (Mendez et al., 2013). This evidence is further supported by postmortem studies that have shown decreased $GABA_A$ and $GABA_B$ receptor subunits in the superior frontal cortex (Fatemi et al., 2014) and reduced GAD65/67 levels in ASD (Fatemi et al., 2002; Yip et al., 2009). However, evidence supporting the benefit of GABAergic drugs in ASD is limited and inconclusive (Brondino et al., 2015) and paradoxical response to treatment with conventional GABAergic agents has also been reported in ASD (Bruining et al., 2015). Lower brain GABA levels in ASD could be the result of a loss of GABAergic interneurons (Barnes et al., 2015). Alternatively, reduced GABA levels may be secondary and compensatory for the paradoxical excitatory effects of GABA described in some ASD patients (Bruining et al., 2015). In contrast to the decreased central GABAergic transmission, most studies report higher peripheral GABA levels of ASD patients compared to controls (Dhossche et al., 2002; El-Ansary et al., 2011; Russo,

2013), although conflicting evidence exists (Rolf et al., 1993). A plausible explanation for this discrepancy of GABA findings in ASD is currently lacking, but underscores the relevance of measuring GABA indices in the brain.

4.3. GABA in schizophrenia

Notwithstanding previous evidence that GABA system functionality is associated with schizophrenia (Gonzalez-Burgos et al., 2015; Lewis et al., 2005; Nakazawa et al., 2012) and the relatively large number of ^{1}H-MRS GABA studies, GABA levels were only significantly lower compared to controls after averaging GABA levels across multiple brain regions from the same study. In light of these equivocal findings, it is important to note that use of antipsychotics may have played a role. Only two studies included medication-free patients (Kegeles et al., 2012; Kelemen et al., 2013), despite the fact that GABAergic transmission may be most prominently impaired in antipsychotic-naïve patients (Frankle et al., 2015). Other sources of clinical heterogeneity may have contributed to the inconclusive evidence such as a wide range of age (25-50 years old), gender (15 to 50% female subjects), and duration of illness, which was reported in only five studies and varied from 5.6 months (Rowland et al., 2015) to 25.5 years (Rowland et al., 2013).

4.4. GABA in bipolar disorder, panic disorder, PTSD and ADHD

Although there is some evidence for altered GABA system functionality in bipolar disorder (Brambilla et al., 2003), panic disorder (Kalueff and Nutt, 2007), PTSD (Geuze et al., 2008), and ADHD (Rivero et al., 2015), our meta-analysis did not show significant differences in brain GABA levels between these patients and healthy controls. The limited number of published ^{1}H-MRS studies may partially account for these results.

4.5. Interpretation of the GABA ^{1}H-MRS signal

For a correct interpretation of this meta-analysis, it is important to understand the background of the ^{1}H-MRS GABA signal. First, the signal originates from both intra- and extracellular GABA, even though the majority probably comes from within GABAergic interneurons (Petroff, 2002). With regard to the biological significance of ^{1}H-MRS GABA signal, two not mutually exclusive hypotheses have been proposed (Hasler et al., 2007): (1) lower GABA signal is indicative of a loss of GABAergic interneurons; (2) lower GABA signal quantifies GABAergic inhibition since intracellular GABA levels regulate extracellular GABA levels (Jackson et al., 2000). If the ^{1}H-MRS GABA signal indeed reflects GABAergic inhibition, it is likely that GABA levels are dynamic and responsive to environmental challenges. In support, GABA levels are decreased in response to psychological stress (Hasler et al., 2010) and changes in GABA levels have been reported after gabapentin administration (Cai et al., 2012). Nevertheless, the ^{1}H-MRS GABA signal in healthy individuals has been reported to be relatively stable; within-session coefficients of variance (CV) range from 7 to 13% (Bogner et al., 2010; Near et al., 2013; O'Gorman et al., 2011), which is more or less consistent with CVs of measurements carried out up to 7 months apart (3.5-21%) (Evans et al., 2010; Near et al., 2014; Stephenson et al., 2011; Wijtenburg et al., 2013). Overall, we do not know to what degree the GABA signal varies as a result of normal physiological variation and whether absolute GABA levels and variability are specific for certain brain regions.

4.6. Methodological ^{1}H-MRS considerations

In addition to the interpretation of the ^{1}H-MRS GABA signal, there are several methodological issues that need to be considered. First, GABA levels probably differ across brain regions. For example, GABA levels differed two-fold between brain regions measured in the same study (Kegeles et al., 2012; Tayoshi et al., 2010). Therefore, a hypothesis-driven regional approach is essential as long as whole-brain approaches with sufficiently high spatial resolution of the ^{1}H-MRS signal are absent. Many studies have focused on the occipital cortex since the spectral resolution is higher compared to most other brain areas, as a result of a more homogeneous magnetic field (Puts and Edden, 2012). However, this also implies that pragmatic reasons rather than hypothesis-driven arguments (e.g. based on postmortem studies examining GAD67 mRNA levels or neuroimaging studies) may have been decisive in the selection of brain region. Fortunately, a hypothesis-driven approach is increasingly common as illustrated by recent PTSD studies in this meta-analysis which focused on prefrontal-limbic structures that have been implicated in the etiology of this disorder (Koenigs and Grafman, 2009). Moreover, some promising technical advances have been made to increase the ^{1}H-MRS signal-to-noise ratio (Boer et al., 2015) and it may eventually be possible to map GABA levels across the brain with a high spectral resolution. Probably the greatest challenge in deriving a reliable GABA signal from ^{1}H-MRS measurements is the disentanglement of the GABA signal from the macromolecular signal (Mullins et al., 2014). Editing techniques are essential for filtering out relatively large overlapping signals from both creatine and macromolecules (Rothman et al., 1993). However, even editing techniques cannot cancel out all contamination: the proportion of macromolecules in the GABA signal after editing has been estimated at almost 50% using MEGA-PRESS at 3T (Aufhaus et al., 2013). It is also clear that the methods for reducing the macromolecular contamination of the GABA signal differ greatly across studies (for examples of strategies to deal with macromolecular contamination of the GABA signal, see Mullins et al., 2014). As a result, the proportion of actual GABA in the signal differs across studies and its variance will also not be homogeneous. Moreover, it is unclear whether differences in macromolecule concentration exist between patients with specific psychiatric disorders and controls, although there is no reason to assume such difference. Unfortunately, there is currently no consensus on the method to minimize the macromolecular contribution to the GABA signal.

In addition to regional differences and macromolecule contamination, other methodological factors that may have affected quantification of the ^{1}H-MRS GABA signal in this meta-analysis are: i) tissue composition (i.e. the amount of gray matter, white matter and cerebrospinal fluid); ii) whether GABA is reported as a ratio over creatine or water; iii) the specific software used for GABA quantification; iv) the scanner and head coil type; v) pulse sequence acquisition parameters; and vi) the specific rules for quality control of the acquired spectra (e.g. using linewidth as an indicator for the quality of the shimming procedure) and of the fitting (e.g. evaluating the Cramér-Rao lower bound). The importance of these methodological issues cannot be underestimated since they may result in an increased variability in GABA levels across ^{1}H-MRS studies. In summary, obtaining accurate *in vivo* GABA levels remains a challenge.

4.7. Future directions

Although efforts have been made to provide guidelines for minimal best practice for MEGA-PRESS at 3T regarding acquisition, processing and analysis framework (Mullins et al., 2014), overall consensus in the GABA ^{1}H-MRS field remains an important goal. In addition, evidence for disease-related changes in GABA ^{1}H-MRS levels would greatly benefit from longitudinal studies that provide more information about disease course and stability of the GABA signal over time. Importantly, longitudinal studies are unaffected by many confounders such as genetically determined differences in GABA levels (Berrettini et al., 1982; Luykx et al., 2012b). There is also a need for studies with larger and more detailed samples to obtain more robust results as well as to investigate possible confounders such as disease history and medication use. In this context, a more integrative approach taking neuroimmune, stress and epigenetic markers into account may be of particular interest. Finally, increased spatial resolution of the ^{1}H-MRS signal may specifically improve our understanding of how regional brain GABA levels relate to psychopathology. With new developments to suppress the lipid signal in the skull, whole brain MRSI (magnetic resonance spectroscopic imaging) is possible within much shorter acquisition times (Boer et al., 2015). This would enable MRSI with GABA-editing, given a homogeneous magnetic field throughout the brain.

4.8. Conclusions

The present ^{1}H-MRS meta-analysis shows that *in vivo* GABA levels are lower in depressed MDD patients and in ASD patients compared to controls. These results substantiate the importance of GABA in the etiology of both developmental disorders and disorders with a greater environmental component. The evidence suggests that the GABA system remains a promising target for pharmacological interventions in MDD and ASD. However, future studies could benefit from increased fundamental knowledge of the physiological variation in GABA levels, longitudinal studies, and consensus on the preferred methodology and minimal standards of human ^{1}H-MRS studies. Beyond these improvements lies the promise that detailed and accurate brain GABA level measures may advance diagnostic precision and improve personalized medicine.

CONFLICT OF INTEREST

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SUPPLEMENTARY INFORMATION

Title/abstract (OR)	All fields (OR)
MRS	GABA
Spectroscopy	"Gamma-aminobutyric acid"
Spectroscopic	"Gamma aminobutyric acid"
Mrsi	GABAergic

AND

	Title/abstract (OR)		
AND	Psychiatry	Depressive	OCD
	Psychiatric	MDD	Autism
	Mental	Bipolar	ASD
	Disorder	Panic	ADHD
	Schizophrenia	Anxiety	Dependence
	Schizophrenic	Phobia	Addiction
	Depression	PTSD	Nervosa

Supplementary Table S1. Search terms

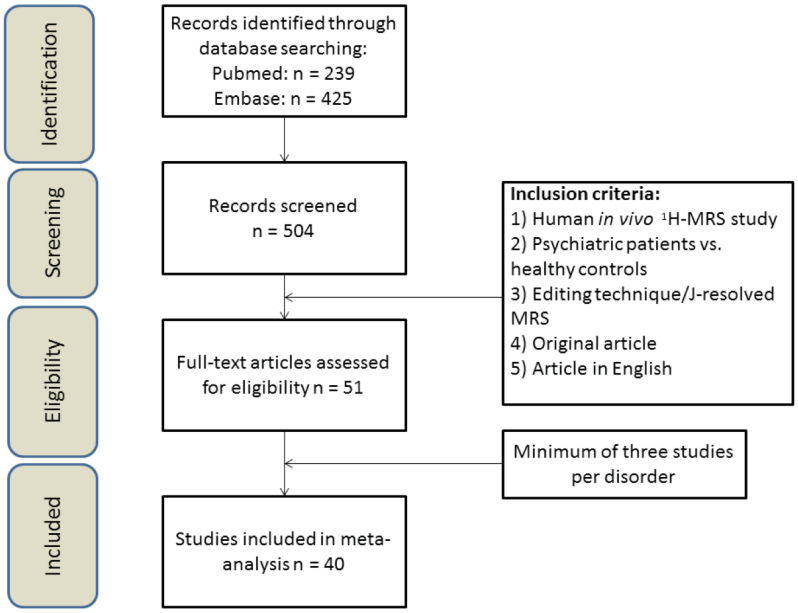
Study	Diagnosis	Instruments	Region[3]	Voxel Size (cm)	Editing technique	Ratio over	Tissue Correction	Software
MAJOR DEPRESSIVE DISORDER								
Kugaya 2003	MDD	SCID	OCC	1.5x3x3	J-editing	Cr	No	In-house program
Epperson 2006	MDD	HAM-D	OCC	3.5x1.5x3.9[6]	J-editing	Cr	No[7]	LCModel
Bhagwagar 2008	MDD-R	DSM-IV[1]+SCID+HAM-D	ACC	3x3x2	MEGA-PRESS	Cr	No[7]	LCModel
Walter 2009	MDD	DSM-IV[1]+HAM-D+BDI	ACC (R)	2x2.5x3.5	JPRESS	Cr	No	ProFit
Hasler 2005	MDD-R	DSM-IV[1]+SCID	dm/daPFC vmPFC	5x3x2 3x3x2	J-editing	Cr	No	MRUI
Sanacora 1999	MDD	SCID	OCC	1.5x3x3	J-editing	Cr	No	In-house program
Bhagwagar 2007	MDD-R	DSM-IV[1]+SCID+HAM-D	OCC	3x3x2	MEGA-PRESS	Cr	No[7]	LCModel
Shaw 2013	MDD-R	MINI	Subcortical (L) OCC PFC (L)	3x3.5x3.5 3x3x3 3x3x3	MEGA-PRESS	Water	Yes	Gannet
Hasler 2007	MDD	DSM-IV[1]+SCID+MADRS	dm/daPFC vmPFC	5x3x2 3x3x2	J-editing	Cr	No[7]	In-house program
Abdallah 2014	MDD	DSM-IV[1]+SCID+HAM-D	OCC	3x1.5x3	J-editing	Cr	No[7]	LCModel
Gabbay 2012	MDD	DSM-IV-TR[1]+CDRS-R	ACC	2.5x2.5x3	J-editing	Water	No (WM as covariate)	MPFIT
Price 2009	MDD	DSM-IV[1]+SCID	OCC ACC	2x3x3 2.5x2.5x3	J-editing	Water	No	In-house program
Sanacora 2004	MDD	SCID	OCC	3x3x1.5	J-editing	Cr	No	In-house program
SCHIZOPHRENIA								
Rowland Old and Young 2013	SZ	SCID	ACC CSO	3.5x3.5x3.5 5x3x5	MEGA-PRESS	Water	Yes	LCModel
Yoon 2010	SZ	SCID	OCC	3.5x3x2.5	MEGA-PRESS	Cr	No	jMRUI
Marsman 2014	SZ	CASH	mPFC POC[4]	2.5x2.5x2.5	MEGA-sLASER	Cr	Yes	In-house program
Stan 2015	SZ	DSM-IV[1]	Hippocampus (L)	5x1.5x1.5	J-editing	Cr	Yes	LCModel
Goto 2009	SZ	SCID	Basal ganglia (L) FL POC[4]	3x3x3	MEGA-PRESS	Cr	No	Not mentioned
Ongur 2010	SZ or SZAD	SCID	POC ACC	2.3x2.2x3.3	MEGA-PRESS	Cr	Yes	LCModel
Kelemen 2013	SZ	SCID	OCC	3.5x3x2.5	J-editing	Cr	No	jMRUI

Kegeles 2012	SZ or SZAD	DIGS	dlPFC (L) mPFC	1x2x4.8 2.5x3x2.5	J-editing	Water	Yes	In-house program
Tayoshi 2010	SZ	DSM-IV-TR[1]	Basal ganglia (L) ACC	3x3x3	MEGA-PRESS	Water	No[7]	LCModel
Rowland Old and Young 2015	SZ or SZAD	SCID	mPFC[4]	4x3x2	MEGA-PRESS	Water	Yes	Gannet
AUTISM SPECTRUM DISORDER								
Harada 2011	ASD	DSM-IV[2]	Basal ganglia (L)[5] FL (L)	3×3×3	MEGA-PRESS	Water	No[7]	LCModel
Cochran 2015	5 autism, 6 Asperger's, 2 PDD-NOS	DSM-IV[2] and ADOS	ACC	3×3×2	MEGA-PRESS	Cr	No[7]	In-house program
Gaetz 2014	ASD	ADOS&SCQ, ADI-R when inconsistent	OCC[5] Temp (L)[5] PMC (L)[5]	3×3×3 4×3×2 3×3×3	MEGA-PRESS	Cr	Yes	AMARES- jMRUI
Rojas 2014	9 autism, 7 Asperger's, 1 PDD-NOS	DSM-IV[2], ADOS and either SCQ or ADI-R	Temp (L)	3×3×4	MEGA-PRESS	Cr	No[7]	SAGE
Brix 2015	ASD	DSM-5[2]	ACC (L)	3×3×3	MEGA-PRESS	Water and Cr	No[7]	LCModel

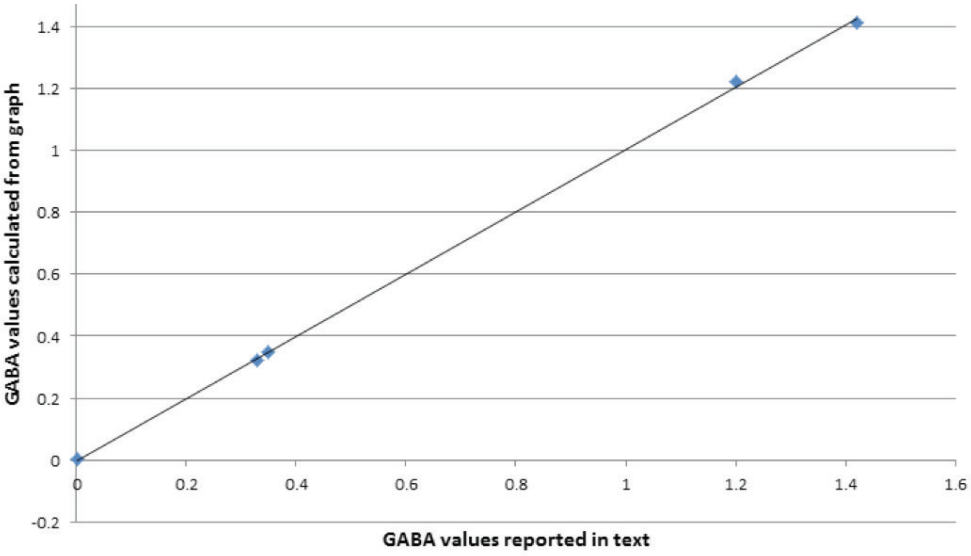
Supplementary Table S2. Additional clinical and technical characteristics of ^{1}H-MRS studies in Major Depressive Disorder, Schizophrenia and Autism Spectrum Disorder. MDD(-R) = Major Depressive Disorder (in remission); SZ = Schizophrenia; SZAD = Schizoaffective Disorder; ASD = Autism spectrum disorder; PDD-NOS = Pervasive Developmental Disorder, not otherwise specified; SCID = Structured Clinical Interview for DSM-IV (DSM-IV); DSM = Diagnostic and Statistical Manual for Mental Disorders; HAM-D = Hamilton Depression Rating Scale; BDI = Beck Depression Inventory; MINI = Mini International Neuropsychiatric Interview; MADRS = Montgomery-Asberg Depression Rating Scale; CDRS-R = Children's Depression Rating Scale - Revised; CASH = Comprehensive Assessment of History and Symptoms; DIGS = Diagnostic Interview for Genetic Studies; ADOS = Autism Diagnostic Observation Schedule; SCQ = Parent report on the Social Communication Questionnaire; ADI-R = Autism Diagnostic Interview-Revised; OCC = Occipital cortex; ACC = Anterior cingulate cortex; R = Right; dm/daPFC = Dorsomedial dorsal anterolateral prefrontal; (vm)PFC = (Ventromedial) prefrontal cortex; CSO = Centrum semiovale; mPFC = Medial prefrontal cortex; POC = Parieto-occipital cortex; L = Left; FL = Frontal lobe; dlPFC = Dorsolateral prefrontal cortex; Temp = Temporal lobe; PMC = Primary motor cortex; Cr = Creatine; WM = White matter. 1. Diagnosis established by psychiatrist according to DSM-IV(-TR); 2. Clinical interview with a child psychiatrist (Cochran)/clinical psychologist (Rojas)/pediatrician (Harada)/clinical ASD specialist (Brix) meeting criteria for autism spectrum disorder according to DSM-IV/5; 3. Region is midline unless otherwise specified; 4. Referred to in original article as medial parieto-occipital cortex, parieto-occipital lobe and medial frontal cortex, respectively; 5. Referred to in original article as lenticular nucleus, visual, auditory and motor, respectively. 6. For 6 healthy controls the voxel size was 3x1.5x3 cm; 7. No significant difference between groups in tissue composition.

Study	Diagnosis	Instruments	Region[3]	Voxel Size (cm)	(Editing) Technique	Ratio over	Tissue Correction	Software
				BIPOLAR DISORDER				
Wang 2006	5 BD-I, 9 BD-II, 1 BD-NOS *(Eu: 8, D: 7)* 9 BD-I, 7 BD-II *(Eu: 10, D: 3, I/H: 3)*	DSM-IV[1]	mPFC[4] OCC	2.6x2.4x2 2x3x3	Edited pulse[5]	Cr	No	SAGE
Kaufman 2009	BD *(Eu: 10, D: 2, M: 1)*	MINI+MADRS	Basal ganglia Whole brain	MRSI slabs: 2 cm thick	J-resolved-^{1}H-MRS	Cr	No	LCModel
Brady 2013	BD-I *(Eu: all)*	SCID+YMRS+ MADRS+PANSS	ACC POC	2.3x2.2x3.3	MEGA-PRESS	Cr	No (GM as covariate)	LCModel
Bhagwagar 2007	BD-I *(Eu: all)*	DSM-IV[1]+YMRS+ SCID+HAM-D	OCC	3x3x2	MEGA-PRESS	Cr	No[6]	LCModel
				PANIC DISORDER				
Long 2013	PD	SCID	mPFC[4] OCC	20.8 mL 18.8 mL	MEGA-PRESS	Cr	No[6]	LCModel
Goddard 2001	PD	ADIS-IV-L Or SCID	OCC	1.5x3x3	MEGA-PRESS	Cr	No	LCModel
Hasler 2009	PD	DSM-IV[1]+SCID	dm/daPFC vmPFC	5x3x2 3x3x2	J-editing	Water	No[6]	In-house program
				PTSD				
Rosso 2014	PTSD	SCID	ACC Insula (R)	3x2x2 1.5x3x2	MEGA-PRESS	Cr	No[6]	LCModel
Michels 2014	PTSD	CAPS	ACC dlPFC (L)	2.8x0.30225 cm2 2.5x4x3	MEGA-PRESS	Cr	No	LCModel
Pennington 2014	PTSD	CAPS	Temp ACC POC (R)	2x4x2 3.5x2.5x2 2x4x2	J-editing	Water	No[6]	In-house program
				ADHD				
Edden 2012	ADHD: 10 C, 3 IA	DICA-IV, CPTRS-R and ADHD-RS-IV (HV/SV)[2]	PMC (L)[4]	3x3x3	J-editing	Water	No	In-house program
Bollmann Children 2015	ADHD	K-SADS-PL	Basal ganglia (L)	2.8×4×2.5	MEGA-PRESS	Water	No[6]	LCModel
Ende 2015	ADHD	WURS-k, CAARS, WRARS	ACC	4x3x2	MEGA-PRESS	Water	No[6]	jMRUI
Bollmann Adults 2015	ADHD	DSM-IV[1]	Basal ganglia (L)	2.8×4×2.5 2.5×4×3	MEGA-PRESS	Water	No[6]	LCModel

Supplementary Table S3. Additional clinical and technical characteristics of ^{1}H-MRS studies in Other Disorders (Bipolar Disorder, Panic Disorder, Posttraumatic Stress Disorder, Attention Deficit/Hyperactivity Disorder).

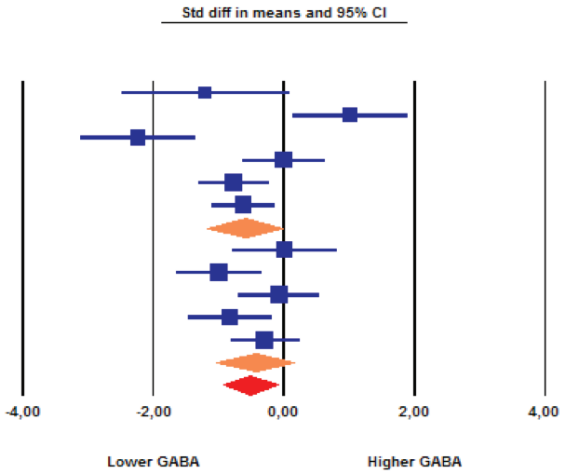


Supplementary Figure S1. PRISMA diagram of the literature search.



Supplementary Figure S2. Correlation between calculated values from graphs and reported values in 5 selected studies. GABA values from Rowland et al. 2015 (older healthy volunteers, anterior cingulate), Stan et al. 2015 (normal controls, hippocampus), Sanacora et al. 2004 (healthy controls, occipital), Price et al. 2009 (healthy volunteers, anterior cingulate), Rojas et al. 2014 (healthy controls, temporal lobe).

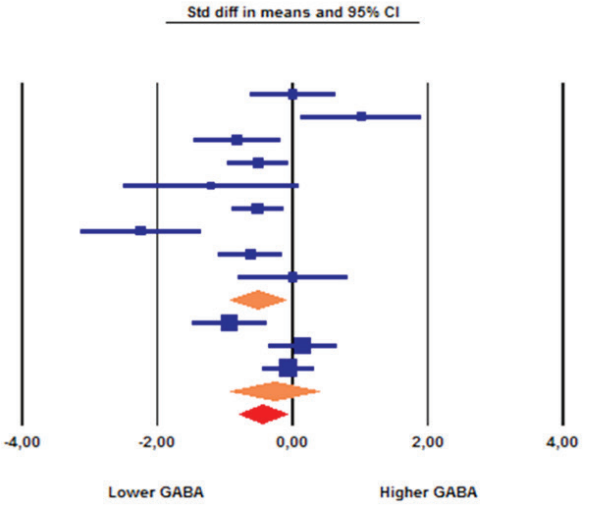
Study year	Region of interest	Outcome	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
			Statistics for each study				
Kugaya 2003	OCC	Occipital region	-1,206	-2,495	0,084	-1,833	0,067
Epperson 2006	OCC	Occipital region	1,008	0,121	1,895	2,228	0,026
Sanacora 1999	OCC	Occipital region	-2,244	-3,132	-1,355	-4,948	0,000
Abdallah 2014	OCC	Occipital region	0,000	-0,627	0,627	0,000	1,000
Price 2009	OCC	Occipital region	-0,766	-1,311	-0,222	-2,760	0,006
Sanacora 2004	OCC	Occipital region	-0,625	-1,103	-0,148	-2,566	0,010
			-0,597	-1,175	-0,020	-2,026	0,043
Walter 2009	ACC	Prefrontal region	0,000	-0,803	0,803	0,000	1,000
Hasler 2007	dm/daPFC	Prefrontal region	-1,000	-1,657	-0,343	-2,981	0,003
Hasler 2007	vmPFC	Prefrontal region	-0,077	-0,697	0,543	-0,242	0,808
Gabbay 2012	ACC	Prefrontal region	-0,827	-1,465	-0,189	-2,541	0,011
Price 2009	ACC	Prefrontal region	-0,291	-0,820	0,237	-1,080	0,280
			-0,445	-1,049	0,160	-1,442	0,149
			-0,524	-0,942	-0,107	-2,461	0,014



Supplementary Figure S3. Forest plot of subanalysis of GABA levels in occipital and prefrontal brain regions in Major Depressive Disorder (depressed). Diamond shaped orange symbols represent occipital regions (upper symbol) and prefrontal regions (lower symbol). Diamond shaped red symbol represents summary of occipital and prefrontal regions. Size of the blue squares is proportionate to the sample size used. OCC = Occipital cortex; ACC = Anterior cingulate cortex; dm/daPFC = Dorsomedial dorsal anterolateral prefrontal; vmPFC = Ventromedial prefrontal cortex.

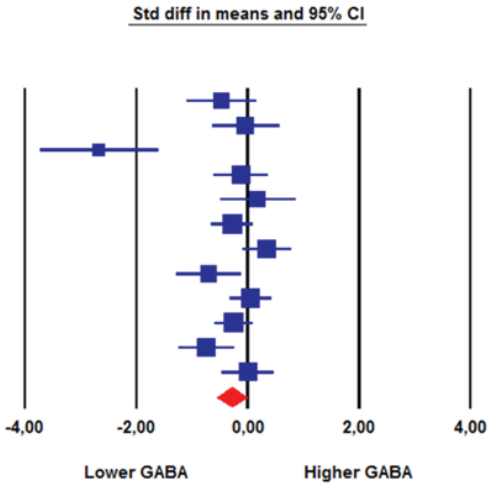
A. Major Depressive Disorder

Study year	Region of interest	Outcome	Statistics for each study				
			Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Abdallah 2014	OCC	MDD	0,000	-0,627	0,627	0,000	1,000
Epperson 2006	OCC	MDD	1,008	0,121	1,895	2,228	0,026
Gabbay 2012	ACC	MDD	-0,827	-1,465	-0,189	-2,541	0,011
Hasler 2007	Combined	MDD	-0,511	-0,962	-0,060	-2,222	0,026
Kugaya 2003	OCC	MDD	-1,206	-2,495	0,084	-1,833	0,067
Price 2009	Combined	MDD	-0,522	-0,901	-0,143	-2,698	0,007
Sanacora 1999	OCC	MDD	-2,244	-3,132	-1,355	-4,948	0,000
Sanacora 2004	OCC	MDD	-0,625	-1,103	-0,148	-2,566	0,010
Walter 2009	ACC	MDD	0,000	-0,803	0,803	0,000	1,000
			-0,516	-0,926	-0,106	-2,464	0,014
Bhagwagar 2007/8	Combined	MDD-R	-0,933	-1,486	-0,379	-3,305	0,001
Hasler 2005	Combined	MDD-R	0,148	-0,352	0,649	0,581	0,561
Shaw 2013	Combined	MDD-R	-0,068	-0,458	0,323	-0,340	0,734
			-0,269	-0,917	0,379	-0,814	0,416
			-0,445	-0,792	-0,099	-2,517	0,012



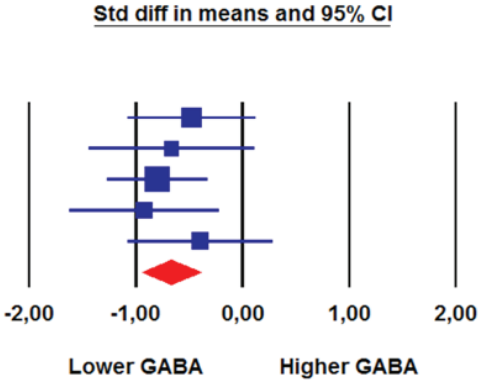
B. Schizophrenia

Study year	Region of interest	Outcome	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Rowland Old 2013	Combined	Combined	-0,468	-1,097	0,160	-1,461	0,144
Rowland Young 2013	Combined	Combined	-0,038	-0,644	0,568	-0,123	0,902
Yoon 2010	OCC	Other	-2,668	-3,725	-1,611	-4,948	0,000
Marsman 2014	Combined	Combined	-0,122	-0,611	0,366	-0,491	0,623
Stan 2015	Hippocampus	Other	0,180	-0,495	0,855	0,523	0,601
Goto 2009	Combined	Combined	-0,281	-0,660	0,098	-1,451	0,147
Ongur 2010	Combined	Combined	0,348	-0,094	0,791	1,545	0,122
Kelemen 2013	OCC	Other	-0,700	-1,290	-0,109	-2,321	0,020
Kegeles 2012	Combined	Frontal	0,052	-0,332	0,436	0,264	0,792
Tayoshi 2010	Combined	Combined	-0,257	-0,601	0,086	-1,471	0,141
Rowland Old 2015	mPFC	Frontal	-0,744	-1,238	-0,251	-2,957	0,003
Rowland Young 2015	mPFC	Frontal	0,000	-0,478	0,478	0,000	1,000
			-0,288	-0,562	-0,014	-2,061	0,039



c. Autism Spectrum Disorder

Study year	Region of interest	Statistics for each study				
		Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Harada 2011	Combined	-0,479	-1,081	0,123	-1,560	0,119
Cochran 2015	ACC	-0,667	-1,442	0,109	-1,685	0,092
Gaetz 2014	Combined	-0,798	-1,272	-0,324	-3,302	0,001
Rojas 2014	Temp	-0,924	-1,631	-0,216	-2,560	0,010
Brix 2015	ACC	-0,394	-1,077	0,288	-1,133	0,257
		-0,666	-0,943	-0,389	-4,714	0,000



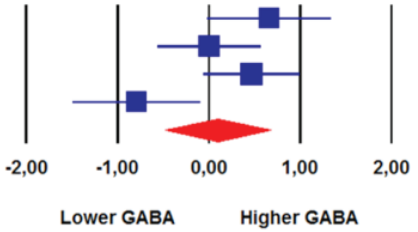
Supplementary Figure S4. Forest plots of brain GABA levels in Major Depressive Disorder, Schizophrenia and Autism Spectrum Disorder after averaging GABA levels across multiple brain regions from the same study. Diamond shaped orange symbols represent depressed (upper symbol) and remitted MDD (lower symbol). Size of the blue squares is proportionate to the sample size used. OCC = Occipital cortex; ACC = Anterior cingulate cortex; MDD(-R) = Major Depressive Disorder (remitted); mPFC = Medial prefrontal cortex; Temp = Temporal lobe.

A Bipolar Disorder

Study year	Region of interest	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Wang 2006	Combined	0,659	-0,022	1,340	1,897	0,058
Kaufman 2009	Combined	0,000	-0,568	0,568	0,000	1,000
Brady 2013	Combined	0,469	-0,062	1,000	1,733	0,083
Bhagwagar 2007	OCC	-0,792	-1,491	-0,092	-2,219	0,027
		0,099	-0,483	0,681	0,333	0,739

Statistics for each study

Std diff in means and 95% CI

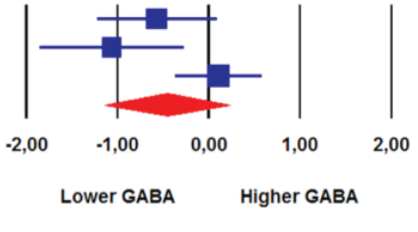


B Panic Disorder

Study year	Region of interest	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Long 2013	Combined	-0,570	-1,226	0,087	-1,701	0,089
Goddard 2001	OCC	-1,068	-1,859	-0,276	-2,643	0,008
Hasler 2009	Combined	0,104	-0,372	0,579	0,427	0,669
		-0,455	-1,140	0,229	-1,304	0,192

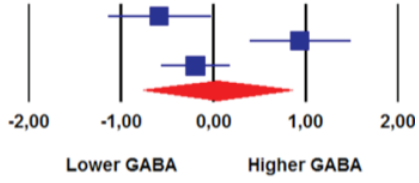
Statistics for each study

Std diff in means and 95% CI



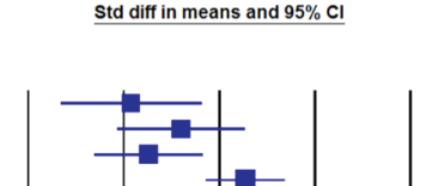
C PTSD

Study year	Region of interest	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Rosso 2014	Combined	-0,584	-1,139	-0,029	-2,062	0,039
Michels 2014	Combined	0,940	0,390	1,490	3,351	0,001
Pennington 2014	Combined	-0,194	-0,566	0,178	-1,024	0,306
		0,047	-0,762	0,857	0,115	0,909



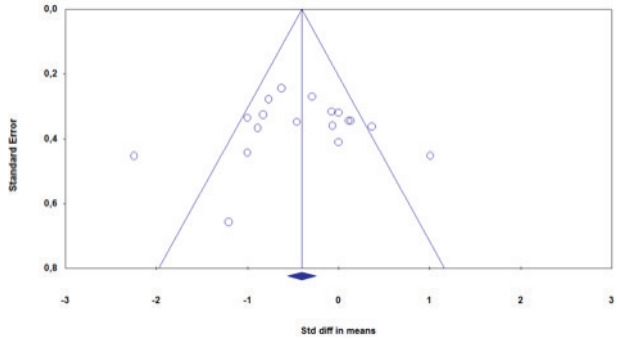
D ADHD

Study year	Region of interest	Std diff in means	Lower limit	Upper limit	Z-Value	p-Value
Edden 2012	PMC	-0,924	-1,665	-0,183	-2,444	0,015
Bollmann Children 2015	Basal ganglia	-0,401	-1,073	0,270	-1,171	0,242
Ende 2015	ACC	-0,741	-1,309	-0,172	-2,555	0,011
Bollmann Adults 2015	Combined	0,274	-0,141	0,688	1,294	0,196
		-0,409	-1,001	0,184	-1,352	0,176

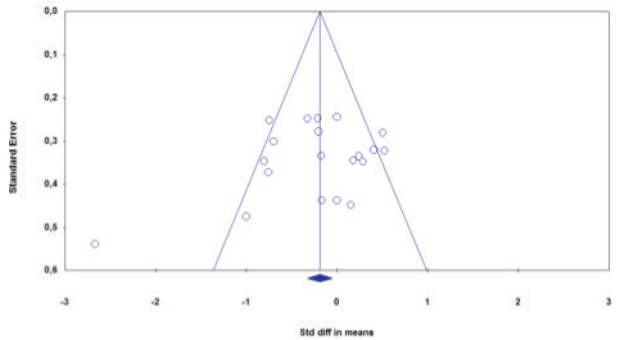


Supplementary figure S5. Forest plots of brain GABA levels in Bipolar Disorder, Panic Disorder, PTSD and ADHD after averaging GABA levels across multiple brain regions from the same study. Size of the blue squares is proportionate to the sample size used. OCC = Occipital cortex; ACC = Anterior cingulate cortex; Temp = Temporal lobe.

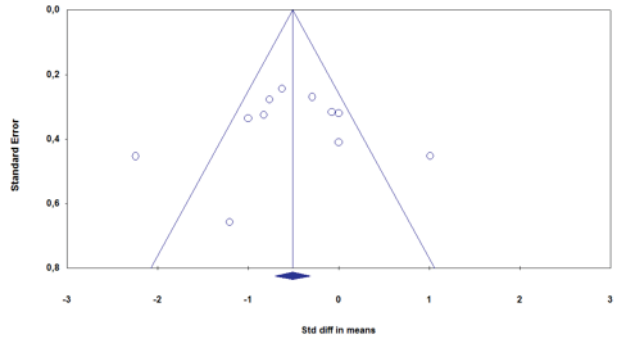
Major Depressive Disorder



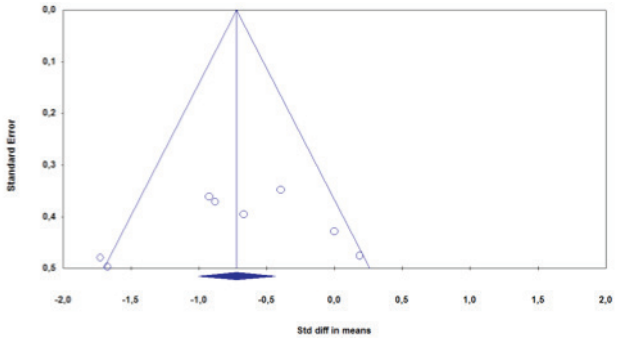
Schizophrenia



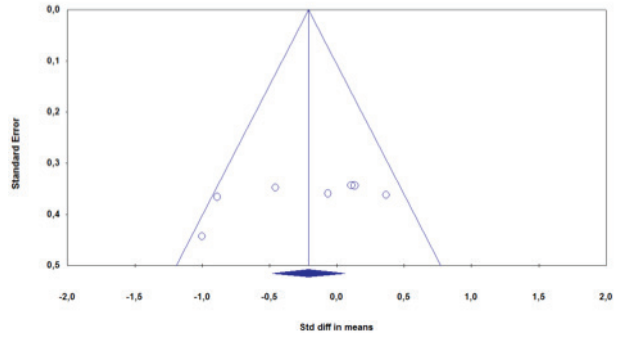
Major Depressive Disorder (depressed)



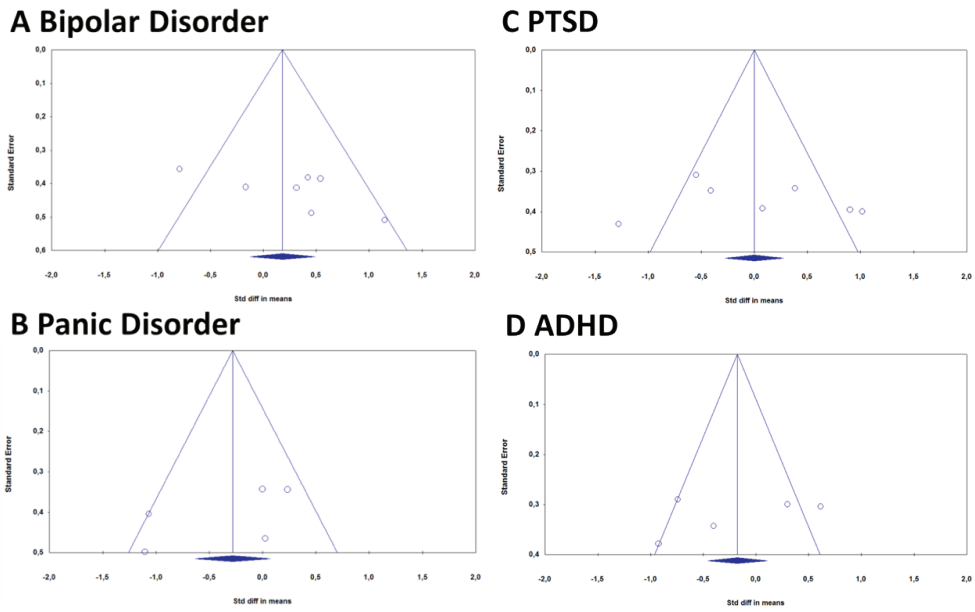
Autism Spectrum Disorder



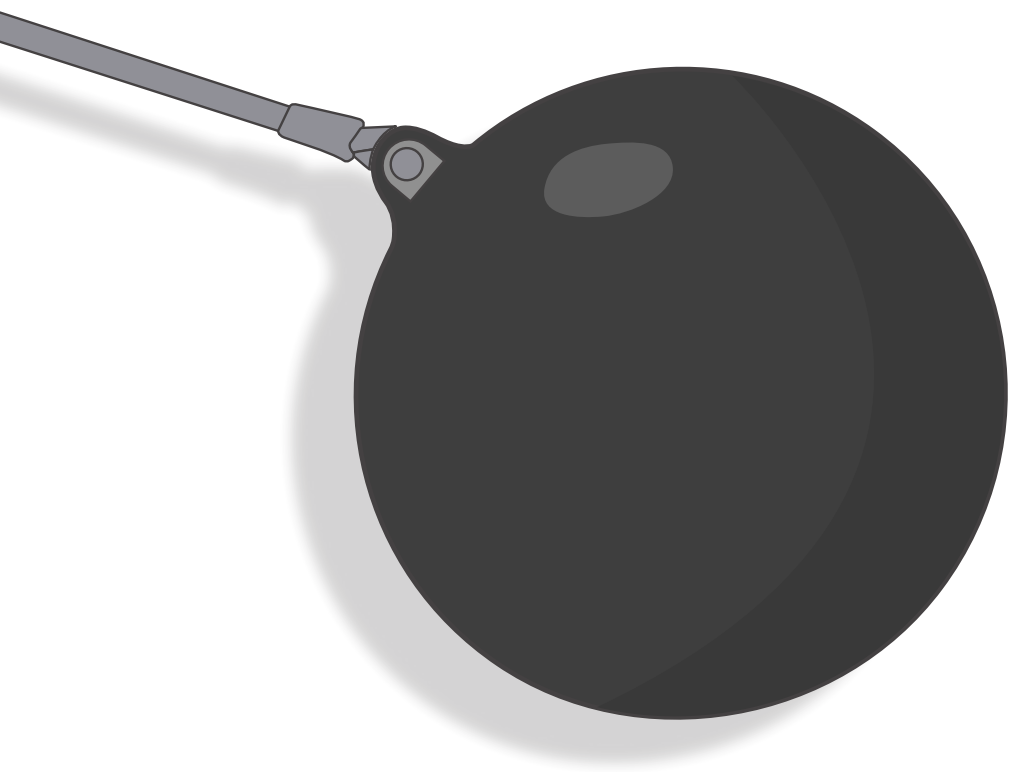
Major Depressive Disorder (remitted)



Supplementary Figure S6. Funnel plots for the studies on Major Depressive Disorder (all, depressed and remitted), Schizophrenia and Autism Spectrum Disorder showing the relation between the standardized difference in means and the standard error for each study. Egger's test is not significant for Major Depressive Disorder (2-tailed p-value all = 0.807, depressed = 0.822, remitted = 0.144), Schizophrenia (2-tailed p-value = 0.279) and Autism Spectrum Disorder (2-tailed p-value = 0.562).



Supplementary Figure S7. Funnel plots for the studies on Bipolar Disorder, Panic Disorder, PTSD and ADHD showing the relation between the standardized difference in means and the standard error for each study. Egger's test is not significant for Bipolar Disorder (2-tailed p-value = 0.099), Panic Disorder (2-tailed p-value = 0.279), PTSD (2-tailed p-value = 0.700) and ADHD (2-tailed p-value = 0.458).



CHAPTER 5

Development of psychopathology in deployed armed forces in relation to plasma GABA levels

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ABSTRACT

The GABA system is pivotal for an adequate response to a stressful environment but has remained largely unexplored in this context. The present study investigated the relationship of prospectively measured plasma GABA levels with psychopathology symptoms in military deployed to Afghanistan at risk for developing psychopathology following trauma exposure during deployment, including posttraumatic stress disorder (PTSD) and major depressive disorder (MDD). Plasma GABA levels were measured in military personnel (n = 731) one month prior to deployment (T0), and one (T1) and six months (T2) after deployment using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Mental health problems and depressive symptoms were measured with the Dutch revised Symptom Checklist (SCL-90) and PTSD symptoms with the Dutch Self-Rating Inventory for PTSD (SRIP). Six months after deployment increases in GABA concentrations were present in individuals who had developed mental health problems (T2: $\beta = 0.06$, $p = 1.6 \times 10^{-2}$, T1: $\beta = 4.7 \times 10^{-2}$, $p = 0.13$), depressive symptoms (T2: $\beta = 0.29$, $p = 7.9 \times 10^{-3}$, T1: $\beta = 0.23$, $p = 0.072$) and PTSD symptoms at T2 (T2: $\beta = 0.12$, $p = 4.3 \times 10^{-2}$, T1: $\beta = 0.11$, $p = 0.13$). Plasma GABA levels prior to and one month after deployment poorly predicted a high level of psychopathology symptoms either one or six months after deployment. The number of previous deployments, trauma experienced during deployment, childhood trauma, age and sex were not significantly associated with plasma GABA levels over time. Exclusion of subjects who either started or stopped smoking, alcohol or medication use between the three time points rendered the association of increasing GABA levels with the emergence of psychopathology symptoms more pronounced (mental health problems at T2: $\beta = 0.09$, $p = 4.2 \times 10^{-3}$; depressive symptoms at T2: $\beta = 0.35$, $p = 3.5 \times 10^{-3}$, PTSD symptoms at T2: $\beta = 0.17$, $p = 1.7 \times 10^{-2}$). To our knowledge, this is the first study to provide prospective evidence that the development of psychopathology after military deployment is associated with increasing plasma GABA levels. Our finding that plasma GABA rises after the emergence of psychopathology symptoms suggests that GABA increase may constitute a compensatory mechanism and warrants further exploration of the GABA system as a potential target for treatment.

1. INTRODUCTION

Military deployment increases the risk for mental health problems, including major depressive disorder (MDD) and posttraumatic stress disorder (PTSD) (Hoge et al., 2006, 2004; Reijnen et al., 2015). However, there are considerable interindividual differences and many individuals do not develop mental health problems after deployment and are resilient. Whereas most studies have focused on the hypothalamic-pituitary-adrenal (HPA) axis as the main determinant of this resilience (Russo et al., 2012; Van Zuiden et al., 2012), several converging lines of evidence underscore the importance of the GABA system in stress resilience and vulnerability. GABAergic circuits are pivotal for stress reactivity and can directly affect HPA axis activity (Cullinan et al., 2008; Herman et al., 2004; Ulrich-Lai and Herman, 2009). Moreover, neurosteroids (e.g. allopregnanolone), elevated in response to stress, positively modulate the $GABA_A$ receptor, thereby contributing to the dampening of the stress response (Skilbeck et al., 2010). In humans, the GABA system has been implicated in the etiology of PTSD (Geuze et al., 2008) and evidence suggests that low plasma GABA levels measured directly after a traffic accident can predict the development of PTSD (Vaiva et al., 2006). A review of preclinical studies has shown that traumatic stress has persistent effects on the GABA system, with stress exposure both in early life and during adulthood affecting $GABA_A$ receptor composition in the brain (Skilbeck et al., 2010). There is also convincing evidence that GABA plays a role in the etiology of MDD (for review, see Luscher et al., 2011). Compared with healthy individuals, altered GABA levels in MDD patients have been found in plasma (Honig et al., 1988; Petty, 1994), cerebrospinal fluid (Gerner and Hare, 1981; Honig et al., 1988) and in the brain using proton magnetic resonance spectroscopy (^{1}H-MRS) (Hasler et al., 2007; Sanacora et al., 2004).

However, it is currently unknown whether plasma GABA levels are a trait marker of vulnerability for psychopathology or whether dynamic plasma GABA changes over time are related to the development of psychopathology. We therefore investigated the prospective relationship between the development of psychopathology and plasma GABA levels over time in a large military cohort (Van Zuiden et al., 2009). GABA levels in plasma are readily accessible and have been found to correlate ($\rho = 0.51$) to GABA concentrations in cerebrospinal fluid (Uhlhaas et al., 1986) and to $GABA_A$ receptor binding in the anterior and posterior cingulate cortex, the temporal cortex and the insula (Klumpers et al., 2010). In contrast to the fluctuating nature of central GABA, which depends on the menstrual cycle (Epperson et al., 2002) and disease state (Schür et al., 2016b), plasma GABA concentrations are generally stable throughout the day and the year (Petty and Kramer, 1992), with limited influence of physical activity, diet, gender and menstrual cycle (Petty et al., 1987). In line with previous evidence suggesting stability (Petty et al., 1987; Petty and Kramer, 1992) and predictive value of plasma GABA levels for the development of PTSD following trauma exposure (Vaiva et al., 2006), we hypothesized that low pre-deployment plasma GABA levels would predict the development of psychopathology symptoms after military deployment. Alternatively, we hypothesized that the current sample size in combination with the longitudinal design could demonstrate fluctuating GABA levels linked to psychopathology symptoms, corresponding to central GABA levels in MDD (Schür et al., 2016b).

2. METHODS

2.1. Participants and procedure

The current study was carried out in a large prospective cohort of Dutch military personnel deployed to Afghanistan for a period of 4 months between 2005 and 2008 (Reijnen et al., 2015; Van Zuiden et al., 2011). General information on common duties and exposure during deployment are described in a previous study (Van Zuiden et al., 2012). Military personnel were assessed 1 to 2 months prior to deployment (T0), 1 month after deployment (T1) and 6 months after deployment (T2). All participants gave oral and written informed consent. The Institutional Review Board of the University Medical Center Utrecht (UMCU) approved the study.

2.2. Measurements

2.2.1. Plasma samples and GABA measurement

Venous blood was collected in EDTA containing tubes (Greiner, Bio-One, the Netherlands) between 8.00 and 11.30 A.M. at all three different waves (T0, T1, and T2) in 731 individuals. Samples were centrifuged at 3,500 rpm for 12 minutes at 4°C after which they were stored at –80°C. Plasma GABA levels were determined using a validated method involving ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Details of the analytic procedure for the detection of GABA in plasma, including sample preparation, quality control checks, performance characteristics and instruments used are available in the Supplementary Information (see Supplementary Methods, Supplementary Results and Supplementary Figure S1).

2.2.2. Questionnaires

Mental health problems were assessed at T0, T1 and T2 using the Dutch version of the Symptom Checklist-90 (SCL-90) (Arrindell and Ettema, 2003), which has good reliability and is frequently applied in clinical and population settings (Arrindell et al., 2003; Marres et al., 2011). Depressive symptoms were assessed with the SCL-90 depression subscale which has been extensively validated for the screening of depression in various population samples (Aben et al., 2002; Schmitz et al., 1999; Strik et al., 2001). The Self-Report Inventory for PTSD (SRIP) was used to measure PTSD symptoms at T0, T1 and T2 (Hovens et al., 2002, 1994), which was implemented after the first 85 individuals had completed the first visit. For secondary analyses, validated cutoff scores of 124 for SCL-90 total score, 24 for the depression subscale and 38 for the SRIP were used to separate a high from a low level of symptoms (Arrindell et al., 2003; Van Zuiden et al., 2009; Van Zuiden et al., 2012). These previously defined cutoff scores for the SCL-90 total score and the depression subscale delineate individuals with above average symptoms in the general population (Arrindell and Ettema, 2003). The prespecified cutoff of 38 for the SRIP (Van Zuiden et al., 2012) is in the range with the highest sensitivity and specificity for the DSM-IV classification of PTSD (Van Zelst et al., 2003). All cutoffs corresponded with the 95th percentile of scores before deployment in the present study. Deployment-related trauma exposure was quantified with a 19-item checklist at T1 (Reijnen et al., 2015). Childhood trauma (including physical, sexual and emotional abuse) was established at T0

using the Dutch version of the self-report short form Early Trauma Inventory (ETISR-SF; range 0-62) (Bremner et al., 2007).

2.3. Statistical analysis

Mixed models for repeated measures (MMRM) were implemented using the nlme package in R (Pinheiro et al., 2015). We modeled changes in plasma GABA levels over time and analyzed the interaction of time with continuous outcomes of mental health problems, depressive symptoms and PTSD symptoms, with the pre-deployment GABA level as a covariate. In post hoc analyses, we investigated changes in subscales of the SRIP (re-experiencing, avoidance and numbing, and arousal) in relation to change in plasma GABA levels. Only data from individuals with a baseline GABA level and an SCL-90, depression subscale or SRIP value at T2 were used for primary analyses. In addition, we compared the pattern of GABA change in subjects who developed psychopathology symptoms at T2 with subjects who developed these symptoms at T1. Assuming randomness, missing values in baseline SCL-90 and SRIP data were imputed for mental health problems (n = 127), depressive symptoms (n = 111) and PTSD symptoms (n = 198), using multiple imputation with the MICE package in R (Van Buuren and Groothuis-Oudshoorn, 2011). Variables used for multiple imputation are listed in Supplementary Figure S2.

In the secondary analyses, focusing on dichotomous outcomes, individuals with a high level of pre-deployment mental health problems, depressive symptoms, or PTSD symptoms (as determined by the predefined cutoff values) were excluded to focus on healthy individuals who developed a high level of symptoms at T2. Again, plasma GABA change in subjects who had developed a high level of symptoms at T1 was explored to compare the patterns with subjects who had developed a high level of symptoms at T2. Additionally, to compare post- with pre-deployment symptoms, change in GABA levels of subjects with a high level of pre-deployment psychopathology symptoms was investigated. Moreover, individuals with a high and a low level of pre-deployment mental health problems, depressive symptoms and PTSD symptoms were compared cross-sectionally using a linear model, adjusting for age and sex. Finally, to compare the characteristics of individuals with a high level of symptoms at T2 with those without, we used a chi square test to evaluate gender differences and a Mann-Whitney U test to examine differences in childhood trauma, deployment-related trauma and age (Table 1).

Assumptions for the MMRM were evaluated by inspection of plots of residuals and random effects. Plasma GABA levels were corrected for storage time in all analyses since storage time (range 5.1-9.7 years) significantly decreased plasma GABA levels ($\beta = 6.2 \times 10^{-3}$, $p = 1.7 \times 10^{-7}$). When the storage time at a time point was missing (for one individual at T0, for 60 individuals at T1 and for 22 at T2), missing values were replaced by the mean interval duration between the different time points (183 days between T0-T1 and 325 days for T0-T2).

Possible confounding effects of childhood trauma, number of previous deployments, deployment-related trauma, age and sex were investigated by analyzing the association with GABA levels and psychopathology outcomes. In addition, covariates were investigated for their influence on coefficients of the main determinants. To prevent overfitting of the models, covariates were not included in case they did not meet criteria for confounding. The influence of smoking, alcohol, and medication use was investigated in sensitivity

analyses, by inspecting the change in the coefficients of the main determinants after excluding subjects who either started or stopped the use of any of these substances.
For prediction analyses with plasma GABA levels at T0 and T1, receiver operation curves (ROC) were obtained with mental health problems, depressive and PTSD symptoms 1 and 6 months after deployment as outcomes. The verification package in R was used to calculate AUCs and corresponding p-values (NCAR - Research Applications Laboratory, 2015). The Epi package was used to calculate the sensitivity, specificity, positive and negative predictive values (Carstensen B, Plummer M, Laara E, 2013). Age and sex were included in all prediction models.

Characteristics	All	Mental health problems 6 months after deployment		Depressive symptoms 6 months after deployment		PTSD symptoms 6 months after deployment	
Sample size	731	47		52		56	
Gender (male, %)	90.7	91.5	X^2=0, p=0.96	94.2	X^2=0.28, p=0.60	94.6	X^2=0.51, p=0.47
Age (mean, SD)	29.0 (9.2)	27.6 (8.5)	W=155 76, p=0. 35	29.3 (8.8)	W=15 140, p=0.41	27.0 (9.1)	W=21 088, $p=3.5\cdot10^{-2}$
Early Trauma Inventory (mean, SD)	3.4 (3.0)	3.9 (3.4)	W=11 622, p=0.17	4.8 (3.8)	W=11 130, $p=2.1\cdot10^{-3}$	4.9 (3.4)	W=11 746, $p=1.2\cdot10^{-4}$
Number of deployments (mean, SD)	0.9 (1.2)	0.9 (1.2)	W=12 126, p=0.93	1.1 (1.3)	W=12 386, p=0.23	0.7 (1.1)	W=16 358, p=0.06
Number of deployment-related trauma events (mean, SD)	4.6 (3.2)	5.9 (3.2)	W=6 096, $p=4.7\cdot10^{-3}$	5.9 (3.0)	W=7 443, $p=3.7\cdot10^{-3}$	6.8 (3.1)	W=6 195, $p=4.4\cdot10^{-6}$
Antidepressant use (at any time)	4	0		0		0	

Table 1. Characteristics of different subgroups used for analyses

3. RESULTS

3.1. General

A total of 2,090 blood samples from 731 individuals were available for primary analyses and samples at all three time points were available for 633 individuals. The mean plasma GABA concentration was 122 ± 23 nmol/L, which is in agreement with published data in healthy individuals (Petty et al., 1992). Plasma GABA levels displayed a normal distribution (see Supplementary Figure S3). Within individuals, plasma GABA levels were significantly correlated over the three time points (T0-T1: $\rho = 0.57$, T1-T2: $\rho = 0.63$, T0-T2: $\rho = 0.57$, all p-values $< 2.2 \times 10\text{-}16$), indicating a relative stability of plasma GABA levels over time. Application of the predefined cutoffs for mental health problems, depressive symptoms, and PTSD symptoms six months after deployment yielded three partly overlapping groups (Supplementary Figure S4) which differed with regard to age, childhood trauma and trauma exposure during deployment but were comparable in gender distribution (Table 1). Uncorrected plasma GABA levels and GABA levels adjusted for storage time, age and sex in these groups are displayed in Table 2.

Group	*Time*					
	T0		*T1*		*T2*	
	Uncorrected	*Residual (SD)*	*Uncorrected*	*Residual (SD)*	*Uncorrected*	*Residual (SD)*
Low levels of symptoms at T2	120.4	0.8 (22.4)	124.1	0.0 (22.7)	123.0	-0.5 (23.0)
High level of mental health problems at T2	117.6	-2.7 (21.6)	121.1	-3.4 (19.8)	128.2	4.4 (26.8)
High level of depressive symptoms at T2	117.2	-2.6 (19.3)	123.7	-0.4 (23.5)	131.0	7.6 (24.4)
High level of PTSD symptoms at T2	120.9	0.4 (19.3)	126.1	1.5 (22.7)	128.7	4.7 (27.6)

Table 2. Plasma GABA levels (mean in nmol/L and mean residuals adjusted for storage time, age and sex). Uncorrected plasma GABA levels, residuals adjusted for storage time, age and sex, and standard deviations (SD) before deployment (T0), 1 month after deployment (T1) and 6 months after deployment (T2) in individuals who developed a high level of mental health problems/depressive symptoms/posttraumatic stress disorder (PTSD) symptoms at T2 compared with individuals with a low level of these symptoms.

3.2. Mental health problems and plasma GABA level changes

The increase in mental health problems (total score of the SCL-90) six months after deployment was significantly associated with an increase in plasma GABA levels over time ($\beta = 0.06$, $p = 1.6 \times 10^{-2}$). Post hoc analyses showed a significant positive association between mental health problems at T2 and an increase in GABA levels, both compared with pre-deployment GABA levels (T0-T2, $\beta = 0.13$, $p = 1.5 \times 10^{-2}$) and compared with GABA levels one month after deployment (T1-T2, $\beta = 0.16$, $p = 3.6 \times 10^{-3}$).

In secondary analyses, using cutoff scores, a similar association was seen between GABA increase and subjects who developed a high level of mental health problems six months after deployment ($\beta = 3.6$, $p = 2.0 \times 10^{-2}$) (Figure 1), with a similar time pattern (T0-T2, $\beta = 7.3$, $p = 1.9 \times 10^{-2}$; T1-T2, $\beta = 8.0$, $p = 1.1 \times 10^{-2}$).

Although subjects developing a high level of mental health problems at T2 reported higher levels of trauma exposure during deployment ($W = 6{,}096$, $p = 4.7 \times 10^{-3}$), adding this covariate to the model did not significantly contribute to altered plasma GABA levels over time ($\beta = 0.12$, $p = 0.37$), nor did it affect the relationship between the prospective increase in GABA levels and post-deployment mental health problems (data not shown).

The same pattern in GABA level change was observed in subjects who developed (a high level) of mental health problems at T1 (continuous measure: $\beta = 4.7 \times 10^{-2}$, $p = 0.13$, cutoff: $\beta = 3.3$, $p = 3.9 \times 10^{-2}$), whereas post hoc analyses in subjects with a high level of pre-deployment mental health problems showed an elevation in GABA levels between T0 and T1 compared with the other individuals (cutoff: $\beta = 7.3$, $p = 1.1 \times 10^{-2}$) (see Supplementary Figures S5 and S6).

At baseline, GABA levels of individuals with a high level of pre-deployment mental health problems ($n = 55$) did not significantly differ from those without ($\beta = -2.7$, $p = 0.37$).

3.3. Depressive symptoms and plasma GABA level changes

Increasing depressive symptoms six months after deployment were significantly associated with an increase in GABA levels over time ($\beta = 0.29$, $p = 7.9 \times 10^{-3}$). Comparable to the results for mental health problems, the increase in depressive symptoms at T2 was significantly associated with higher GABA levels compared with both the pre-deployment situation (T0-T2, $\beta = 0.58$, $p = 7.6 \times 10^{-3}$) and compared with GABA levels one month after deployment (T1-T2, $\beta = 0.64$, $p = 3.4 \times 10^{-3}$). The association between GABA levels and depression was more pronounced in individuals who developed a high compared with a low level of post-deployment depressive symptoms at T2 ($\beta = 5.2$, $p = 4.4 \times 10^{-4}$) (Figure 1) and had a similar time pattern (T0-T2, $\beta = 10.5$, $p = 4.2 \times 10^{-4}$; T1-T2, $\beta = 7.8$, $p = 9.5 \times 10^{-3}$). Individuals who developed high levels of depressive symptoms after deployment reported more trauma exposure during deployment ($W = 7{,}443$, $p = 3.7 \times 10^{-3}$) and increased levels of childhood trauma ($W = 11{,}130$, $p = 2.1 \times 10^{-3}$). These covariates, however, did not significantly contribute to plasma GABA levels over time ($\beta = 0.17$, $p = 0.20$ and $\beta = -3.0 \times 10^{-2}$, $p = 0.82$, respectively), nor did they affect the relationship between the prospective increase in GABA levels and post-deployment depressive symptoms.

A comparable change in plasma GABA levels was present in individuals who developed (a high level) of depressive symptoms at T1 (continuous measure: $\beta = 0.23$, $p = 0.072$, cutoff: $\beta = 5.2$, $p = 1.7 \times 10^{-3}$). Similar to mental health problems, post hoc analyses revealed that subjects with a high level of depressive symptoms at baseline demonstrated a rise in GABA levels between T0 and T1 compared with the subjects with a low level of depressive symptoms at baseline, which was borderline significant (cutoff: $\beta = 5.3$, $p = 0.096$) (see Supplementary Figures S5 and S6).

At baseline, individuals with a high level of pre-deployment depressive symptoms ($n = 45$) showed a tendency towards lower rather than higher pre-deployment plasma GABA levels compared with the rest of the sample ($\beta = -6.3$, $p = 0.056$).

3.4. PTSD symptoms and plasma GABA level changes

For PTSD symptoms, a similar but attenuated pattern compared to mental health problems and depressive symptoms was found. The increase in PTSD symptoms at T2 was significantly associated with a prospective GABA increase over time ($\beta = 0.12$, $p = 4.3 \times 10^{-2}$). Similar to the previous measures, this association was apparent for GABA levels at T2 compared with pre-deployment GABA levels (T0-T2, $\beta = 0.25$, $p = 4.2 \times 10^{-2}$) and compared with GABA levels one month after deployment (T1-T2, $\beta = 0.25$, $p = 4.9 \times 10^{-2}$). The arousal subscale of the SRIP showed a significant link to increasing plasma GABA levels ($\beta = 0.28$, $p = 4.6 \times 10^{-2}$), whereas increases in re-experiencing ($\beta = 0.33$, $p = 0.13$) or avoidance and numbing ($\beta = 0.21$, $p = 0.11$) showed no association. Comparable to all previous post hoc analyses, change in arousal was associated with change in plasma GABA levels between time points T0-T2 ($\beta = 0.55$, $p = 4.5 \times 10^{-2}$) and at trend level between T1-T2 ($\beta = 0.57$, $p = 0.053$).

In secondary analyses, the association between the development of a high level of PTSD symptoms and GABA levels over time did not reach statistical significance, although the directionality was comparable ($\beta = 2.7$, $p = 0.061$) (Figure 1).

Individuals who developed a high level of post-deployment PTSD symptoms were on average younger ($W = 21{,}088$, $p = 3.5 \times 10^{-2}$), reported more trauma exposure during

deployment ($W = 6{,}195$, $p = 4.4 \times 10^{-6}$) and higher childhood trauma levels ($W = 11\,746$, $p = 1.2 \times 10^{-4}$). These covariates did not affect the relationship between GABA increase over time and post-deployment PTSD symptoms, nor did they significantly contribute to plasma GABA levels over time ($\beta = -2.3 \times 10^{-2}$, $p = 0.57$; $\beta = 0.16$, $p = 0.23$; $\beta = -3.0 \times 10^{-2}$, $p = 0.82$, respectively).

Subjects who developed a high level of PTSD symptoms one month after deployment showed a tendency towards increased plasma GABA levels six months after deployment, whereas the link between an increase in plasma GABA with the continuous measure of the SRIP was absent (continuous measure: $\beta = 0.11$, $p = 0.13$, arousal subscale: $\beta = 0.29$, $p = 0.070$; cutoff: $\beta = 2.9$, $p = 0.054$). Post hoc analyses showed that individuals with a high level of PTSD symptoms at baseline did not have a significant increase in plasma GABA levels between T0 and T1 compared with subjects with a low level of PTSD symptoms

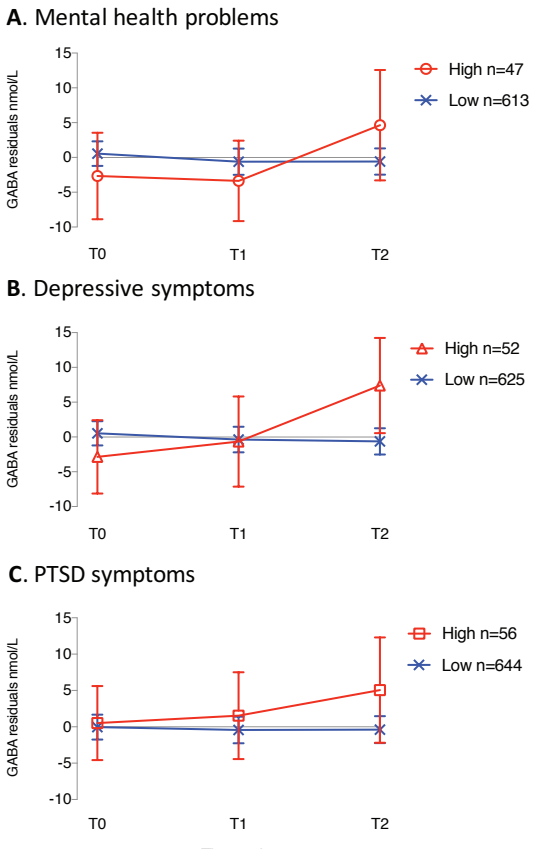


Figure 1. Plasma GABA levels adjusted for storage time (±95% Confidence Interval of the mean) before deployment (T0), 1 month after deployment (T1) and 6 months after deployment (T2) in individuals who developed a high compared with a low level of mental health problems/depressive symptoms/PTSD symptoms at T2.

(cutoff: $\beta = 0.70$, $p = 0.86$) (see Supplementary Figures S5 and S6).
At baseline, GABA levels of individuals with a high level of pre-deployment PTSD symptoms ($n = 29$) did not significantly differ from those without ($\beta = -4.0$, $p = 0.33$).

3.5. Factors influencing the relation between GABA and psychopathology

To further understand the relation between the increase in post-deployment psychiatric symptoms and plasma GABA levels, we next investigated the role of various possible confounders. Since some data were missing on these possible confounders, their influence on the relation between GABA and psychopathology was investigated in subgroups of all available data. Childhood maltreatment, the number of previous deployments (45.6% of the sample had previously been deployed), the number of traumatic events during deployment, age and sex were not significantly associated with plasma GABA levels over time (all p-values > 0.10) and change in coefficient of the main indicators (mental health problems, depressive symptoms, and PTSD symptoms) were all below 5 percent, suggesting that these variables did not significantly confound the reported associations (data not shown).
Associations of change in plasma GABA levels with the development of post-deployment psychopathology symptoms were more pronounced in sensitivity analyses, in which subjects were excluded who either started or stopped smoking ($n = 33$), using alcohol ($n = 28$) or medication ($n = 109$) between T0 and T2. Betas of change in all three symptom scales were altered more than 10% when excluding subjects with a changed medication or alcohol status, while only the beta of change in PTSD symptoms was altered when individuals who either started or stopped smoking were excluded. Exclusion of subjects with a change in any of the three parameters ($n = 152$) yielded significant associations of GABA level increase (T0-T2) with the development of (a high level of) mental health problems (continuous: $\beta = 0.09$, $p = 4.2 \times 10^{-3}$; cutoff: $\beta = 5.2$, $p = 2.4 \times 10^{-3}$), depressive symptoms (continuous: $\beta = 0.35$, $p = 3.5 \times 10^{-3}$; cutoff: $\beta = 5.4$, $p = 9.2 \times 10^{-4}$) and PTSD symptoms (continuous: $\beta = 0.17$, $p = 1.7 \times 10^{-2}$; arousal subscale: $\beta = 0.47$, $p = 4.1 \times 10^{-3}$; cutoff: $\beta = 6.1$, $p = 4.2 \times 10^{-4}$) at T2 (see Supplementary Figure S7).

3.6. Prediction of psychopathology with GABA levels prior to and one month after deployment

Prediction models using pre-deployment plasma GABA levels, sex and age were significant for a high level of PTSD symptoms at T1 (AUC = 0.57, $p = 3.1 \times 10^{-2}$) and at T2 (AUC = 0.60, $p = 8.7 \times 10^{-3}$). In contrast, these models were not significant for mental health problems at T1 (AUC = 0.51, $p = 0.44$) or at T2 (AUC = 0.56, $p = 0.07$), or for depressive symptoms at T1 (AUC = 0.55, $p = 0.12$) or at T2 (AUC = 0.54, $p = 0.18$). In addition, GABA levels at T1 in combination with sex and age significantly predicted PTSD symptoms at T2 (AUC = 0.59, $p = 1.2 \times 10^{-2}$), but not mental health problems (AUC = 0.54, $p = 0.16$) or depressive symptoms (AUC = 0.57, $p = 0.06$) (see Supplementary Table S8, for sensitivity, specificity, positive and negative predictive values).

4. DISCUSSION

In the present study we investigated the prospective relationship between psychopathology after deployment in relation to plasma GABA levels in a large military cohort. We found that increases in mental health problems, depressive symptoms and PTSD symptoms six months after military deployment were related to increasing plasma GABA levels (compared with levels before and one month after deployment). Interestingly, these associations became more pronounced after excluding subjects who either started or stopped smoking, using alcohol or medication between baseline and six months after deployment. A delay between psychopathology symptoms one month after deployment and elevated plasma GABA levels appearing only six months after deployment may suggest that GABA levels rise as a compensatory mechanism for post-deployment psychopathology. In contrast, plasma GABA levels prior to or one month after deployment poorly predicted these outcomes either one or six months after deployment. We found no relationship between alterations in GABA levels and quantitative measures of trauma exposure during deployment or during childhood. Increasing GABA levels are therefore likely to reflect the development of (general) psychopathology rather than a response to traumatic stress. To the best of our knowledge, our study constitutes the largest longitudinal study of plasma GABA levels in relation to psychopathology.

4.1. Compensatory mechanism vs. trait marker

Our finding that post-deployment psychopathology was followed and accompanied by increasing plasma GABA levels indicates that plasma GABA is state-dependent rather than a trait vulnerability as previously proposed (Petty et al., 1995). This finding is in line with ^{1}H-MRS data that show altered GABA levels in currently depressed but not in remitted MDD patients (Schür et al., 2016b). The present study further extends this evidence, as it suggests that GABA levels may rise as a compensatory mechanism to the development of psychopathology symptoms. The dynamic properties of plasma GABA levels have implications for the interpretation of previous and future GABA studies and point to a close relationship with current psychopathology.

4.2. GABA in MDD

Our results further subs tantiate the large body of evidence linking the GABA system to MDD (Emrich et al., 1980; Gerner and Hare, 1981; Klumpers et al., 2010; Luscher et al., 2011). The evidence, however, is fairly heterogeneous. Several studies have shown lower plasma and CSF GABA levels in MDD (Gerner et al., 1984; Gold et al., 1980; Kasa et al., 1982; Petty et al., 1995, 1992), but the results are not consistent (Klumpers et al., 2010; Post et al., 1980; Roy et al., 1991; Zimmer et al., 1980). This inconsistency is also present in ^{1}H-MRS studies measuring *in vivo* GABA levels in the occipital cortex that report lower, higher, or comparable GABA levels in MDD patients compared with healthy controls (Abdallah et al., 2014; Epperson et al., 2006; Sanacora et al., 2004). This discrepancy in GABA findings may be at least partially due to the large clinical heterogeneity in MDD, the smaller sample sizes in these studies and the cross-sectional design. In light of the present study, it is also possible that post-deployment psychopathology has distinct underlying biological mechanisms. In support, individuals with a high level of pre-

deployment depressive symptoms (n = 45) had a tendency towards lower rather than higher pre-deployment plasma GABA levels compared with individuals with a low level of pre-deployment depressive symptoms. Interestingly, however, subjects with a high level of pre-deployment mental health problems or depressive symptoms also demonstrated an increase in plasma GABA levels between T0 and T1, suggesting a similar compensatory mechanism as seen in post-deployment psychopathology symptoms (see Supplementary Figure S6).

4.3. Relation between plasma GABA and central GABAergic activity

Although we found convincing evidence that plasma GABA levels are state-dependent and possibly compensatory to post-deployment psychopathology symptoms, more evidence about the central nervous system correlates of plasma GABA levels is critical for clinical involvement of plasma GABA in detection, prevention and treatment of psychopathology emerging after deployment. There are some indications that plasma GABA levels may be informative of central processes. For instance, Klumpers et al. (Klumpers et al., 2010) used positron emission tomography (PET) with flumazenil to quantify $GABA_A$ receptor binding in relation to plasma GABA levels in MDD patients and healthy individuals. They found an inverse relation between plasma GABA levels and $GABA_A$ receptor binding in the right insula of MDD patients as well as a positive correlation between plasma GABA levels and $GABA_A$ receptor binding in the bilateral anterior cingulate cortex, the left temporal cortex and the right posterior cingulate cortex. Also, changes in plasma GABA levels have been found to correlate significantly with changes in GABA levels in cerebrospinal fluid in a study of 14 alcohol-dependent patients undergoing alcohol withdrawal (Adinoff B, Kramer GL, Petty F, 1995). When evaluating the link between peripheral and central GABA, it is of note that there is limited GABA synthesis outside of the brain and therefore plasma GABA levels (at µM concentrations) are likely to rely on central GABAergic activity (at mM concentrations) (Petty et al., 1987).

4.4. Strengths and limitations

The present study has several strengths. First, our cohort is unique in its size and homogeneity. All individuals were generally healthy at baseline and deployed to a combat zone with no antidepressant use in any of the subjects who developed psychopathology. Moreover, we explored the associations of both trauma exposure during deployment and childhood trauma with prospective plasma GABA levels. Importantly, in contrast to the vast majority of previous studies, plasma GABA levels were prospectively measured. This is informative since within-subject analyses are not influenced by confounders such as the genetic underpinnings that partly determine both GABA levels (Luykx et al., 2012b) and psychopathology. Moreover, investigating both psychopathology symptoms and plasma GABA levels at three time points around deployment provides some indications about the order in which symptoms and GABA levels change and possibly about causality. Finally, plasma GABA levels were quantified using a sensitive and validated analytic method, which can accurately measure GABA levels at the low nanomolar level.

Nevertheless, the results of this study should be interpreted in light of its limitations. Benzodiazepine use was unknown in the present study. However, benzodiazepine use is unlikely in this overall healthy population in which only four individuals used antidepressants. Moreover, both chronic and acute use of benzodiazepines has been associated with decreased

rather than increased GABA levels (Goddard et al., 2004; Roy-Byrne et al., 1992). Consequently, it is implausible that the increase in plasma GABA levels after deployment in individuals developing psychiatric symptoms was the result of benzodiazepines use for their symptoms. Another limitation is the use of self-report questionnaires to determine PTSD symptoms and depressive symptoms. However, the cutoff used for the depressive symptom subscale of the SCL-90 is very comparable in sensitivity and specificity to the optimal cutoffs of specific depression questionnaires such as the Hamilton Rating Scale for Depression and the Beck Depression Inventory (Aben et al., 2002; Strik et al., 2001).

4.5. Future directions and conclusions

A more thorough understanding of the central GABAergic mechanisms underlying these peripheral findings remains critical for clinical progress in the detection, prevention and treatment of psychopathology emerging after deployment (Klumpers et al., 2010). Moreover, genetic and epigenetic studies are pivotal to elucidate the role of genetic variation and DNA methylation in determining plasma GABA levels and GABA system functionality in relation to susceptibility to psychopathology. The present study suggests that a rise in plasma GABA levels may constitute a compensatory mechanism for (post-deployment) psychopathology symptoms. In line with these findings, rodent studies show that HPA axis hyperactivity can be at the origin of changes in GABA system functionality (Orchinik et al., 2001). Other preclinical studies, however, point to the GABA system in the etiology of mood disorders, either directly or by influencing HPA axis activity (Liu et al., 2007), and it is not known to what extent intertwinement with other neurotransmitter systems, such as the serotonergic system, is important (Campos et al., 2012). From a treatment perspective, it is particularly interesting that only the development of arousal, and not re-experiencing or avoidance, was associated with an elevation in plasma GABA levels. This may point to a compensatory elevation of GABAergic neurotransmission in response to the aroused state, similar to benzodiazepine treatment. Considering the potential for pharmacological manipulation of the GABAergic system the question to what extent this compensation is possible, maintainable and desirable is particularly relevant from a clinical viewpoint.

To our knowledge, this is the first study to provide prospective evidence that the development of psychopathology after deployment is accompanied and followed by increasing plasma GABA levels. Together, these findings provide suggest a role of the GABA system in the compensation of (post-deployment) psychopathology and warrant additional exploration of the GABA system as a potential target for treatment.

ACKNOWLEDGEMENTS

We thank M.W. Roeleveld for performing UPLC-MS/MS experiments.

CONFLICT OF INTEREST

This study was funded by a grant from the Dutch Ministry of Defence. Plasma GABA analyses were funded by the VENI fellowship from the Netherlands Organisation for Scientific Research (NWO, grant number 451.13.001) to CHV.

Funders had no role in design and reporting of the study. All authors reported no biomedical financial interests or potential conflicts of interest.

SUPPLEMENTARY INFORMATION

Methods

Reagents
GABA, $^{2}H_{6}$-GABA, acetylchloride and ammoniumformiate were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Formic acid was purchased from VWR (Amsterdam, the Netherlands), Acetonitrile (ACN) UPLC-grade was purchased from Biosolve (Valkenswaard, the Netherlands) and butanol was purchased from Merck (Amsterdam, the Netherlands).

Sample preparation
Stock solutions of GABA and $^{2}H_{6}$-GABA were prepared in milliQ-water (5 mmol/L), and were diluted to obtain working solutions of 1 µmol/L GABA and 1 µmol/L $^{2}H_{6}$-GABA, respectively. For quantification of GABA, calibration curves were prepared to obtain concentrations of 0, 100, 200, 300, 400 and 500 nmol/L.
Before analysis, samples were thawed and vortexed. Hundred µl plasma was mixed with 20 µL 1 µmol/L $^{2}H_{6}$-GABA after which 500 µL ACN was added. The samples were vortexed, centrifuged at 13,000 rpm for 5 min at room temperature and the supernatant was transferred to a 96 wells-plate (Waters, Etten-Leur). The samples were evaporated to dryness under a stream of nitrogen at 40°C. After addition of 100 µL 3M butylation reagent (consisting of 4:1 butanol and acetylchloride), the 96 wells-plate was placed on a shaker (Scientific Industries, New York, USA) for 1 minute and subsequently placed for 15 minutes at 60°C. Next, the samples were evaporated to dryness under a stream of nitrogen at 40°C. Samples were dissolved in 100 µl ACN, after which the 96-wells plate was placed at the shaker again for 1 minute. After this step, the samples were ready for UPLC-MS/MS analysis.

Quality Control (QC)
Two calibrators (QC-high and QC-low) were prepared for quality control by spiking a plasma sample to obtain GABA concentrations in the range of approximately one-third and two-third of the calibration curve.

Performance characteristics
Performance characteristics that were tested include carry-over, detection limit (LOD), limit of quantification (LOQ), linearity, within-run variation, between run variation, stability, analytical sensitivity, interferences and uncertainty of measurement. The uncertainty of measurement was defined as twice the between run variation. LOD was calculated as three times signal to noise (S/N)-ratio, while LOQ was calculated as ten times signal to noise (S/N)-ratio.

Instrument
The chromatographic separation for plasma was carried out on an Acquity UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 µm particle size) including a Van GuardTM UPLC BEH Amide pre-column (2.1 × 5 mm, 1.7 µm particle size) (Waters, Milford, USA) (see

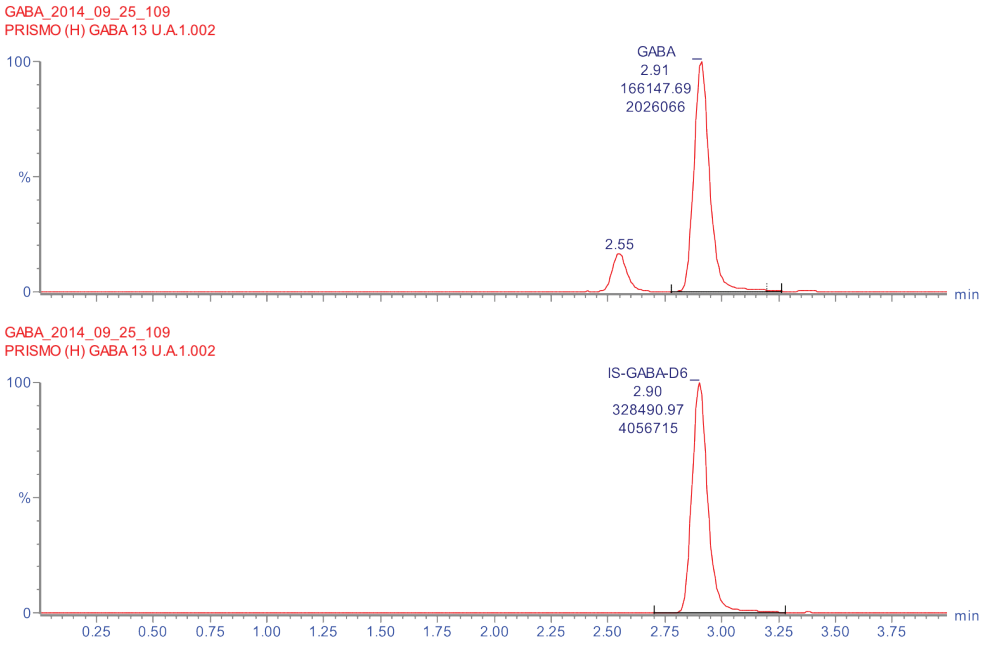
Figure S1). The column was maintained at a temperature of 40°C and the sample volume injected was 5 µL. Optimal chromatographic separation was achieved at a flow-rate of 0.4 ml/min using a isocratic gradient with 5% v/v ammoniumformiate (50 mM pH 3 and 95 % ACN). Total run time was 5 minutes. The column was coupled to the mass spectrometer. A Xevo-TQ MS triple quadrupole mass spectrometer with an electrospray ionization (ESI) source and an Acquity UPLC-system (Waters, Manchester, United Kingdom) were used. Masslynx software (v4.1; Waters, Manchester, United Kingdom) was used for instruments' control and data acquisition. The mass spectrometer operated in ESI-positive mode, capillary voltage 0.5 kV, desolvation temperature 600°C, source temperature 150°C, cone gas flow 0L/h, desolvation gas flow was 700 L/h. Collision energy and cone voltage were optimized for GABA. The dwell time was set automatically. Positively singly charged ions $[M + H]^+$ of GABA (m/z 160.2) and $^2H_6$-GABA (m/z 166.2) were selected as parent ions for Collision Induced Dissociation (CID). The daughter ion m/z 87.0 and 93.0 were the most abundant ions for GABA and $^2H_6$-GABA, respectively. For multiple reaction monitoring (MRM), the transitions m/z 160.2 → 87.0 (GABA) and m/z 166.2→ 93.0 ($^2H_6$-GABA) were measured.

Results

Several isomers of GABA (molecular weight of 103.1198 g/mol) are known, including alfa-amino-isobutyric acid, beta-amino-isobutyric acid, 2-amino butyric acid and 3-amino butyric acid. These compounds did not interfere in the analysis as they were baseline separated from GABA (data not shown).During the validation experiments no relevant carry-over was noticed.

The LOD (0.1 nmol/L) and LOQ (0.8 nmol/L) were calculated and were low enough for accurate analysis of GABA in the physiological range, thereby fulfilling the criteria of analytical sensitivity.

The linearity of the calibration curve ranged from 1-500 nmol/L and was not further tested. The within run variation and between run variation for GABA was determined by analyzing QC-samples. The within run variation (n = 10) was 2.0 % for QC-low and 3.0 % for QC-high, while the between run variation (n = 10) was 3.7 % for QC-low and 4.9 % for QC-high. The uncertainty of measurement for GABA was 9.8%. For stability experiments GABA was analyzed at day 1 and at day 5. In between, the samples were stored at 4°C and data were similar. Additional stability experiments (e.g. freeze/thaw experiments, long-term stability) were not performed.

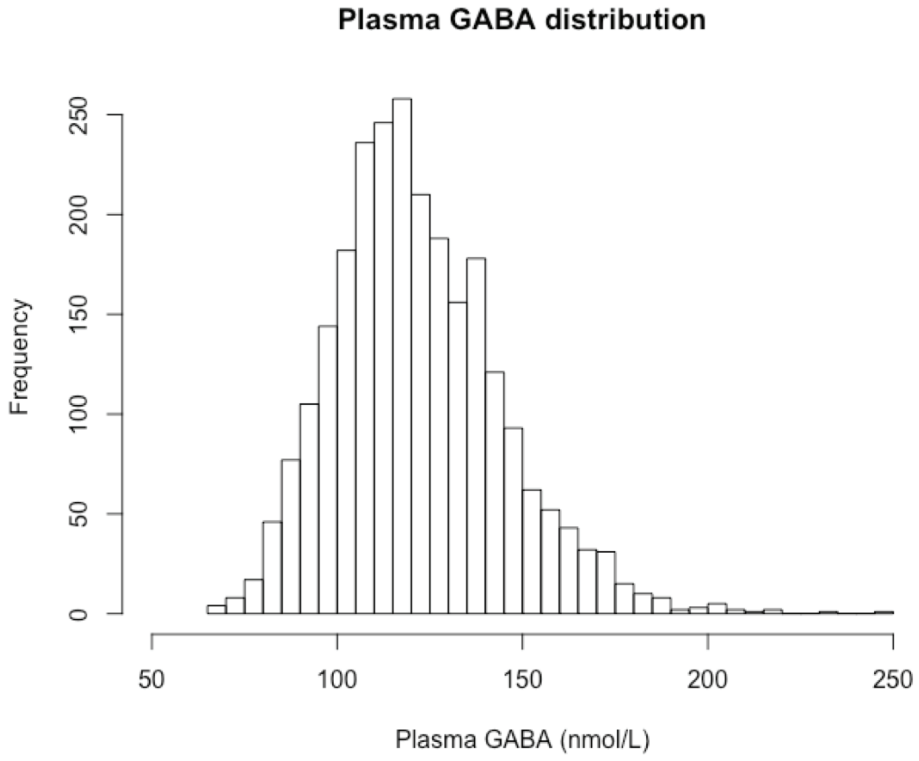


Supplementary Figure S1. Chromatographic separation of GABA.

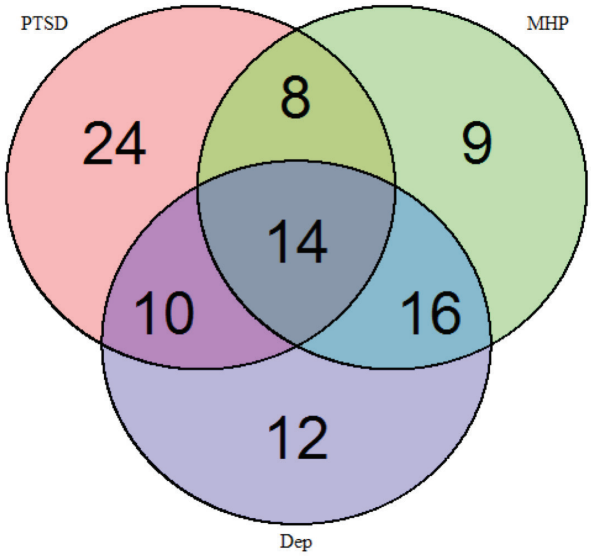
Mental health problems	Depressive symptoms	PTSD symptoms
Age	Age	Age
Gender	Gender	Gender
Education	Education	Education
Rank	Rank	Rank
Number of previous deployments	Number of previous deployments	Number of previous deployments
Childhood trauma	Childhood trauma	Childhood trauma
Mental health problems at T1	Depressive symptoms at T1	PTSD symptoms at T1
Mental health problems at T2	Depressive symptoms at T2	PTSD symptoms at T2
Change in PTSD symptoms between T0 and T2	Change in PTSD symptoms between T0 and T2	Change in mental health problems between T0 and T2
Subject ID*	Subject ID*	Subject ID*
Time point*	Time point*	Time point*
GABA concentration at all available time points*	GABA concentration at all available time points*	GABA concentration at all available time points*
GABA concentration at baseline*	GABA concentration at baseline*	GABA concentration at baseline*

* **Included in the mixed models for repeated measures and therefore taken into account performing multiple**

Supplementary Table S2. Parameters used for multiple imputation of psychopathology symptoms at baseline. Random seed of 245,435, number of multiple imputations and number of iterations: 50.

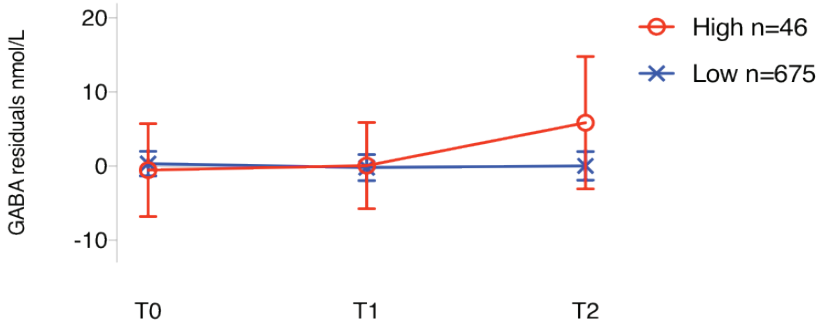


Supplementary Figure S3. Distribution of plasma GABA levels (2,090 samples of 731 individuals).

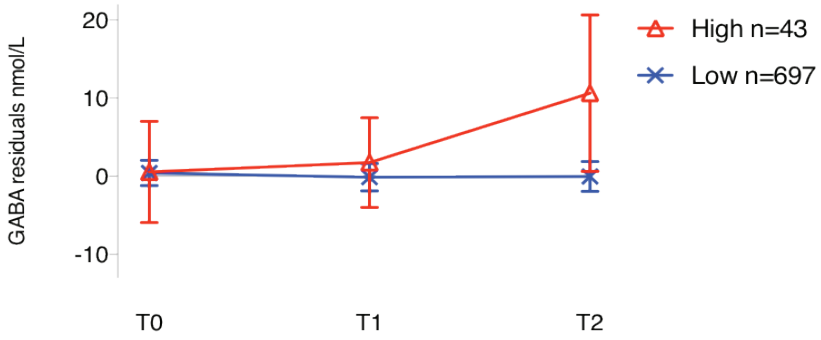


Supplementary Figure S4. Venn diagram displaying the overlap in symptom groups six months after deployment. MHP = Mental Health Problems, PTSD = Posttraumatic Stress Disorder symptoms, Dep = Depressive symptoms.

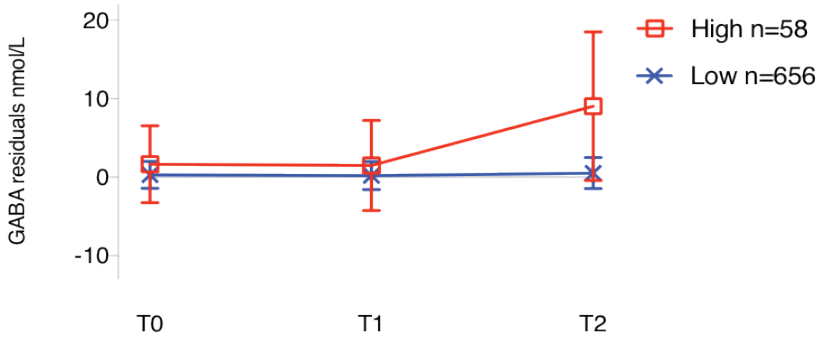
A. Mental health problems at T1



B. Depressive symptoms at T1

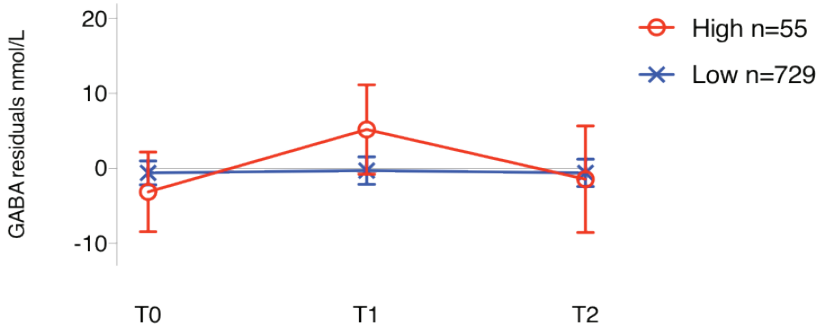


C. PTSD symptoms at T1

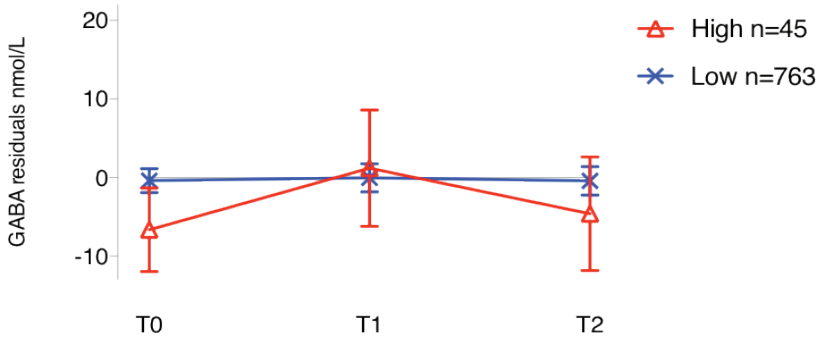


Supplementary Figure S5. Plasma GABA levels adjusted for storage time (±95% Confidence Interval of the mean) before deployment (T0), 1 month after deployment (T1) and 6 months after deployment (T2) in individuals who developed a high compared with a low level of mental health problems/depressive symptoms/PTSD symptoms at T1.

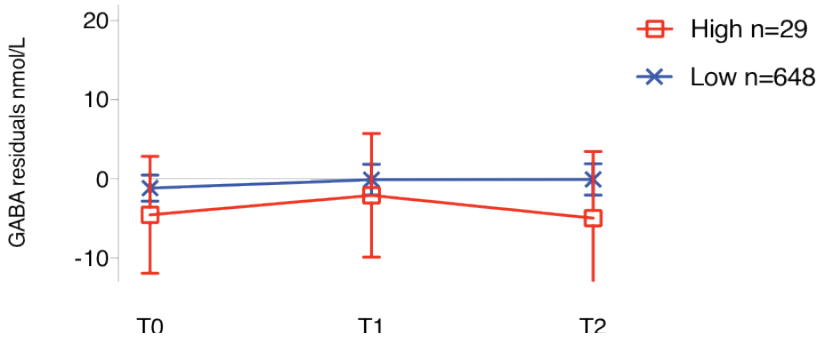
A. Mental health problems at To



B. Depressive symptoms at To

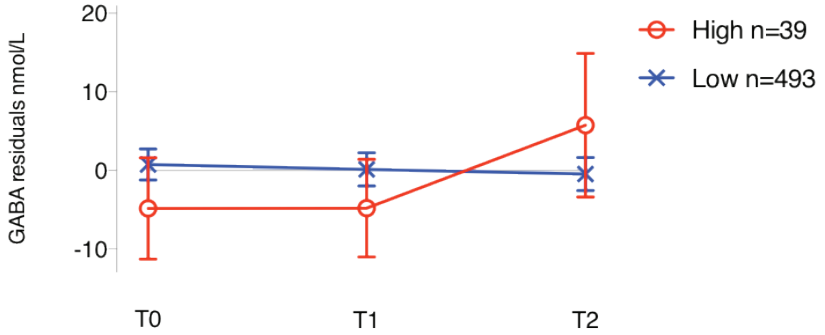


C. PTSD symptoms at To

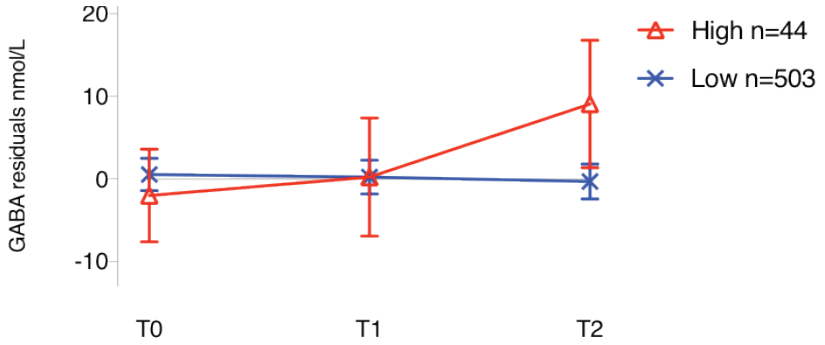


Supplementary Figure S6. Plasma GABA levels adjusted for storage time (±95% Confidence Interval of the mean) before deployment (To), 1 month after deployment (T1) and 6 months after deployment (T2) in individuals who had a high compared with a low level of mental health problems/depressive symptoms/PTSD symptoms at To.

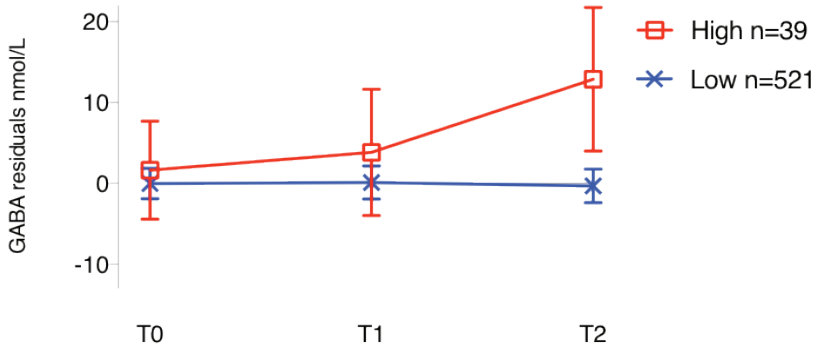
A. Mental health problems no substance switch



B. Depressive symptoms no substance switch



C. PTSD symptoms no substance switch



Supplementary Figure S7. Plasma GABA levels adjusted for storage time (±95% Confidence Interval of the mean) before deployment (T0), 1 month after deployment (T1) and 6 months after deployment (T2) in individuals who had a high compared with a low level of mental health problems/depressive symptoms/PTSD symptoms at T2, without a switch in medication/alcohol/smoking between T0 and T2.

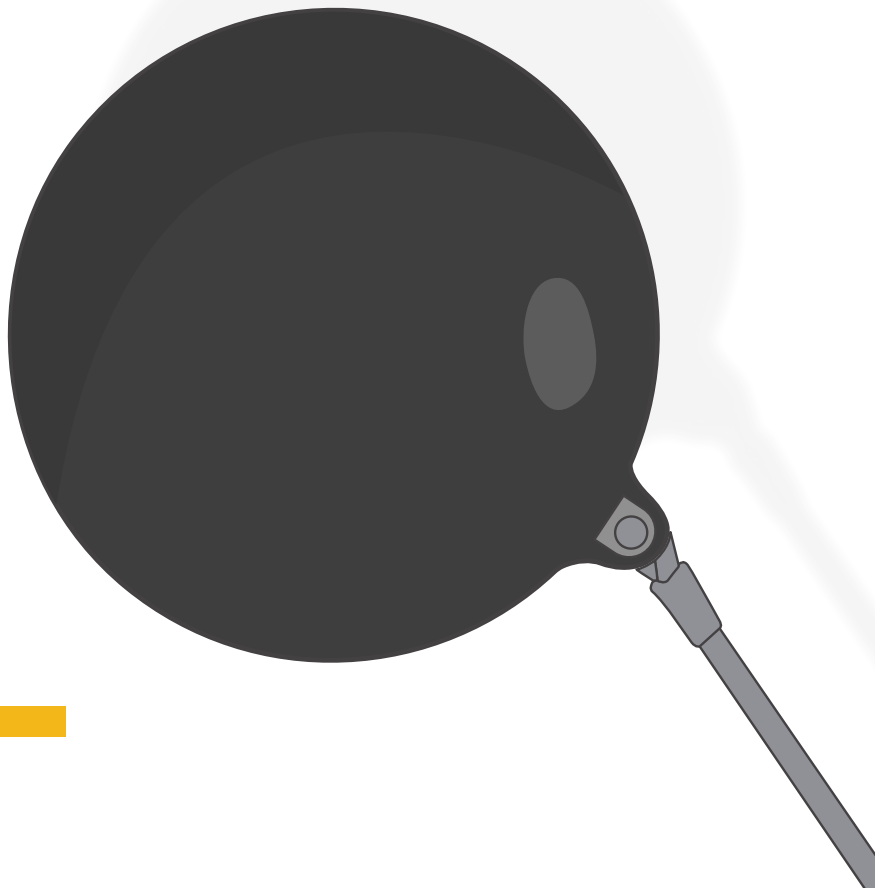
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		Pre-deployment plasma GABA levels		
Mental health problems T1	28.3	82.2	5.6	90.2
Mental health problems T2	70.2	45.7	4.8	91.0
Depressive symptoms T1	81.4	33.6	3.3	93.0
Depressive symptoms T2	96.2	15.2	2.1	91.4
PTSD symptoms T1	67.2	51.1	5.4	89.2
PTSD symptoms T2	73.2	47.4	4.7	89.2
		Plasma GABA levels at T1		
Mental health problems T2	84.4	29.9	4.0	91.3
Depressive symptoms T2	80.0	38.7	4.3	89.9
PTSD symptoms T2	63.6	56.7	5.6	88.1

PPV = Positive Predictive Value, NPV = Negative Predictive Value.

Supplementary Table S8. Characteristics of the prediction models with plasma GABA levels prior to deployment or one month after deployment as predictor and a high level mental health problems, depressive and PTSD symptoms 1 and 6 months after deployment as outcomes. All prediction models were adjusted for sex and age.

PART 3

The association between GABA and the HPA axis



CHAPTER 6

Acute stress effects on GABA and glutamate levels in the prefrontal cortex: A 7T ^{1}H-magnetic resonance spectroscopy study

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ABSTRACT

There is ample evidence that the inhibitory GABA and the excitatory glutamate system are essential for an adequate response to stress. Both GABAergic and glutamatergic brain circuits modulate hypothalamus-pituitary-adrenal (HPA) axis activity, and stress in turn affects glutamate and GABA levels in the rodent brain. However, studies examining stress-induced GABA and glutamate levels in the human brain are scarce. Therefore, we investigated the influence of acute psychosocial stress (using the Trier Social Stress Test) on glutamate and GABA levels in the medial prefrontal cortex of 29 healthy male individuals using 7 Tesla proton magnetic resonance spectroscopy (^{1}H-MRS). *In vivo* GABA and glutamate levels were measured before and 30 min after exposure to either the stress or the control condition. We found no associations between psychosocial stress or cortisol stress reactivity and changes over time in medial prefrontal glutamate and GABA levels. GABA and glutamate levels over time were significantly correlated in the control condition but not in the stress condition, suggesting that very subtle differential effects of stress on GABA and glutamate across individuals may occur. However, overall, acute psychosocial stress does not appear to affect *in vivo* medial prefrontal GABA and glutamate levels, at least this is not detectable with current practice ^{1}H-MRS.

1. INTRODUCTION

Stressful situations require a prompt response of the organism to promote adaptation and survival (McEwen, 2004). Hypothalamus-pituitary-adrenal (HPA) axis functionality is essential for such a response, and depends on many mediators, such as steroid hormones (e.g. cortisol), neurotransmitters (including glutamate and GABA), cytokines, and neuropeptides, which all function in time- and brain area-dependent manners (Joëls and Baram, 2009). The hippocampus, amygdala and prefrontal cortex (PFC) are particularly interesting regions, as they project onto the HPA axis via the inhibitory GABA and excitatory glutamate system (Ulrich-Lai and Herman, 2009), but the stress-related dynamics of these systems largely remain unclear. Of note, stress exposure generally increases prefrontal cortex glutamate levels in the rodent brain (for review see Popoli et al., 2012) and mostly decreases brain GABA levels, depending on the type and duration of stress, and the brain region examined (Acosta and Rubio, 1994; Bedse et al., 2015; Borsini et al., 1988; De Groote and Linthorst, 2007; Gunn et al., 2011; Otero Losada, 1988; Petty and Sherman, 1981). In addition, rapid changes in $GABA_A$ receptors occur after acute stress in animals (Skilbeck et al., 2010).

In contrast to the abundance of animal studies examining the relation between stress and GABA/glutamate levels, human studies are scarce. Currently, the only method to directly measure GABA and glutamate levels in the living human brain is proton magnetic resonance spectroscopy (^{1}H-MRS). Using ^{1}H-MRS to detect stress-related differences in metabolite levels in the PFC, one study reported increased glutamate + glutamine levels after chemically induced panic (Zwanzger et al., 2013) and another study showed decreasing GABA levels under threat of shock (Hasler et al., 2010). However, to the best of our knowledge, the influence of acute psychosocial stress on GABA and glutamate levels in the human brain is unknown. Investigating the mechanisms underlying psychosocial stress is relevant in light of the impact of repeated psychosocial stress exposure on the risk for and course of psychiatric disorders (Brenner et al., 2009; Lange et al., 2013).

Recent technical developments at a field strength of 7 Tesla (T) enable improved measurement of *in vivo* glutamate and GABA levels in the human brain (Boer et al., 2011; Mullins et al., 2014). Scanning at higher field strength yields greater spectral dispersion and thereby more reliable signal quantification (Govindaraju et al., 2000), which is of particular interest since glutamate and especially GABA are present at low concentrations in the brain (5-15 mmol/kg (Govindaraju et al., 2000) and ±1 mmol/kg (Wijtenburg et al., 2015), respectively).

Therefore, we aimed to investigate acute psychosocial stress-induced changes in glutamate and GABA levels in the human medial PFC (mPFC) as measured with ^{1}H-MRS in a 7T MRI scanner. Based on the available studies in rodents (Drouet et al., 2015; Otero Losada, 1988; Popoli et al., 2012; Skilbeck et al., 2010), we hypothesized that, compared to the control condition, stress would increase glutamate levels and decrease GABA levels in the human mPFC.

2. METHODS

2.1. Participants

Healthy non-smoking male individuals (age 18-40, n = 30) were recruited from the general population in The Netherlands (see Table 1). Participants did not take any medication and had not previously been enrolled in any stress-related research. The absence of mental disorders according to DSM-IV criteria was confirmed using the Mini International Neuropsychiatric Interview (MINI)-plus (Sheehan et al., 1998) conducted by a trained rater. On the day of the test, participants did not take heavy meals or drinks other than water and they abstained from heavy exercise for at least 2 h prior to arrival. Absence of psychoactive substance use (amphetamines, MDMA, barbiturates, cannabinoids, benzodiazepines, cocaine, and opiates) was determined by self-report and verified with a urine multi-drug screening device (InstantView) (Vinkers et al., 2013).

Variable	Total (n=29)	Control (n=14)	Stress (n=15)
Mean age in years (SD)	24 (5)	23 (5)	25 (5)
Childhood maltreatment (mean, range)	31 (25-44)	31 (27-39)	32 (25-44)
Major life events (mean,range)	2.5 (0-6)	2.6 (0-5)	2.5 (0-6)
Daily hassles (mean, range)	17.6 (5-44)	16.9 (5-44)	18.5 (6-44)

Table 1. Baseline sample characteristics in the total sample and per condition

2.2. General

All experimental procedures were approved by the ethical review board of the University Medical Center Utrecht and performed according to the ICH guidelines for Good Clinical Practice and the Declaration of Helsinki. We measured GABA and glutamate levels in the mPFC of participants who were randomized to either the validated stress (n = 15) or control (n = 15) condition of the Trier Social Stress Test (TSST) (Kirschbaum et al., 1993). During a first visit, participants were familiarized with the 7T MRI scanner environment and scanning procedure with a 15-minute scan session to reduce any potential stressful associations with the scanning environment. Throughout the second visit, participants completed a 120-minute protocol during which GABA and glutamate levels were quantified in the mPFC before (time point 1) and 30 min after (time point 2) exposure to either the stress or the control condition (see Figure 1). Scanning around 30 min after stress exposure (time point 2) was selected to coincide with the cortisol peak of the stress response (Vinkers et al., 2013).

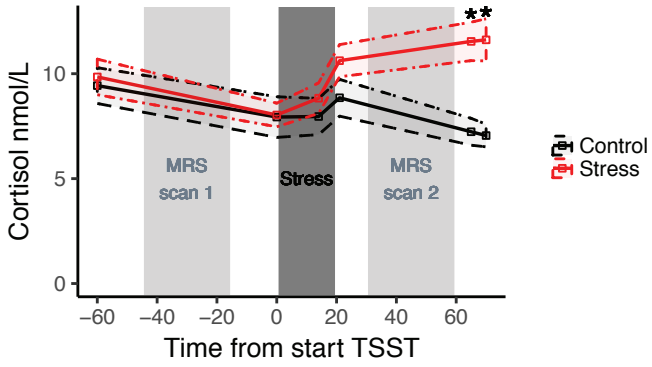

Figure 1. Cortisol levels over time before and after exposure to the control condition (n = 15) or the stress condition (n = 14). The dotted lines represent the standard error. * = p-value < 0.01 (comparing the stress to the control condition in the post hoc test per time point).

2.3. Stress and control conditions

All experimental conditions were carried out between 2 P.M. – 9 P.M. to minimize diurnal variations of cortisol secretion. The stress condition was carried out in accordance with previously published methods (Kirschbaum et al., 1993). Five minutes before the stress or control intervention, all participants received written instructions. In the stress condition, participants delivered a public speech and performed a challenging mental arithmetic while being seemingly videotaped and recorded in front of an evaluative panel that did not show any signs of social support. The combination of an evaluated public speech and cognitive task reliably stimulates the HPA axis by integrating uncontrollability with threat to the social self and self-esteem. The control condition consisted of a speech and simple arithmetic without the presence of a video camera or evaluative panel. Thus the control task has a comparable cognitive load without the social evaluative aspects that stimulate the HPA axis (Het et al., 2009). Salivary cortisol levels were measured using six saliva samples (Salivettes) collected over a 120-minute time period (from 60 min prior to the experimental condition up to 60 min afterwards, Figure 1). Cortisol was measured using an in-house radioimmunoassay as previously published (Vinkers et al., 2013). For three individuals one saliva sample was missing due to insufficient saliva for reliable detection. For these three missing samples (that were all prior to the experimental condition), a value was imputed based on all other cortisol measurements, age and experimental condition. The area under the curve with respect to the increase (AUCi) of cortisol was calculated as previously described (Pruessner et al., 2003). Moreover, the cortisol peak response was calculated representing a more dynamic measure of temporal changes as previously published (5^{th} sample – 2^{nd} sample) (Vinkers et al., 2013).

2.4. Magnetic resonance spectroscopy

All scans were performed on a 7T MRI scanner (Philips, Cleveland, OH, USA) with a birdcage transmit head coil driven by two amplifiers in combination with a 32 channel receive coil (Nova Medical, Inc.). A T1-weighted MP-RAGE sequence was acquired for voxel placement (174 slices, TR = 4ms, TE = 1.8 ms, flip angle = 7°, field of view = 246 × 246 × 174 mm). Glutamate levels were detected in a 20 × 20 × 20 mm^3 voxel using an sLASER sequence (semi-localized by adiabatic selective refocusing; TE = 30-36 ms, TR = 5000 ms, 32 averages, max B1 = 17-20 μT, no OVS (Boer et al., 2011)). The TE was either 30 ms in case we could reach a local B1 of 20 μT, or 36 ms in case the local B1 was between 17 and 20 μT. J-difference spectral editing was used to differentiate the GABA signal from other metabolites. The macromolecular contribution to the GABA signal was minimized by using symmetric editing around the macromolecule resonance at 1.7 ppm, alternating the editing pulse between 1.9 ppm (GABA refocused) and 1.5 ppm (GABA undisturbed) (Andreychenko et al., 2012). GABA-edited ^{1}H-MRS spectra were obtained using a MEGA-sLASER sequence (TE = 74 ms, TR = 4000 ms, 64 averages, no OVS (Andreychenko et al., 2012)) in a 25 × 25 × 25 mm^3 voxel. Non-water suppressed spectra were obtained in order to calculate absolute concentrations of metabolites. Prior to ^{1}H-MRS acquisition, RF shimming on the region of interest was used to optimize phase settings of the individual transmit channels. Second order B0 shimming was automatically performed before data acquisition. For tissue segmentation purposes, a whole-brain three-dimensional fast field echo T1-weighted scan was obtained (450 slices, slice thickness = 0.8 mm, TR = 7 ms, TE = 3 ms, flip angle = 8°, field of view = 250 × 200 × 180 mm, 312 × 312 acquisition matrix, SENSE factor 2.7, scan duration = 408 s). The voxel was placed in the mPFC with the posterior edge adjacent to the corpus callosum and the anterior edge placed to avoid signal from the cerebrospinal fluid (25 × 25 × 25mm^3 voxel for GABA; 20 × 20 × 20mm^3 voxel for glutamate Figure 2). To ensure comparable voxel placement before and after the experimental procedure, screenshots of the first scan were used to place the voxel in the second scan session.

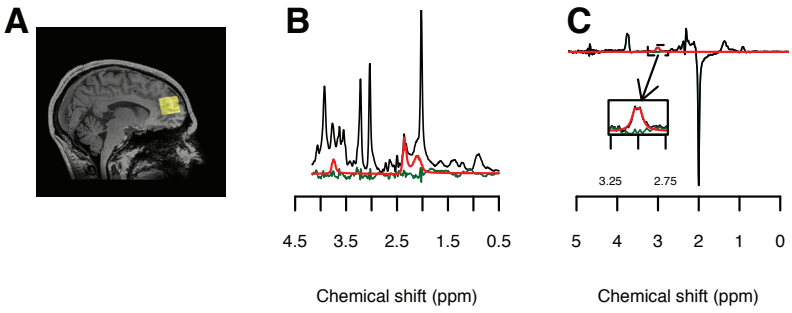

Figure 2. Representative example of voxel placement (yellow rectangle) in the medial prefrontal cortex (panel A), an sLASER spectrum (panel B) and an edited MEGA-sLASER spectrum (panel C). In the spectra, the red line denotes the individual metabolite fit of respectively glutamate (panel B) or GABA (panel C) and the green line is the residual after fitting the metabolites. Insert: zoom of the GABA peak in the edited MEGA-sLASER spectrum.

2.5. Metabolite quantification

Data from 32 receiver coils were combined after amplitude weighting and phasing based on the water reference signal, and noise decorrelation based on a noise scan. The water reference signal was also used for eddy current correction and as an internal standard for GABA and glutamate quantification. Metabolites (including glutamate) were quantified from conventional MR spectra using LCModel-based software implemented in Matlab (Provencher, 1993; NMR Wizard) which relies on a priori knowledge of spectral components of metabolites. Measured macromolecules and sixteen simulated metabolite profiles were fitted to each spectrum: taurine (Tau), myo-inositol (m-Ino), glutathione (GSH), glutamine (Gln), glutamate (Glu), GABA, N-acetyl aspartyl glutamate (NAAG), N-acetyl aspartate (NAA), phosphocreatine (PCr), creatine (Cr), phosphoethanolamine (PE), glycerophosphocholine (GPC), phosphocholine (PCh), lactate (Lac), aspartate (Asp) and glycine (Gly). The baseline of the spectral fit was adjusted by incorporating possible lipid and water artifacts. GABA-edited MR spectra were frequency-aligned with the singlet resonance of choline prior to subtraction of odd and even acquisitions. Fitting of the GABA-edited spectra was performed by frequency-domain fitting of the GABA and creatine resonances to Lorentzian line shapes using in-house Matlab tools (Andreychenko et al., 2013).

Spectral fitting was assessed based on (i) visual inspection by two independent investigators and (ii) a Cramér-Rao lower bound (CRLB) estimate lower than 10% for GABA and glutamate, which is lower than the generally recommended CRLB of 20% (Provencher, 2015). The CRLB represents estimates of the standard deviations of the fit for each metabolite. Based on these criteria, one MEGA-sLASER scan was excluded. A typical example of metabolite fits has been included in Figure 2. Due to data transfer problems, GABA data was missing for three individuals and we did not have an anatomical scan to calculate GABA and glutamate concentrations for one individual. Glutamate and GABA data were available for 29 and 26 individuals, respectively.

To correct for partial volume effects in the voxel, grey matter (f_{GM}), white matter (f_{WM}) and CSF(f_{CSF}) fractions per voxel were obtained using segmentation of the anatomical images with statistical parametric mapping software (SPM8) according to the unified segmentation method (see Supplementary Method 1 for full description) (Ashburner and Friston, 2005). In short, the sum value for each of the three tissue masks was divided by the sum of all three tissue masks for each voxel, resulting in $f_{GM}+f_{WM}+f_{CSF} = 1$ (see Supplementary Table 1). Correction for partial volume differences did not change any of the results and we used the corrected values for all analyses (see Supplementary Note 1 for the analyses without partial volume corrections).

2.6. Questionnaires

To investigate possible confounding by childhood maltreatment, life events, and daily hassles on cortisol stress reactivity, participants completed validated self-report questionnaires of childhood trauma (Childhood Trauma Questionnaire (CTQ) (Bernstein et al., 2003)), major life events (Lifetime Stressor Checklist-Revised (LSC-R) (Wolfe et al., 1996)) and current daily hassles (Dutch Everyday Problem Checklist (Vinkers et al., 2014)).

2.7. Statistical Analysis

General
All statistical analyses were carried out using R version 3.2.1 (R Core Team, 2014). For regression modelling, the Limma package was used (Smyth, 2004). There were no outliers (defined as having a Cook's Distance > 1). Age was included as a covariate to adjust for age variation in brain metabolite levels (Marsman et al., 2013). In all regression models, GABA or glutamate levels after the experimental condition, adjusted for baseline GABA or glutamate levels, were used as primary outcome. Since trauma exposure can influence cortisol stress reactivity, we examined if group differences existed for childhood trauma, major life events or daily hassles.

Stress exposure: effects on GABA and glutamate levels
The main aim of the current study was to investigate the effects of stress on GABA and glutamate levels. Therefore, we examined the association between GABA or glutamate levels after the experimental condition (stress versus control) in a linear regression model while adjusting for age and baseline GABA or glutamate levels. We also calculated the correlations between GABA and glutamate concentrations before and after the experimental condition to examine whether these correlations would differ in the stress compared to the control condition.

Stress-induced cortisol levels: effects on GABA and glutamate levels
First we examined whether the cortisol response over time differed between the stress and the control condition using Mixed Model Repeated measures with the nlme package in R (Pinheiro et al., 2015). In this model condition, time, age and the interaction between time and condition were modeled as fixed effects and we included a by-subject random effect of intercepts and slopes. If a significant interaction was present between the experimental condition and time, the specific time points between the control and stress condition were identified in planned post hoc tests with Bonferroni adjustment for multiple comparisons. Next, we examined the association between cortisol stress reactivity (expressed as $AUCi_{CORTISOL}$ or peak cortisol response) and longitudinal change in GABA or glutamate levels after the experimental condition in a linear regression with age and baseline GABA or glutamate levels as covariates.

2.8. Reliability ^{1}H-MRS measurement
To evaluate the reproducibility of ^{1}H-MRS measurements over time, we calculated the intraclass correlation coefficient (ICC) for GABA and glutamate in the control condition. Consistent with previous neuroimaging studies, an ICC of 0.7 was deemed acceptable (Cai et al., 2012).

3. RESULTS

3.1. Group characteristics

No significant group differences were present for age, baseline GABA or glutamate levels in the mPFC, partial volumes in the mPFC voxels, childhood trauma, major life events and minor stressors (Table 1 and 2).

Variable	Total (n=29)**	Control (n=14)**	Stress (n=15)**
Glutamate (mM) before (mean,SD)	8.7 ± 1.5	8.6 ± 1.6	8.8 ± 1.4
Glutamate (mM) after (mean,SD)	8.0 ± 1.4	8.3 ± 1.0	8.0 ± 1.5
GABA (mM) before (mean,SD)	1.6 ± 0.5	1.6 ± 0.6	1.6 ± 0.4
GABA (mM) after (mean,SD)	1.4 ± 0.5	1.3 ± 0.5	1.5 ± 0.4

** For GABA total N= 26, stress N=12 and control N= 14

Table 2. Glutamate and GABA levels in the total sample and per condition.

3.2. Stress related differences in prefrontal GABA and glutamate levels

Stress did not significantly affect prefrontal GABA and glutamate levels (glutamate: β = -0.1, t = -0.2, p = 0.86, model fit: $F(3,25) = 0.49$, $R^2 = 0.06$; GABA: $\beta = 0.22$, t = 1.3, p = 0.20, model fit: $F(3,22) = 3.9$, $R^2 = 0.26$) (Figure 3). Both for GABA and glutamate, the levels before and after the control condition were significantly correlated (GABA: $\rho = 0.45$, p = 0.03, Glutamate: $\rho = 0.43$, p = 0.04). In contrast, before-after levels were not significantly correlated in the stress condition (GABA: $\rho = -0.09$, p = 0.69, Glutamate: $\rho = 0.18$, p = 0.46).

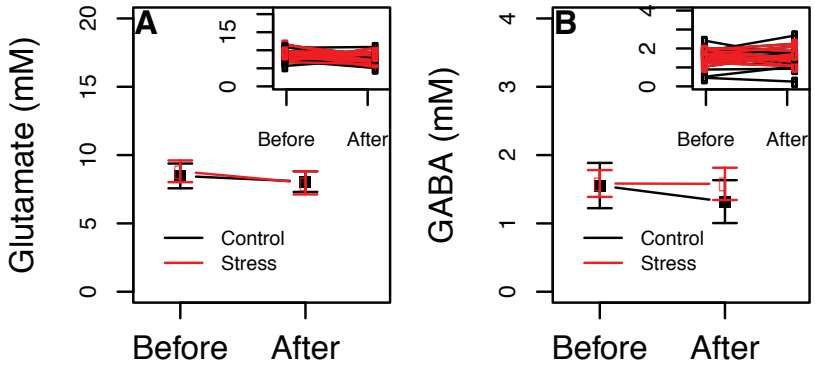

Figure 3. Mean glutamate (A) and GABA (B) levels before and after the task in either the control (black) or stress (red) condition. Error bars indicate the standard error per condition. Insert: individual GABA and glutamate levels for each participant.

3.3. Cortisol stress reactivity, GABA and glutamate levels

Cortisol levels over time were significantly higher in the stress condition compared to the control condition (Condition × Time interaction: $F(4,112) = 9.89$, $p < 0.001$). Post hoc tests indicated higher cortisol levels in the stress condition at the time points immediately after the second ^{1}H-MRS measurement (t_{65}min: $\beta = 4.6$, $p = 0.002$; t_{70}min: $\beta = 4.8$, $p < 0.001$) (Figure 1). As expected, stress exposure resulted in a larger cortisol peak response ($\beta = 3.9$, $t = 3.2$, $p = 0.003$, model fit: $F(2,27) = 7.0$, $R^2 = 0.29$) and a trend towards a higher $AUCi_{CORTISOL}$ ($\beta = 149$, $t = 2.04$, $p = 0.05$, model fit: $F(2,27) = 2.1$, $R^2 = 0.07$). However, cortisol release was not associated with changes in either glutamate ($AUCi_{CORTISOL}$: $\beta = 4.7 \times 10^{-4}$, $t = -0.3$, $p = 0.73$, model fit: $F(3,25) = 0.52$, $R^2 = -0.05$; cortisol increase: $\beta = -0.02$, $t = -0.3$, $p = 0.79$, model fit: $F(3,25) = 0.5$, $R^2 = -0.06$) or GABA levels ($AUCi_{CORTISOL}$: $\beta = 3.4 \times 10^{-5}$, $t = 0.08$, $p = 0.93$, model fit: $F(3,22) = 3.1$, $R^2 = 0.20$; cortisol increase: $\beta = -0.009$, $t = -0.3$, $p = 0.73$, model fit: $F(3,22) = 3.1$, $R^2 = 0.20$).

3.4 Reliability ^{1}H-MRS signal

In the control group the ICC estimates were similar for GABA (ICC = 0.60) and glutamate (ICC = 0.57), but lower than the 0.7 cut-off deemed acceptable in previous neuroimaging studies that aimed to establish reproducibility between scans (Cai et al., 2012).

4. DISCUSSION

In the current study, we investigated the influence of acute psychosocial stress on glutamate and GABA levels in the human prefrontal cortex using 7T ^{1}H-MRS. Stress exposure did not significantly alter GABA and glutamate levels compared to the control condition. Moreover, the peak and AUCi cortisol response were not associated with changes in prefrontal GABA or glutamate levels. Nonetheless, whereas both GABA and glutamate before and after the control condition were significantly correlated, this was not the case in the stress condition, possibly indicating very subtle stress effects differing across individuals.

4.1. GABA and glutamate changes in response to stress

GABAergic and glutamatergic neurotransmission are pivotal for restoring homeostasis after acute stress, with the mPFC and hippocampus constituting two key regions affecting HPA axis activity (Ulrich-Lai and Herman, 2009). Rodent studies indicate increased stress-related prefrontal glutamate levels, primarily based on studies carried out in synaptosomes (for review see Popoli et al., 2012). In the hippocampus either no effect (Popoli et al., 2012) or a rapid increase in glutamate levels or release probability was observed (Karst et al., 2005; Venero and Borrell, 1999). Also, several hours after acute stress glutamatergic transmission was found to be enhanced, both in the PFC (Yuen & Yan, 2009; Yuen et al., 2011) and in the hippocampus (Karst and Joëls, 2005). In contrast, acute stress generally decreased frontal and hippocampal GABAergic transmission (Biggio et al., 2007). Some evidence suggests that the direction of GABAergic transmission change after acute stress is stressor dependent, both in the hippocampus (for review see Linthorst and Reul, 2008) and in the frontal cortex (Acosta and Rubio, 1994; Bedse et al., 2015).

Although many rodent studies report GABA and glutamate differences after stress, human

studies investigating stress-induced GABA and glutamate levels are scarce. In contrast to our findings of no stress-related differences in GABA and glutamate levels after acute psychosocial stress, two previous ^{1}H-MRS studies reported increased glutamate (Zwanzger et al., 2013) and decreased GABA (Hasler et al., 2010) levels in the prefrontal cortex after chemically induced panic and threat of shock, respectively. However, it is important to note several differences in study methodology. First, we used an extensively validated psychosocial stressor with a social evaluative aspect which induces a robust cortisol response (for review see Foley and Kirschbaum, 2010). Nevertheless, it is possible that GABA and glutamate levels are not as susceptible to this type of stressor as to chemically induced panic or threat of shock. In addition, since the stress task needs to be carried out outside of the MR scanner, voxel placement, shimming and voxel localization were done twice, which may have led to more within-subject variation. Moreover, while the previously reported glutamate increase was detected 10 min after stress (Zwanzger et al., 2013) and the GABA decrease 15 min after stress (Hasler et al., 2010), we measured GABA and glutamate levels at the peak of the cortisol response (30 min after stress) in line with a bidirectional relationship between cortisol levels and GABA and glutamate (Mody and Maguire, 2012). We cannot exclude that GABA and glutamate levels immediately after stress exposure are more relevant for cortisol stress reactivity than GABA and glutamate levels 30 min after stress. A final difference with previous studies is the use of a 7T scanner enabling better separation of glutamate from glutamine and, in the edited sequence, GABA detection with less macromolecule contamination than at lower field strength. This is particularly relevant as macromolecular content can contribute to > 30% of the GABA signal (Andreychenko et al., 2012; Choi et al., 2010).

4.2. GABA and glutamate in stress-related psychopathology

Notwithstanding the absence of stress or cortisol effects on prefrontal GABA and glutamate levels, adequate functioning of these systems is crucial for maintaining mental health. In support, GABA system abnormalities have been described in a wide range of stress-related disorders, including major depressive disorder (MDD) (Luscher et al., 2011), post-traumatic stress disorder (PTSD) (Geuze et al., 2008), schizophrenia (Gonzalez-Burgos et al., 2015), and general mental health problems after military deployment (Schür et al., 2016a). In addition, differences in the glutamatergic system have also been linked to MDD (Luykx et al., 2012a), PTSD (Pitman et al., 2012), and schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). It remains to be determined to what extend stress-related dynamics of these systems are disturbed in stress-related psychopathology.

4.3. GABA and glutamate quantification

The GABA and glutamate levels we report are in line with the previously reported human brain concentrations of GABA (±1 mmol/kg (Wijtenburg et al., 2015)) and glutamate (5-15 mmol/kg (Govindaraju et al., 2000)). Direct comparison between our values and those of others is complicated by differences in quantification methodology. Important parameters affecting metabolite concentrations include the quantification software, number of metabolites fitted, partial volumes in the voxel location and MRS data quality checks (Alger, 2010; Mullins et al., 2014; Schür et al., 2016b; Van de Bank et al., 2015). Our glutamate

measurement with the sLASER sequence in the mPFC was less consistent (ICC = 0.57) than previously reported for other brain areas (Van de Bank et al., 2015). This lower consistency might be inherent to greater physiological variation in the brain region under study or it could be related to the control task completed in between measurements. Alternatively, it could have resulted from less reliable signal due to magnetic field inhomogeneity, as the region of interest was situated near the paranasal sinuses. Importantly, all CRLBs were below 10% which indicates that the measurements were of good quality.

4.4. Conclusions

In conclusion, we did not find a significant effect of acute stress exposure or cortisol stress reactivity on prefrontal GABA and glutamate levels in the human brain. Although GABA and glutamate levels over time were not correlated in the stress condition, possibly indicating very subtle and differential effects of stress on GABA and glutamate across individuals, our findings suggest that a stress effect on GABA and glutamate levels in the medial prefrontal cortex 30 min after psychosocial stress is absent or at least undetectable using current practice ^{1}H-MRS.

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AUTHOR CONTRIBUTIONS

All authors have written and approved the manuscript. D.W.K, C.H.V. and L.C.H. designed and collected the data for the study. J.P.W. and V.O.B. helped with the spectroscopy analyses. R.R.S. ran the segmentation analyses. L.C.H. performed the statistical analyses under supervision of C.H.V. and M.P.M.B. R.S.K. and M.J. supervised and commented on the manuscript at all stages.

COMPETING FINANCIAL INTERESTS

Dr. Vinkers, Dr. Boks, Dr. Klomp, Dr. Wijnen, Dr. Boer, Mr. Schür, Prof. Joëls, Prof. Kahn and Ms. Houtepen declare no potential conflict of interest.

SUPPLEMENTARY INFORMATION

Supplementary Method 1

The ratios of the area under the metabolite peak to the area under the water peak, $\frac{\mathrm{AREA_{met}}}{\mathrm{AREA_{water}}}$, was corrected for the fraction of grey matter (f_{GM}), white matter (f_{WM}), and CSF (f_{CSF}) using the following equation [Eq. 1].

[Eq. 1] $$\mathrm{Corr_{met}\ (mM)} = \frac{\mathrm{AREA_{met}}}{\mathrm{AREA_{water}}} \times \frac{(\mathrm{WCONC_{GM}} \times \mathrm{ATT_{water\_GM}}) + (\mathrm{WCONC_{WM}} \times \mathrm{ATT_{water\_WM}}) + (\mathrm{WCONC_{CSF}} \times \mathrm{ATT_{water\_CSF}})}{(1\text{-}f_{CSF}) \times \mathrm{ATT_{met}}},$$

where $\mathrm{ATT_{water\_t}}$ is the relaxation attenuation factor of water in each tissue type, *t,* in grey matter (GM), white matter (WM), and CSF, respectively and calculated using the following equation [Eq. 2]:

[Eq. 2] $$\mathrm{ATT_{water\_t}} = \exp\left(-\frac{\mathrm{TE}}{\mathrm{T_{2_{water_t}}}}\right) \times \left(1 - \exp\left(-\frac{\mathrm{TR}}{\mathrm{T_{1_{water_t}}}}\right)\right)$$

In Eq. 2, TE is the sequence echo time, TR is the repetition time. $\mathrm{T_{1_{water_t}}}$ and $\mathrm{T_{2_{water_t}}}$ are based on values reported in Marjanska et al., 2012 (Marjanska et al., 2012) as follows:

	$\mathrm{T_{1_{water}}}$ (ms)	$\mathrm{T_{2_{water}}}$ (ms)
Cortical GM	2130	50
Basal ganglia GM	1523	41.2
WM	1220	55
CSF	4425	141

In Eq. 1, $\mathrm{ATT_{met}}$ is the relaxation attenuation factor of the metabolite, *met*. It was assumed to be the same in GM and WM and calculated using the following equation [Eq. 3]:

[Eq. 3] $$\mathrm{ATT_{met}} = \exp\left(-\frac{\mathrm{TE}}{\mathrm{T_{2_{met}}}}\right)$$

In Eq. 3, TE is the sequence echo time and $\mathrm{T_{2_{met}}}$ is based on values reported by Marjanska et al. (Marjanska et al., 2012) for glutamate and Andreychenko et al. (Andreychenko et al., 2013) for GABA as follows:

	$\mathrm{T_{2_{met}}}$ (ms)
Occipital cortex glutamate	93
Basal ganglia glutamate	88
GABA	87

The T_1 relaxation factor for metabolites was not taken into consideration as an attenuation factor as this would be very small due to the long TR (4000ms-5000 ms).

Finally, in Eq. 1, $\mathrm{WCONC_t}$ refers to the NMR-visible water concentration (mM) in each tissue type, *t*, using the formula:

[Eq. 4] $$\mathrm{WCONC_t} = f_t \times \mathrm{WD_t},$$

where f_t are the individual tissue volume fractions obtained by tissue segmentation and $\mathrm{WD_t}$ is the density of water in each of the three tissue types. This is obtained by multiplying pure water density

Metabolite	Time	Measure	Total	Control	Stress
Glutamate	*Before*	*N*	29	14	15
		Concentration mM (mean,sd)	8.65 +/-1.48	8.47 +/-1.57	8.81 +/-1.44
		CRLB (mean,sd)	2.51 +/-0.82	2.56 +/-0.92	2.46 +/-0.75
		Linewidth (mean,sd)	8.48 +/-3.59	8.58 +/-3.68	8.39 +/-3.64
		GM (mean,sd)	0.8 +/-0.06	0.81 +/-0.08	0.8 +/-0.06
		WM (mean,sd)	0.09 +/-0.03	0.09 +/-0.03	0.09 +/-0.03
		CSF (mean,sd)	0.11 +/-0.06	0.1 +/-0.07	0.12 +/-0.06
	After	*N*	29	14	15
		Concentration mM (mean,sd)	8.01 +/-1.4	8.06 +/-1.31	7.97 +/-1.53
		CRLB (mean,sd)	2.43 +/-0.82	2.37 +/-0.66	2.5 +/-0.97
		Linewidth (mean,sd)	8.31 +/-3.78	7.9 +/-2.57	8.68 +/-4.71
		GM (mean,sd)	0.82 +/-0.06	0.81 +/-0.06	0.83 +/-0.07
		WM (mean,sd)	0.09 +/-0.05	0.09 +/-0.05	0.09 +/-0.04
		CSF (mean,sd)	0.08 +/-0.05	0.09 +/-0.05	0.07 +/-0.05
GABA	*Before*	*N*	26	14	12
		Concentration mM (mean,sd)	1.57 +/-0.46	1.55 +/-0.57	1.58 +/-0.31
		CRLB (mean,sd)	3.62 +/-1.06	3.58 +/-1.07	3.65 +/-1.1
		Linewidth (mean,sd)	19.09 +/-2.49	19.62 +/-2.05	18.48 +/-2.9
		GM (mean,sd)	0.75 +/-0.06	0.75 +/-0.07	0.74 +/-0.05
		WM (mean,sd)	0.16 +/-0.04	0.15 +/-0.04	0.16 +/-0.04
		CSF (mean,sd)	0.1 +/-0.06	0.09 +/-0.06	0.1 +/-0.06
	After	*N*	26	14	12
		Concentration mM (mean,sd)	1.44 +/-0.48	1.32 +/-0.54	1.58 +/-0.37
		CRLB (mean,sd)	4 +/-1.86	4.05 +/-1.91	3.94 +/-1.88
		Linewidth (mean,sd)	19.89 +/-5.3	19.65 +/-6	20.18 +/-4.6
		GM (mean,sd)	0.76 +/-0.06	0.76 +/-0.06	0.77 +/-0.06
		WM (mean,sd)	0.16 +/-0.05	0.15 +/-0.06	0.17 +/-0.04
		CSF (mean,sd)	0.07 +/-0.05	0.09 +/-0.05	0.06 +/-0.05

Supplementary Table 1. Per metabolite, time point and condition, the number of participants per group with usable spectra and measures for spectral quality and voxel decomposition.

Supplementary Note 1. To ensure that the partial volume correction did not influence our findings, we also performed all statistical analyses with the ratio of the area under the metabolite peak to the area under the water peak. These results are reported below.

Stress related differences in prefrontal GABA and glutamate levels

When we use ratio instead of calculated concentration as GABA and glutamate levels, stress still did not significantly affect GABA and glutamate levels in the prefrontal cortex (glutamate: $F(3,25) = 1.68$, $t = 0.4$, $p = 0.72$; GABA: $F(3,22) = 3.9$, $t = 1.8$, $p = 0.09$). Again, GABA and glutamate levels were significantly correlated before and after in the control condition (GABA: $\rho = 0.65$, $p = 0.005$; glutamate: $\rho = 0.59$, $p = 0.02$), but not in the stress condition (GABA: $\rho = 0.20$, $p = 0.43$, glutamate: $\rho = 0.29$, $p = 0.52$).

Cortisol stress reactivity, GABA and glutamate levels

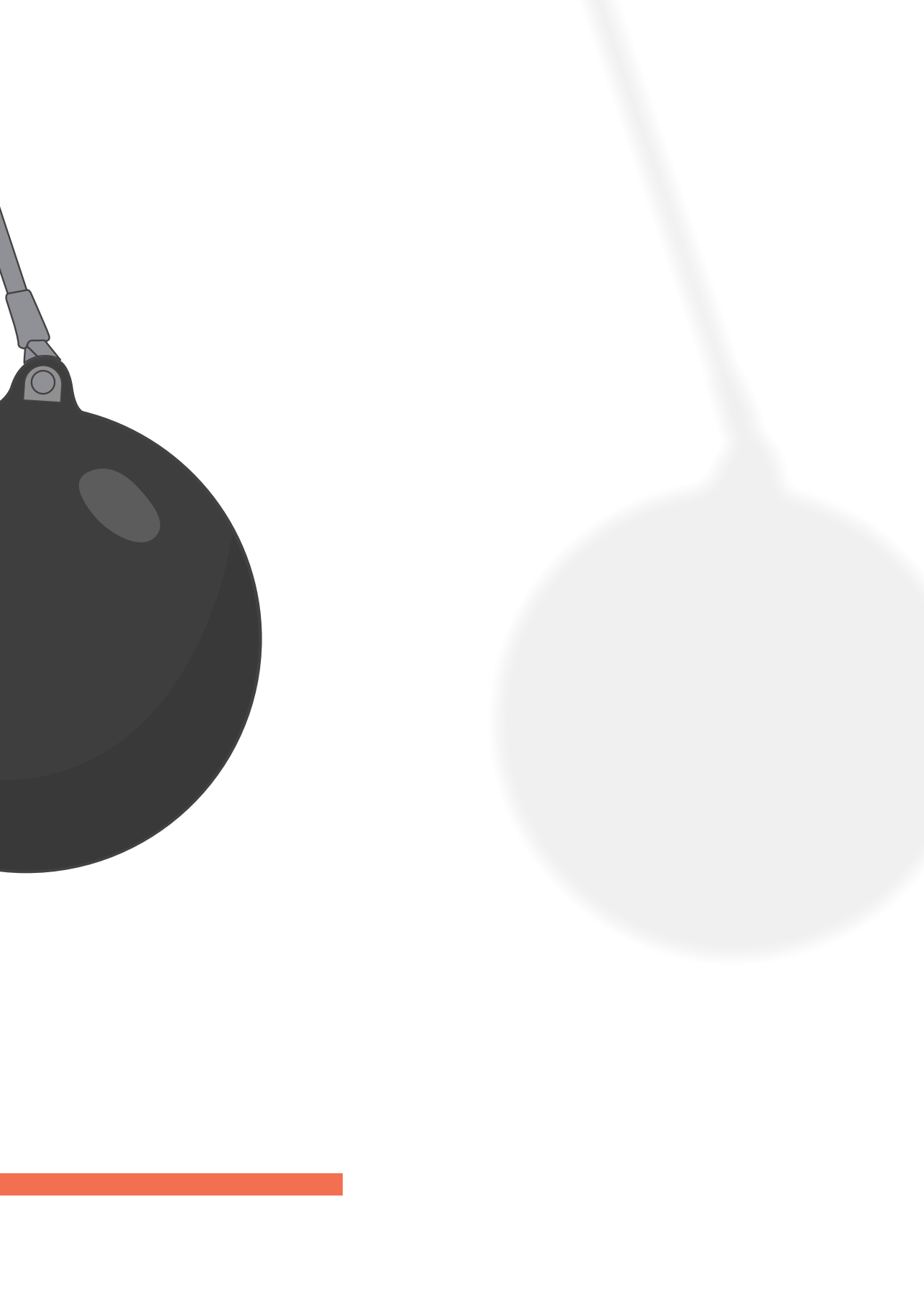
Similarly when we use ratio instead of calculated concentration as GABA and glutamate levels, $AUCi_{CORTISTOL}$ and cortisol increase were not associated with changed glutamate ($AUCi_{CORTISTOL}$: $F(3,25) = 1.63$, $t = -0.03$, $p = 0.97$; cortisol increase: $F(3,25) = 1.7$, $t = 0.5$, $p = 0.62$) or GABA levels ($AUCi_{CORTISTOL}$: $F(3,22) = 4.4$, $t = 0.32$, $p = 0.75$; cortisol increase: $F(3,22) = 4.4$, $t = 3.6$, $p = 0.89$).

Reliability MRS signal in control group

Finally when we use ratio instead of calculated concentration as GABA and glutamate levels, the ICC estimates are similar for GABA (ICC = 0.70) and glutamate (ICC = 0.63) and near the 0.7 cut-off deemed acceptable in previous neuroimaging studies (Cai et al., 2012).

PART 4

Common genetic risk



CHAPTER 7

The interaction between genetic vulnerability and deployment-related trauma in the development of posttraumatic stress disorder and depression

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ABSTRACT

Exposure to trauma strongly increases the risk to develop stress-related psychopathology, such as posttraumatic stress disorder (PTSD) or major depressive disorder (MDD). In addition, liability to develop these moderately heritable disorders is partly determined by common genetic variance, which is starting to be uncovered by genome-wide association studies (GWASs). However, it is currently unknown to what extent genetic vulnerability and trauma exposure interact in the development of PTSD and MDD. We investigated whether genetic risk based on summary statistics of the largest GWASs for PTSD and MDD to date predisposed individuals to develop these disorders in a prospective military cohort (n = 516) at five time points after deployment to Afghanistan: one month, six months and one, two and five years. Linear regression was used to analyze the contribution of polygenic risk scores (at multiple p-value thresholds of association) and their interaction with deployment-related trauma to the development of PTSD and depressive symptoms. We found no main effects of polygenic risk scores nor evidence for interaction with trauma on the development of PTSD or depressive symptoms at any of the available time points in the five years after military deployment. Our results based on a unique long-term follow-up of a deployed military cohort suggest limited validity of current PTSD and MDD polygenic risk scores. Even though PTSD GWASs will likely benefit from larger sample sizes, progress will probably also depend on (endo)phenotype refinement that reduces etiological heterogeneity.

1. INTRODUCTION

Deployment to a combat zone constitutes a major risk factor for the development of several debilitating psychiatric disorders such as posttraumatic stress disorder (PTSD) and major depressive disorder (MDD) (Hoge et al., 2006; Reijnen et al., 2015). Nevertheless, only a relatively small proportion of individuals develop psychopathology after traumatic experiences during deployment and the majority is resilient and remains unaffected. These different outcomes can partly be explained by genetic variation. Compelling evidence suggests that both PTSD and MDD have a heritable component, with estimates ranging from 0.28 to 0.46 (Stein et al., 2016) and from 0.31 to 0.42 (Sullivan et al., 2000), respectively. However, it is currently unknown to what extent genetic risk interacts with traumatic experiences in increasing the risk for MDD or PTSD.

Recently, the largest genome-wide association studies (GWASs) to date were published for both PTSD (Stein et al., 2016) and MDD (Hyde et al., 2016), aiming to elucidate the role of common genetic variation in these disorders. Results from these studies provide the best estimates of genetic risk for these disorders currently available and can be used to generate polygenic risk scores (PRSs) in other, independent, cohorts (Wray et al., 2014). The use of PRSs for PTSD and MDD in a large longitudinal cohort exposed to trauma may further elucidate the interaction of genetic vulnerability and trauma exposure on the development of psychopathology and could be of value to predict vulnerability.

Here, we hypothesized that genetic susceptibility for PTSD and MDD (as indicated by higher PRSs) in interaction with trauma exposure during military deployment determines the degree to which PTSD and MDD symptoms become manifest in a period of up to five years after deployment. In addition, we explored relations of PRSs with symptom levels prior to deployment and the interaction of PRSs with childhood trauma on pre- and the development of post-deployment symptom levels.

2. METHODS

2.1. Participants and general procedure

Participants of the present study were military deployed to Afghanistan for 4 months between 2005 and 2008, either as part of a Provincial Reconstruction Team or as part of the International Security Assistance Force see outlined in (Reijnen et al., 2015; Van Zuiden et al., 2011). All 1,032 participants gave both written and oral informed consent. Assessments and data collection took place prior to, as well as one month, six months and one, two and five years after deployment. A previous study provides general information about common duties and deployment-related potentially traumatic events (Reijnen et al., 2015). Study approval was granted by the University Medical Center Utrecht (UMCU) Institutional Review Board.

2.2. Genetic data

DNA isolated from blood of 1,015 participants was genotyped on the Illumina Human OmniExpress-24 v1.1 BeadChip array. A total number of 713,040 markers was present on this chip. Data underwent extensive quality control (see Supplementary Tables S1 and

S2). First, subjects were removed when they had a genotyping rate < 0.95, discordant genetic and phenotypic sex, heterozygosity rate over 3 standard deviations from the mean and a relatedness coefficient (PI-HAT) > 0.1 (one of a pair of related individuals was removed at random if this were the case). Genetic markers were removed when they were non-autosomal, their genotyping rate was < 0.95, allele frequencies did not meet Hardy-Weinberg Equilibrium (p-value in HWE test < 1×10^{-6}), had an ambiguous AT/CG genotype, were not present in the reference panel for imputation, or had a difference in minor allele frequency compared to the reference panel exceeding 0.15. A total number of 963 individuals and 675,453 remained after quality control.
We imputed the data on the Michigan Imputation Server (Das et al., 2016) using the Haplotype Reference Consortium Release 1.1 (McCarthy et al., 2016) as a reference panel and Eagle version 2.3 as a phasing algorithm (Loh et al., 2016). A total number of 39,127,565 SNPs was available after imputation. We applied another round of quality control, where SNPs were removed from the imputed data when they had a minor allele frequency < 0.01, imputation R^2 (INFO score) < 0.3, minor allele frequency difference from the reference panel exceeding 0.15, or ambiguous AT/CG genotype. A total of 6,414,953 SNPs remained for analyses. We extracted genotype dosages from the imputed data for individuals with a Central European genetic ancestry according to the first two principal components of the genetic data (see Supplementary Figure S1). We only investigated individuals with European ancestry (constituting the vast majority of our total sample), as the GWAS by Stein et al. has shown that effects of common genetic variance are population-specific (Stein et al., 2016).

2.3. Polygenic risk score calculation

We obtained summary statistics from the largest GWASs on PTSD (2,812 patients and 6,244 controls of European descent) (Stein et al., 2016) and MDD (121,380 cases and 338,101 controls, 97% of European descent) (Hyde et al., 2016). We ruled out sample overlap between our current dataset and these GWAS cohorts.
For the PTSD GWAS dataset (Stein et al., 2016), we performed a meta-analysis of three cohorts consisting of individuals of European ancestry described in the paper using METAL (release 25-03-2011) (Willer et al., 2010), in order to include as many samples as possible and thereby increase power of the discovery dataset (see Supplementary Figure S2). We excluded SNPs with imputation INFO scores < 0.3. For the MDD GWAS, we removed SNPs from the GWAS summary statistics which were marked by the authors as not passing QC. This included SNPs with an average INFO score < 0.5 and a minimum INFO score < 0.3 across cohorts included in their meta-analysis. For both GWAS datasets, we extracted marker names, effect alleles, effect sizes and association p-values. We retained only SNPs that were also present in our imputed dataset. Prior to PRS calculation, we removed linkage disequilibrium (LD) from the GWAS datasets using a clumping procedure in PLINK version 1.90b3z (Chang et al., 2015), thereby removing all correlated SNPs in a genetic window except for the SNP with the lowest association p-value (a first round applying a genetic window of 250 kilobase and an LD $R^2 > 0.5$, and a second round applying a genetic window of 5000 kb and an LD $R^2 > 0.2$). We used the phase 1 integrated release of the 1000 genomes consortium to calculate LD structures (Abecasis et al., 2012). Using the score function in PLINK, we calculated PRS for both PTSD and MDD based on

13 sets of SNPs selected on different association p-value thresholds (P_T): $< 5 \times 10^{-8}$, $< 5 \cdot 10^{-7}$, $< 5 \times 10^{-6}$, $< 5 \times 10^{-5}$, $< 5 \times 10^{-4}$, $< 5 \times 10^{-3}$, < 0.05, < 0.1, < 0.2, < 0.3, < 0.4, < 0.5 and all). For PTSD, we were only able to calculate PRS for the latter ten P_Ts, as an insufficient number of SNPs was available for the most stringent P_Ts.

2.4. Psychopathology outcomes

PTSD symptoms were measured prior to and one month, six months and one, two and five years after deployment, whereas for depressive symptoms all but the five year post-deployment measurement were available. PTSD and depressive symptoms were evaluated using the Self-Report Inventory for PTSD (SRIP) (Hovens et al., 2002) and the depression subscale of the symptom checklist-90 (SCL-90) (Arrindell et al., 2003), respectively. The correlation of change in depressive symptoms and PTSD symptoms was significant from pre- to post-deployment (one month: Pearson's rho (ρ) = 0.11, p = 0.03; six months: ρ = 0.41, p = 1.3×10^{-15}; one year: $\rho = 0.62$, $p < 2.2 \times 10^{-16}$; two years: $\rho = 0.56$, $p < 2.2 \times 10^{-16}$). A self-report checklist described in a previous study (Reijnen et al., 2015) was used to assess exposure to potentially traumatic events, including direct combat. The self-report Early Trauma Inventory was used to assess potentially traumatic events during childhood (subdivided in physical, sexual and emotional abuse (Bremner et al., 2007)).

2.5. Statistical analyses

Paired two-tailed t-tests were used to compare pre- and post-deployment psychopathology symptom levels.

No gene-environment effects of PTSD or MDD PRSs on trauma during deployment (or childhood trauma) were found in linear models (e.g. *trauma during deployment* ~ PRS $PTSD_{PT\ 0.05}$, all p-values > 0.05).

For primary analyses, linear regression models were used with post-deployment PTSD symptom levels as the dependent variable and as indicators: pre-deployment PTSD symptom levels, a PRS for PTSD (10 different P_TS, see above), trauma during deployment, childhood trauma, sex, age, and the five first principal components (adjusting for ethnicity) (e.g. $PTSD\ symptoms_{post\_six\_months} \sim PTSD\ symptoms_{pre} + PRS\ PTSD_{PT\ 0.05} + trauma\ during\ deployment + childhood\ trauma + sex + age + PC1 + PC2 + PC3 + PC4 + PC5$). Subsequently, the interaction of the PTSD PRSs with trauma exposure during deployment was added to the model. Identical models were used for depressive symptoms and MDD PRSs (13 P_TS). In secondary analyses, we examined the association of PRSs with pre-deployment psychopathology levels and the interaction of PRSs and childhood trauma on pre- and the development of post-deployment psychopathology levels.

Homogeneity of variance, absence of outliers (Cook's Distance < 1) and normal distribution of the residuals were confirmed by inspecting plots of the main models.

To correct for multiple testing, significance threshold was set at 0.05 / 18 = 0.0028 to adjust for analyses of five time points for PTSD symptoms and four for depressive symptoms using two models (one with only main and one including interaction).

3. RESULTS

3.1. General

Table 1 lists sample characteristics, including age, sex, number of traumatic events during military deployment and childhood, number of previous deployments, and pre- and post-deployment levels of PTSD and depressive symptoms. PTSD symptoms were significantly higher one ($p < 0.001$) and six ($p = 0.03$) months and five years ($p = 0.002$) after deployment compared to pre-deployment, but not one ($p = 0.95$) and two ($p = 0.21$) years after deployment. In contrast, depressive symptoms were significantly higher one ($p = 0.008$) and two years ($p = 0.0001$) post-deployment, whereas one month ($p = 0.13$) and six months ($p = 0.07$) post-deployment no significant increases were present compared to pre-deployment depressive symptoms (see Table 1).

3.2. PTSD symptoms

The development of post-deployment PTSD symptoms at any time point (one month, six months and one, two and five years) was not associated with PTSD PRSs at any P_T. In addition, there were no significant interaction effects of PTSD PRSs with trauma during deployment on the development of PTSD symptoms after deployment (see Table 2, all p-values > 0.03). The added explained variance of PTSD PRSs in the main models was very low or absent (e.g. P_T 0.05 included as main effect, with PTSD symptoms six months post-deployment as the dependent variable: $R^2 = 0.2563$; P_T 0.05 excluded from the model: $R^2 = 0.2572$).

In secondary analyses, PTSD PRSs were not associated with pre-deployment PTSD symptoms (neither direct nor in interaction with childhood trauma; see Supplementary Table S3, all p-values > 0.05). In addition, there were no significant interactions of PTSD PRSs with childhood trauma on the development of post-deployment PTSD symptoms at any time point (see Supplementary Table S4, all p-values > 0.01).

3.3. Depressive symptoms

No significant main or interaction effects with deployment-related trauma were observed for the MDD PRSs at any P_T on the development of depressive symptoms at any time point after deployment (see Table 3, all p-values > 0.005). Similar to PTSD, MDD PRSs added minimally or not at all to the explained variance of the main models (e.g. P_T 0.05 included as main effect, with depressive symptoms six months post-deployment as the dependent variable: $R^2 = 0.3034$; P_T 0.05 excluded from the model: $R^2 = 0.3051$).

In secondary analyses, PRSs for MDD were not associated with baseline depressive symptoms (neither main nor in interaction with childhood trauma; see Supplementary Table S3, all p-values > 0.05). Furthermore, there were no interaction effects of MDD PRSs and childhood trauma on the development of post-deployment depressive symptoms at any time point (see Supplementary Table S5, all p-values > 0.02).

Age (mean, SD)		29.5 (9.3)			
Sex (% male)		91.5			
Number of deployment-related trauma events (mean, SD)		4.6 (3.2)			
Number of early trauma events (mean, SD)		3.3 (3.0)			
Number of previous deployments (mean, SD)		0.9 (1.2)			
	Pre-deployment	*One month post-deployment*	*t*	*p*	*n*
PTSD symptoms (SRIP) (mean, SD)	26.8 (4.9)	27.7 (5.9)	-3.71	*< 0.001*	421
Depressive symptoms (SCL-90 depression subscale) (mean, SD)	17.9 (3.0)	18.1 (3.7)	-1.50	0.13	486
	Pre-deployment	*Six months post-deployment*	*t*	*p*	*n*
PTSD symptoms (SRIP) (mean, SD)	26.7 (4.8)	27.4 (6.8)	-2.22	*0.03*	372
Depressive symptoms (SCL-90 depression subscale) (mean, SD)	17.9 (3.0)	18.2 (4.0)	-1.80	0.07	425
	Pre-deployment	*One year post-deployment*	*t*	*p*	*n*
PTSD symptoms (SRIP) (mean, SD)	26.8 (5.1)	26.8 (6.8)	-0.06	0.95	296
Depressive symptoms (SCL-90 depression subscale) (mean, SD)	18.2 (3.4)	19.0 (5.6)	*-2.67*	*0.008*	*342*
	Pre-deployment	*Two years post-deployment*	*t*	*p*	*n*
PTSD symptoms (SRIP) (mean, SD)	26.7 (5.1)	26.3 (5.6)	1.24	0.21	266
Depressive symptoms (SCL-90 depression subscale) (mean, SD)	17.8 (2.8)	19.0 (5.4)	*-3.91*	*0.0001*	*328*
	Pre-deployment	*Five years post-deployment*	*t*	*p*	*n*
PTSD symptoms (SRIP) (mean, SD)	26.9 (5.2)	28.1 (7.2)	*-3.07*	*0.002*	*290*

Table 1. Sample characteristics (total n = 516).

P_T PRSs	Total SNPs PTSD	One month post-deployment (N = 421)				Six months post-deployment (N = 372)				One year post-deployment (N = 296)				Two years post-deployment (N = 266)				Five years post-deployment (N = 290)			
		Main effect		PRS · deployment trauma		Main effect		PRS · deployment trauma		Main effect		PRS · deployment trauma		Main effect		PRS · deployment trauma		Main effect		PRS · deployment trauma	
		p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β
$< 5 \cdot 10^{-5}$	82	0.90	0.0301	0.14	0.1230	0.78	-0.0905	0.87	0.0166	0.40	-0.3070	0.26	0.1385	0.49	-0.2118	0.21	0.1239	0.14	0.5522	0.85	0.0227
$< 5 \cdot 10^{-4}$	668	0.49	0.0618	0.39	-0.0242	0.46	0.0834	0.84	-0.0072	0.20	-0.1755	0.16	-0.0650	*0.04*	*-0.2399*	0.84	-0.0084	0.24	0.1647	0.95	-0.0029
$< 5 \cdot 10^{-3}$	6,015	0.87	-0.0055	0.41	0.0087	0.82	-0.0094	0.88	-0.0020	0.34	-0.0477	0.87	-0.0027	0.47	-0.0316	0.93	-0.0014	0.34	-0.0506	0.16	-0.0249
< 0.05	49,159	1.00	-0.0000	0.14	0.0051	0.44	-0.0114	0.62	0.0021	0.61	-0.0087	0.78	-0.0015	0.72	-0.0053	0.74	0.0016	0.97	-0.0007	0.78	-0.0016
< 0.1	91,254	0.75	-0.0028	0.12	0.0040	0.38	-0.0099	0.66	0.0014	0.58	-0.0072	0.57	-0.0024	0.71	-0.0042	0.87	0.0006	0.95	0.0009	0.80	0.0010
< 0.2	164,982	0.99	-0.0001	0.06	0.0038	0.86	-0.0016	0.59	0.0014	0.66	0.0046	0.83	0.0007	0.51	0.0060	0.55	0.0017	0.63	0.0053	0.47	0.0023
< 0.3	229,984	0.95	-0.0004	0.07	0.0033	0.97	-0.0003	0.67	0.0010	0.78	0.0027	0.91	0.0003	0.58	0.0046	0.58	0.0015	0.73	0.0035	0.61	0.0015
< 0.4	289,089	0.80	-0.0015	0.14	0.0025	0.90	-0.0010	0.85	0.0004	0.89	0.0013	0.87	-0.0004	0.73	0.0027	0.68	0.0010	0.85	0.0018	0.67	0.0012
< 0.5	341,462	0.87	-0.0009	0.15	0.0024	0.79	-0.0020	0.93	0.0002	0.92	0.0009	0.94	-0.0002	0.81	0.0018	0.71	0.0009	0.81	0.0023	0.67	0.0011
All	509,999	0.91	-0.0007	0.19	0.0021	0.76	-0.0022	0.96	0.0001	1.00	0.0000	0.81	-0.0006	0.76	0.0023	0.71	0.0009	0.78	0.0025	0.77	0.0008

Table 2. PTSD PRSs at all significance thresholds (P_TS) in relation to the development of PTSD symptoms after deployment.

P_T PRSs	Total SNPs PTSD	One month post-deployment (N = 486)				Six months post-deployment (N = 425)				One year post-deployment (N = 342)				Two years post-deployment (N = 328)			
		Main effect		PRS · deployment trauma		Main effect		PRS · deployment trauma		Main effect		PRS · deployment trauma		Main effect		PRS · deployment trauma	
		p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β
$< 5 \cdot 10^{-8}$	14	0.30	-1.2534	*0.01*	*-0.9419*	0.22	-1.8042	0.15	-0.6000	0.51	-1.7848	0.51	-0.5461	0.70	1.0288	0.67	-0.3447
$< 5 \cdot 10^{-7}$	43	0.12	-1.0462	*0.01*	*-0.5004*	0.24	-0.9515	0.15	-0.3336	0.85	0.2841	0.93	0.0403	0.50	0.9685	0.96	0.0223
$< 5 \cdot 10^{-6}$	110	0.28	-0.4638	0.07	-0.2422	0.38	-0.4595	0.13	-0.2508	0.62	-0.4793	0.93	-0.0278	0.53	0.5770	0.66	0.1304
$< 5 \cdot 10^{-5}$	376	0.06	-0.4869	*0.04*	*-0.1635*	0.65	-0.1472	0.36	-0.0909	0.83	-0.1248	0.93	0.0171	0.57	0.3220	0.80	-0.0480
$< 5 \cdot 10^{-4}$	1,779	0.09	-0.2375	0.44	-0.0341	0.14	-0.2530	0.40	-0.0456	0.31	-0.3107	0.77	-0.0297	0.89	0.0397	0.98	-0.0020
$< 5 \cdot 10^{-3}$	10,222	0.76	-0.0197	0.78	-0.0058	0.79	-0.0210	0.66	-0.0110	0.84	0.0282	0.84	-0.0086	0.16	0.1861	0.49	-0.0294
< 0.05	61,859	0.86	0.0053	0.41	-0.0075	0.99	-0.0004	0.52	-0.0073	0.14	0.0966	0.69	-0.0081	0.10	0.1005	0.42	-0.0154
< 0.1	106,827	0.84	-0.0045	0.15	-0.0103	0.90	0.0036	0.61	-0.0046	0.11	0.0826	0.38	-0.0141	0.17	0.0669	0.16	-0.0214
< 0.2	183,941	0.88	0.0027	0.33	-0.0057	0.61	0.0117	0.78	-0.0020	0.15	0.0618	0.52	-0.0085	0.38	0.0355	0.23	-0.0150
< 0.3	250,878	0.90	0.0021	0.21	-0.0067	0.71	0.0077	0.78	-0.0018	0.07	0.0708	0.40	-0.0101	0.36	0.0335	0.27	-0.0124
< 0.4	310,698	0.93	0.0014	0.33	-0.0049	0.77	0.0058	0.80	-0.0016	0.07	0.0664	0.53	-0.0071	0.41	0.0285	0.35	-0.0101
< 0.5	364,011	0.85	0.0031	0.31	-0.0051	0.63	0.0093	0.76	-0.0019	0.07	0.0663	0.45	-0.0084	0.45	0.0250	0.34	-0.0099
All	543,671	0.81	0.0038	0.34	-0.0047	0.67	0.0080	0.77	-0.0018	0.07	0.0636	0.40	-0.0091	0.47	0.0233	0.37	-0.0093

Table 3. MDD PRSs at all significance thresholds (P_TS) in relation to the development of depressive symptoms after deployment.

4. DISCUSSION

In a large prospective military cohort, we calculated PRSs derived from the largest GWASs on PTSD and MDD to date to investigate both main effects of genetic vulnerability and the interaction with deployment-related trauma on the development of PTSD and depressive symptoms at five time points over five years after deployment to Afghanistan. In secondary analyses, we examined the association of these PRSs with baseline depressive and PTSD symptoms and their interaction with childhood trauma. We did not find significant main or interaction effects of PRSs on PTSD or depressive symptoms at any time point after deployment. In addition, there were no significant associations with baseline symptom levels and no interactions with childhood trauma on pre- and the development of post-deployment symptoms levels.

4.1. PTSD PRSs

The absence of evidence of a relation between PTSD PRSs and our outcomes may have several explanations. First, the SNP-based heritability in the European descent subsample of the original study was not significant (Stein et al., 2016), indicating that our PRSs may have been insufficiently refined to discover significant associations. Second, this GWAS (Stein et al., 2016) was carried out in trauma-exposed controls, thereby excluding genetic variation associated with liability to exposure to trauma. Exposure to trauma is itself moderately heritable (True et al., 1993), which is possibly mediated by specific personality traits (e.g. harm avoidance and/or novelty seeking). As such, the PTSD PRSs do not include genetic variation associated with PTSD through specific personality traits, whereas our military cohort was not selected on these traits. This may have contributed to a difference in genetic background of PTSD in the discovery sample (Stein et al., 2016) compared with our target sample. Lastly, PTSD in the majority of the discovery sample had been caused by pre-military traumatic events, as only 24% of the soldiers had been deployed and most participants were in the age range of 18-20 years (Stein et al., 2016). Of note, PTSD caused by childhood trauma may have a different genetic profile than PTSD caused by deployment-related trauma.

4.2. MDD PRSs

The lack of significant findings with MDD PRSs is noteworthy given the results of two previous studies confirming the validity of an MDD PRS based on a maximum of 9,240 cases and 9,519 controls (Ripke et al., 2013) in two different populations (Musliner et al., 2015; Peyrot et al., 2014). Peyrot et al. (Peyrot et al., 2014) found both main and interaction effects of the MDD PRS and childhood trauma on risk for MDD in a study sample of 1,645 patients with MDD and 340 controls. In addition, Musliner et al. (Musliner et al., 2015) found a main effect of the MDD PRS and stressful life events on depressive symptoms in a population of 8,761 mainly older adults. The MDD PRSs in the present study were based on over 120,000 cases and 337,000 controls. This would expectedly lead to more valid PRSs than the ones based on the previous, much smaller GWAS (Ripke et al., 2013), even though depressive symptoms in the recent GWAS (Hyde et al., 2016) were ascertained using self-report. In contrast to PTSD, the SNP-based heritability in the MDD GWAS (Hyde et al., 2016) was significant and estimated at 5.9-6.9%, indicating better validity of

these data compared to the PTSD GWAS (Stein et al., 2016). Possibly, the null finding in the present study, with very low or no added explained variances of the PRSs in our main models, was due to a mismatch in depressive phenotypes of the military cohort after deployment versus patients with a depressive episode in the general population. More specific phenotyping of heterogeneous disorders such as MDD and PTSD in both the discovery and target cohorts may be effective in this regard, as some studies have shown that this increases power to find genome-wide significant associations (CONVERGE consortium, 2015; Milaneschi et al., 2016).

4.3. Strengths and limitations

The longitudinal design around military deployment, with psychopathology outcomes at six time points and quantification of deployment-related trauma, is the main strength of this study. Importantly, the MDD PRSs were based on the largest GWAS in psychiatry thus far, thereby providing a far better estimate for genetic risk to develop MDD than the second largest MDD GWAS (Ripke et al., 2013).

The relatively healthy population represents this study's most important limitation. The increase in PTSD and depressive symptoms after deployment was small and at some time points non-significant, probably hampering the power in our analyses. Furthermore, self-report questionnaires were used to evaluate symptom severity, which may have led to some social desirability bias. Moreover, trauma exposure questionnaires did not take subjective experience of these events into account, while this subjective experience is probably more important than the actual event (Conway et al., 2012). In addition, the present sample size may have been insufficient. This issue, however, is partly compensated for by the longitudinal design. Lastly, as mentioned above, the PRSs of PTSD were based on a relatively small sample (Stein et al., 2016), compromising their validity.

4.4. Future directions

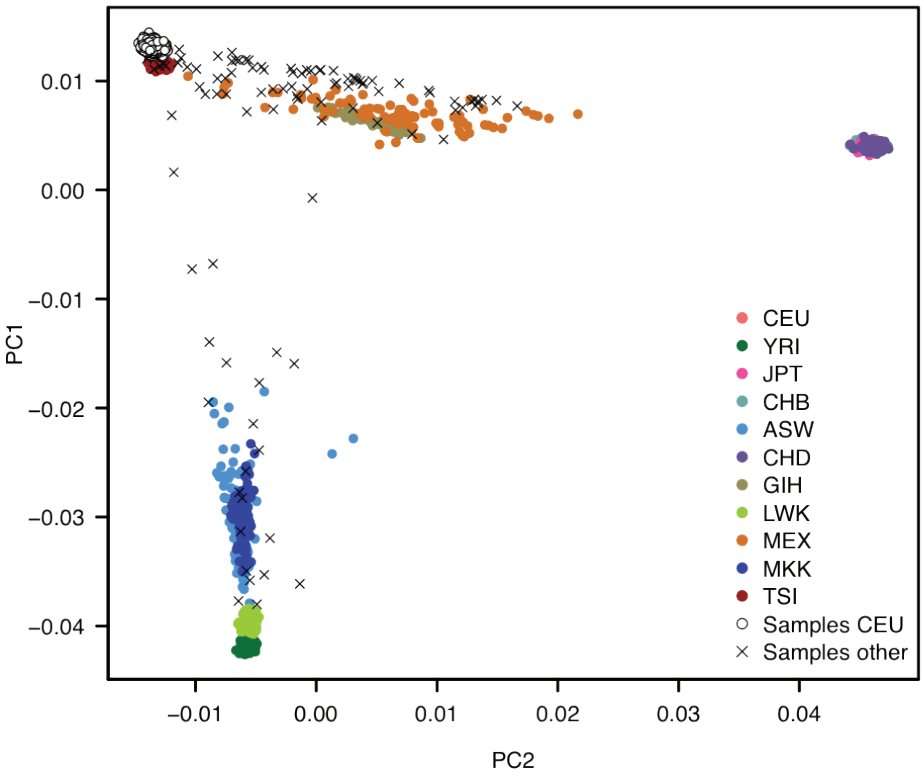
Future studies may further shed light upon the validity of MDD PRSs in non-clinical and clinical samples. For PTSD, a large GWAS by the Psychiatric Genomics Consortium (PGC) is likely to be published within a year (Logue et al., 2015), which may yield more refined PTSD PRSs than the ones used here. In addition to increasing sample sizes, the focus in GWASs may need to be shifted towards more specific (Milaneschi et al., 2016) and/or more severe (CONVERGE consortium, 2015) phenotypes. As such, PRSs may be most informative about etiology and may ultimately be useful for prediction purposes (Smoller, 2016).

4.5. Conclusions

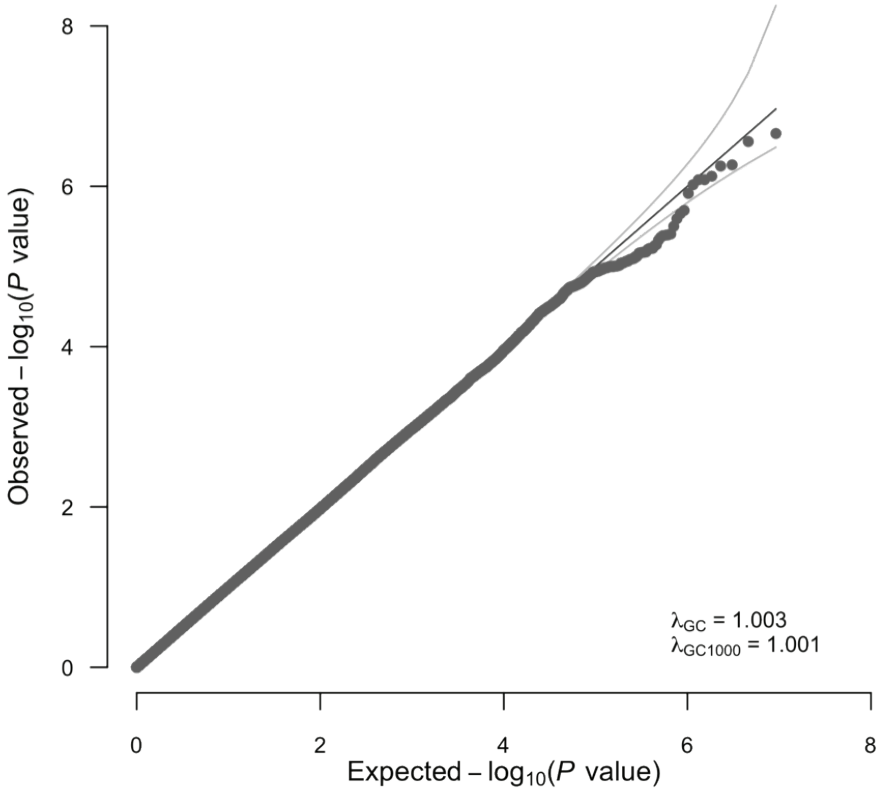
In conclusion, we used polygenic risk scores derived from the largest GWASs for PTSD and MDD so far to investigate the interaction of genetic vulnerability with potentially traumatic events on the development of these disorders in a prospective military cohort deployed to a combat zone. Our results indicate the limited validity of PTSD and MDD PRSs in this relatively healthy military population and highlight the importance of etiological heterogeneity of these disorders. Future studies investigating common genetic risk for PTSD will benefit from increased sample sizes in GWASs, but for both MDD and PTSD phenotype refinement may be of crucial importance for progress in this field.

CONFLICT OF INTEREST
This study was funded by a grant from the Dutch Ministry of Defence.
Funders had no role in design and reporting of the study. All authors reported no biomedical financial interests or potential conflicts of interest.

SUPPLEMENTARY INFORMATION



Supplementary Figure S1. Population structure of the cohort and selection of individuals with a Central-European ancestry. Principal components were calculated from the genetic data and the first two principal components are plotted on a HapMap3 background. Colored dots indicate HapMap3 individuals, white dots indicate individuals with a Central-European ancestry from our cohort that were selected for polygenic risk score analyses, crosses indicate individuals in our cohort that do not have a Central-European genetic ancestry and were excluded from analyses. HapMap3 populations: CEU, Utah residents with Northern and Western European ancestry; YRI, Yoruba in Ibadan (Nigeria); JPT, Japanese in Tokyo; CHB, Han Chinese in Beijing; ASW, African ancestry in Southwest USA; CHD, Chinese in Metropolitan Denver (Colorado); GIH, Gujarati Indians in Houston (Texas); LWK, Luhya in Webuye (Kenya); MEX, Mexican ancestry in Los Angeles (California); MKK, Maasai in Kinyawa (Kenya); TSI, Toscani in Italy.



Supplementary Figure S2. Quantile-quantile plot of meta-analysis on three European ancestry cohorts from the Stein et al. PTSD GWAS. The observed $-\log_{10}$(p-value) of SNPs in the meta-analysis is plotted against the expected $-\log_{10}$(p-value) under the assumption of null inflation of SNP p-values. λ_{GC} indicates the inflation of p-values from the expected null, and λ_{GC1000} the inflation scaled to a study of 1000 cases and 1000 controls.

SNP-LEVEL QUALITY CONTROL STEPS	Number of SNPs
SNPs pre-QC	713,014
SNPs with genotyping rate < 0.95	4,340
SNPs HWE test p < 10^{-6}	2,178
AT/CG SNPs	2,412
Non-autosomal SNPs	19,485
SNPs not in HRC imputation reference panel	6,548
SNPs discordant with HRC	2,096
SNPs MAF difference HRC > 0.15	427
Observed MAF vs. HRC (R^2)	0.984
SNPs post-QC	675,453

INDIVIDUAL-LEVEL QUALITY CONTROL STEPS	Number of individuals
Individuals pre-QC	1,015
Males pre-QC	926
Females pre-QC	89
Individuals genotyping rate < 0.95	10
Individuals discordant sex	4
Individuals heterozygosity rate > +/- 3SD from mean	31
Related pairs of individuals with pi-hat > 0.10	8
Individuals post-QC	1,015
Males post-QC	877
Females post-QC	86
Overall genotyping rate pre-QC	0.995
Overall genotyping rate post-QC	0.999

Abbreviations: QC, Quality control; SNP: Single-nucleotide polymorphism; HWE, Hardy-Weinberg Equilibrium; MAF, Minor allele frequency; HRC: Haplotype Reference Consortium.

Supplementary Table S1. Overview of pre-imputation quality control of genetic data.

POST-IMPUTATION QUALITY CONTROL STEPS	Number of SNPs
SNPs pre-imputation	675,453
SNPs post-imputation	39,127,565
SNPs imputation $r^2 < 0.3$	18,048,435
SNPs MAF < 0.01	31,285,207
AT/CG SNPs	5,835,756
SNPs post-QC	6,414,953

Supplementary Table S2. Overview of post-imputation quality control of genetic data. Abbreviations: QC, Quality control; SNP: Single-nucleotide polymorphism; MAF, Minor allele frequency.

	Total	PTSD PRS in relation to baseline PTSD symptoms (N = 590)				Total	MDD PRS in relation to baseline depressive symptoms (N = 705)			
	SNPs	Main effect		PRS · early trauma		SNPs	Main effect		PRS · early trauma	
P_T PRSs	PTSD	p	β	p	β	PTSD	p	β	p	β
$< 5 \cdot 10^{-8}$	0	NA	NA	NA	NA	14	0.81	-0.2607	0.22	-0.4545
$< 5 \cdot 10^{-7}$	0	NA	NA	NA	NA	43	0.90	-0.0780	0.24	-0.2599
$< 5 \cdot 10^{-6}$	0	NA	NA	NA	NA	110	0.63	-0.1848	0.24	-0.1541
$< 5 \cdot 10^{-5}$	82	0.88	0.0339	0.95	0.0053	376	0.95	-0.0144	0.97	-0.0034
$< 5 \cdot 10^{-4}$	668	0.14	0.1217	0.85	-0.0058	1,779	0.97	-0.0050	0.79	-0.0112
$< 5 \cdot 10^{-3}$	6,015	0.75	-0.0095	0.75	0.0032	10,222	0.83	-0.0122	0.70	-0.0075
< 0.05	49,159	0.69	0.0041	0.10	-0.0061	61,859	0.66	0.0111	0.23	-0.0098
< 0.1	91,254	0.73	0.0028	0.06	-0.0055	106,827	0.58	0.0110	0.24	-0.0077
< 0.2	164,982	0.63	0.0030	0.19	-0.0030	183,941	0.89	0.0023	0.31	-0.0054
< 0.3	229,984	0.47	0.0041	0.12	-0.0032	250,878	0.94	0.0011	0.31	-0.0049
< 0.4	289,089	0.50	0.0036	0.08	-0.0035	310,698	0.96	0.0008	0.29	-0.0049
< 0.5	341,462	0.52	0.0033	0.10	-0.0032	364,011	0.98	-0.0003	0.29	-0.0048
All	509,999	0.55	0.0030	0.12	-0.0029	543,671	0.94	-0.0010	0.23	-0.0053

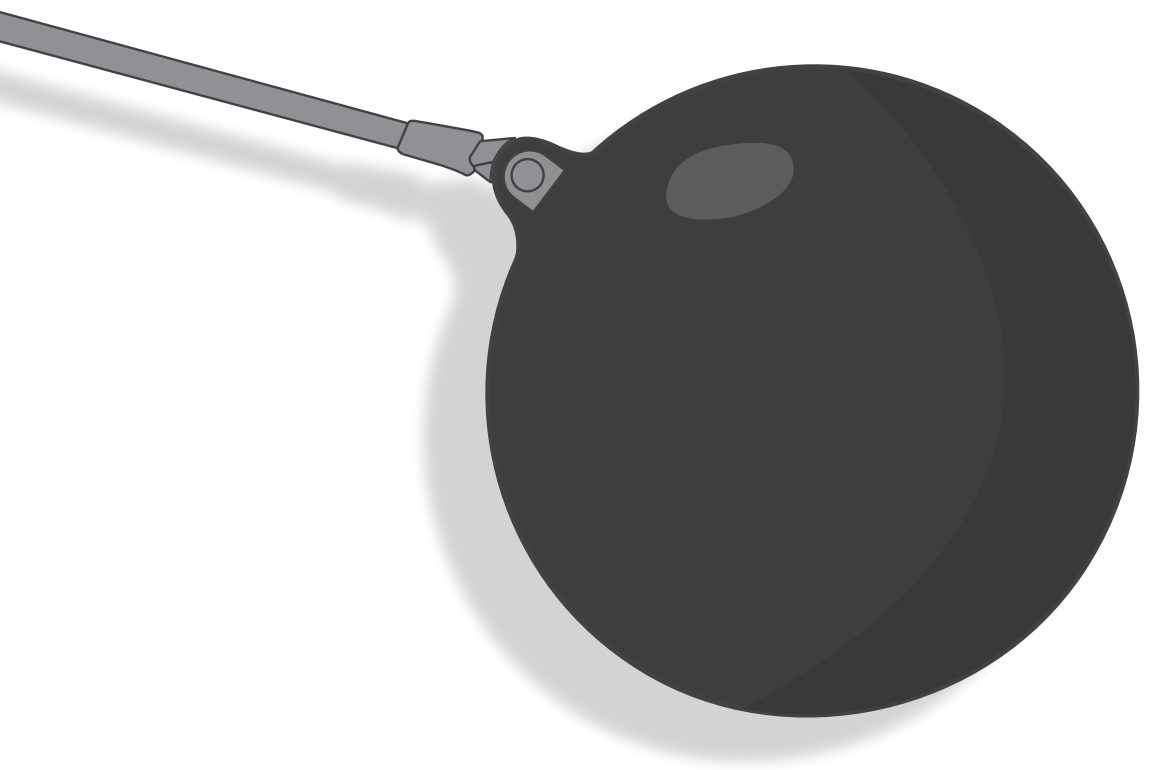
Supplementary Table S3. PRSs at all significance thresholds (P_TS) in relation to pre-deployment PTSD and depressive symptoms.

P_T PRSs	Total SNPs PTSD	One month post-deployment (N = 421)		Six months post-deployment (N = 372)		One year post-deployment (N = 296)		Two years post-deployment (N = 266)		Two years post-deployment (N = 290)	
		PRS · childhood trauma		PRS · childhood trauma		PRS · childhood trauma		PRS · childhood trauma		PRS · childhood trauma	
		p	β	p	β	p	β	p	β	p	β
$< 5 \cdot 10^{-5}$	82	0.57	0.0498	0.46	-0.0794	0.81	0.0313	0.68	-0.0415	0.85	-0.0252
$< 5 \cdot 10^{-4}$	668	0.64	-0.0155	0.43	-0.0338	0.31	-0.0514	*0.02*	*-0.0986*	0.85	0.0098
$< 5 \cdot 10^{-3}$	6,015	0.28	-0.0118	0.79	-0.0037	0.66	0.0069	0.78	-0.0036	*0.03*	*0.0361*
< 0.05	49,159	0.47	0.0031	0.91	0.0006	0.91	-0.0007	0.52	-0.0034	0.64	0.0030
< 0.1	91,254	0.50	0.0023	0.67	0.0018	0.56	-0.0030	0.63	-0.0020	0.88	0.0008
< 0.2	164,982	0.22	0.0034	0.23	0.0042	0.61	-0.0021	0.64	0.0017	0.98	-0.0001
< 0.3	229,984	0.31	0.0025	0.13	0.0047	0.78	-0.0010	0.65	0.0015	0.87	0.0006
< 0.4	289,089	0.34	0.0022	0.17	0.0040	0.61	-0.0017	0.97	-0.0001	0.94	0.0003
< 0.5	341,462	0.25	0.0026	0.27	0.0032	0.56	-0.0020	0.96	-0.0002	0.99	0.0000
All	509,999	0.34	0.0021	0.30	0.0029	0.72	-0.0012	1.00	0.0000	0.96	-0.0002

Supplementary Table S4. The interaction of PTSD PRSs at all significance thresholds (P_TS) with childhood trauma on the development of PTSD symptoms after deployment.

P_T PRSs	Total SNPs PTSD	One month post-deployment (N = 486)		Six months post-deployment (N = 425)		One year post-deployment (N = 342)		Two years post-deployment (N = 328)	
		PRS · childhood trauma		PRS · childhood trauma		PRS · childhood trauma		PRS · childhood trauma	
		p	β	p	β	p	β	p	β
$< 5 \cdot 10^{-8}$	14	0.88	-0.0678	0.96	-0.0270	0.74	-0.3211	0.13	-1.3520
$< 5 \cdot 10^{-7}$	43	0.98	-0.0073	0.89	0.0463	0.81	0.1487	0.91	-0.0657
$< 5 \cdot 10^{-6}$	110	0.64	-0.0742	0.42	-0.1575	0.48	-0.2463	0.44	-0.2489
$< 5 \cdot 10^{-5}$	376	0.56	0.0537	0.63	0.0521	0.30	0.1929	0.67	0.0737
$< 5 \cdot 10^{-4}$	1,779	0.85	-0.0096	0.58	-0.0322	0.96	0.0055	0.42	-0.0736
$< 5 \cdot 10^{-3}$	10,222	0.26	0.0251	0.73	0.0091	*0.02*	*0.1056*	0.26	0.0500
< 0.05	61,859	0.40	0.0085	0.44	0.0088	0.15	0.0293	0.41	0.0154
< 0.1	106,827	0.43	0.0066	0.36	0.0083	0.21	0.0020	0.52	0.0095
< 0.2	183,941	0.40	0.0056	0.41	0.0059	0.37	0.0117	0.85	-0.0022
< 0.3	250,878	0.52	0.0039	0.62	0.0033	0.43	0.0094	0.80	-0.0027
< 0.4	310,698	0.56	0.0034	0.66	0.0027	0.47	0.0082	0.82	-0.0024
< 0.5	364,011	0.60	0.0030	0.60	0.0032	0.45	0.0082	0.92	-0.0010
All	543,671	0.70	0.0021	0.67	0.0025	0.51	0.0070	0.94	-0.0008

Supplementary Table S5. The interaction of MDD PRSs at all significance thresholds (P_TS) with childhood trauma on the development of depressive symptoms after deployment.



CHAPTER 8

General discussion

SUMMARY

In this thesis we investigated (traumatic) stress and psychopathology with a focus on the potential roles of the HPA axis (PART 1), the GABA system (PART 2), their association (PART 3) and the general genetic background of major depressive disorder (MDD) and posttraumatic stress disorder (PTSD) (PART 4). Figure 1.3 (reiterated below) recapitulates the sub-questions we addressed in the different chapters. In part 1, chapters 2 and 3 investigated HPA axis parameters in relation to psychopathology (and traumatic stress in chapter 3). In part 2, chapters 4 and 5 focused on the role of the GABA system in psychopathology (and traumatic stress in chapter 5). Chapter 6, in part 3, was aimed at analyzing the association between the HPA axis and the GABA system in shaping the coordinated stress response. Finally, in part 4 (chapter 7) we explored the interaction between trauma exposure during deployment and common genetic variation underlying MDD and PTSD on the development of these disorders.

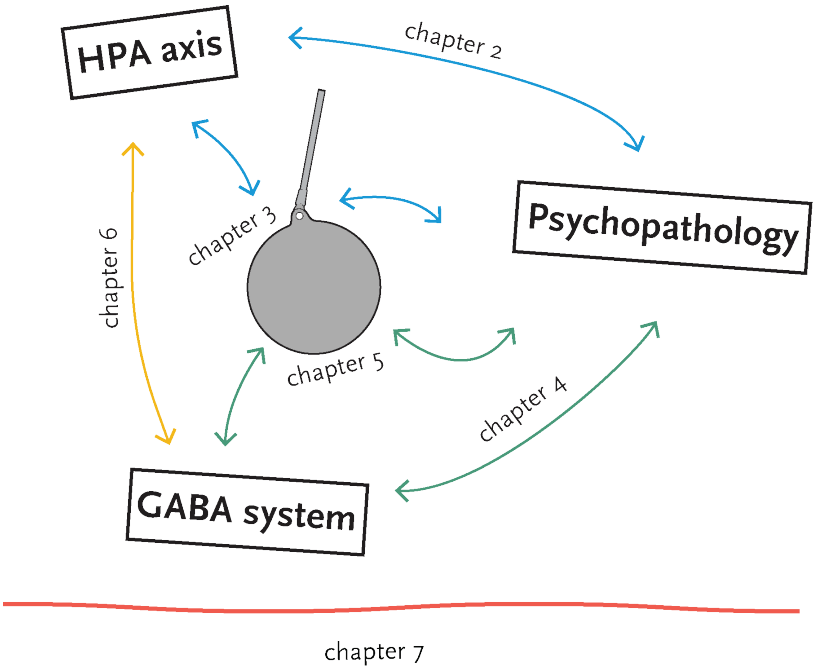


Figure 1.3. Schematic overview of how the chapters in this thesis relate to the main themes.

PART 1

In chapter 2 we reviewed the evidence on the cortisol response to psychosocial stress across psychiatric disorders. The results indicated that sex and symptomatic state are crucial factors to consider when comparing patients with a psychiatric disorder with healthy controls. Whereas women with MDD or an anxiety disorder showed blunting of the cortisol stress response, men with MDD or social anxiety disorder (SAD; the most commonly studied anxiety disorder) showed an increased cortisol stress response. These aberrations were absent (in men) or attenuated (in women) in individuals remitted from MDD compared with controls.
In chapter 3 we investigated changes in glucocorticoid receptor (GR) 1_F methylation over time in a subsample of a military cohort deployed to Afghanistan. We showed that exposure to potentially traumatic events during deployment was associated with an increase in GR-1_F methylation and that specifically increases at functionally relevant sites were associated with the development of post-deployment psychopathology. Importantly, baseline methylation levels did not predict the development of psychopathology and were not related to the amount of childhood trauma.

PART 2

We evaluated the literature on *in vivo* brain GABA levels (as measured with proton magnetic resonance spectroscopy (^{1}H-MRS)) across psychiatric disorders in chapter 4. Lower GABA levels were found in patients with MDD compared with controls, but not in patients remitted from MDD. Moreover, convincingly lower brain GABA levels were observed in patients with autism spectrum disorder and a trend level GABA decrease was seen in patients with schizophrenia compared with healthy individuals. Subsequently, in chapter 5 we examined change in plasma GABA levels in relation to psychopathology and trauma exposure during deployment in a large military cohort. A rise in plasma GABA levels over time was associated with the development of psychopathology (especially depressive symptoms), but not with trauma exposure. As GABA levels rose after the development of psychopathology symptoms, it may serve as a compensatory mechanism for these symptoms. Accordingly, the development of psychopathology after deployment could not be predicted with baseline plasma GABA levels.

PART 3

In Chapter 6 we used ^{1}H-MRS to investigate changes in medial prefrontal GABA and glutamate levels in response to a psychosocial stress test and in relation to salivary cortisol levels over time in healthy individuals. We did not find evidence for an effect of stress on brain GABA or glutamate levels 30 minutes after stress onset, nor did we observe an association between the cortisol stress response and GABA or glutamate levels.

PART 4

Polygenic risk scores (PRS) for MDD and PTSD were created in chapter 7, based on the largest genome-wide association studies (GWASs) for these disorders to date. We found no significant main effects of PTSD and MDD PRSs or interactions with trauma exposure during military deployment on the development of PTSD and depressive symptoms at five time points up to five years post-deployment.

MESSAGES FROM THIS THESIS

The most important findings in the current thesis are discussed more extensively below, as they provide new and/or overlooked perspectives on the relation of our biological measures and stress-related psychopathology.

State- or trait-dependent characteristics (or both)

A recurrent theme in this thesis is whether the investigated marker depends on a symptomatic state or whether it represents a trait (i.e. persistent vulnerability factor). Previous evidence had indicated that abnormalities in both the HPA axis (Van Zuiden et al., 2011) and the GABA system (Petty et al., 1995; Vaiva et al., 2004) may be trait markers, increasing the risk to develop psychopathology following trauma exposure. Such vulnerability factors or intermediate phenotypes may represent 'scars' from traumatic experiences during childhood, which has been suggested in the case of HPA axis abnormalities (Lupien et al., 2009). In addition, these intermediate phenotypes could partly be determined by genetic variation. In contrast, both meta-analyses in the present thesis (chapters 2 and 4) suggest that abnormalities of these systems can also be state-dependent, with altered characteristics in patients with current MDD but (more) normal characteristics in remitted individuals. However, it cannot be concluded that HPA axis and GABA system functionality were restored in remitted individuals, as these individuals may have had an attenuated phenotype compared with currently depressed individuals. Our prospective studies in the military cohort (chapters 3 and 5) give a better indication of possible causality. In these longitudinal studies, change in GABA system and HPA axis parameters occurred over a period of military deployment, thereby showing that both systems are still amenable to change in adults and have state characteristics.

Importantly, in the same military cohort, the trait properties of GR binding has previously been established (Van Zuiden et al., 2011). These seemingly contrasting findings may provide complementary information about both state and trait properties of HPA axis functionality in relation to stress-related psychopathology. Based on the results of this thesis, it is recommended that future studies employ a longitudinal design to further shed light upon the dynamics of these GABA and HPA axis parameters, ideally measuring immediately after stress, in response to treatment and in both symptomatic episodes and remission in the same individuals. Thereby, the value of these parameters for predicting psychopathology as well as their possible causal/compensatory role in etiology and/or pathophysiology can be further elucidated.

Sex-dependent markers

Another important finding in the present thesis regards the influence of sex on the relationship between stress and psychiatric disorders. Sex differences in the cortisol stress response have long been established (Kirschbaum et al., 1999; Kudielka et al., 2004), but have previously largely been disregarded in the context of psychiatric disorders (e.g. in a very recent meta-analysis (Ciufolini et al., 2014)) Our findings presented in chapter 2 suggest that sex differences are crucial to consider in patients with a psychiatric disorder, as women with MDD or an anxiety disorder showed blunting, whereas men with MDD or SAD showed hyperresponsiveness of the cortisol stress response compared with healthy individuals. Although we could not stratify our meta-analysis on brain GABA levels across psychiatric disorders for sex (chapter 4), and

although we found no influence of sex on the relation between plasma GABA levels and the development of post-deployment psychopathology in the military cohort (chapter 5), GABA system functionality is also known to be influenced by sex hormones (Skilbeck et al., 2010).
Evidence from human studies on the influence of sex hormones on HPA axis functionality is limited. In general, men show a higher cortisol stress response than women (Stephens et al., 2016). However, this sex difference is only present when women have low estrogen and progesterone levels (in menopause or in the follicular phase) or when they use oral contraceptives (Kirschbaum et al., 1999). Counterintuitively, estrogen, progesterone and testosterone have all been associated with blunting of the cortisol stress response, although the two largest studies show some contrasting sex-specific effects (Juster et al., 2016; Stephens et al., 2016). Of note, in both women (all follicular phase) and men, testosterone, estradiol and sex-hormone binding globulin rise in response to acute stress, whereas they seem to decrease after chronic stress (Lennartsson et al., 2012), complicating a detailed understanding of the interactions between the HPA axis and sex hormones.
Although the neurobiological mechanisms that sex hormones employ to fine-tune HPA axis and GABA system functionality have not been fully elucidated yet, some mechanisms have been revealed in rodent studies (Handa and Weiser, 2014). For example, estrogen receptor (ER) α agonists seem able to inhibit the negative GABAergic tone on the HPA axis, thereby inducing anxiety-like behavior (Handa and Weiser, 2014). In contrast, ERβ agonists directly reduce the HPA axis response to acute stress and decrease anxiety- and depressive-like behaviors, possibly in part through effects on the serotonergic system (Handa and Weiser, 2014). Testosterone, on the other hand, is only known to reduce the HPA axis response to stress, via androgen receptors (AR) (most potently after conversion to DHT) and predominantly via ERβ receptors (after conversion of DHT to 3β-diol) (Handa and Weiser, 2014). As a result, testosterone reduces depressive- and anxiety-like behaviors, at least in part via the AR (Handa and Weiser, 2014). Evidence is more limited for progesterone, with high levels during pregnancy and the luteal phase, which stimulates tonic inhibition of the GABAergic system, possibly dampening the cortisol stress response (Skilbeck et al., 2010).
The fact that the crucial influence of sex on stress reactivity in MDD and anxiety disorders was only established in our meta-analysis, emphasizes the necessity to form large collaborations dedicated to extensive phenotyping and data sharing. Such large collaborations yield the power to dissect sex-dependent effects into distinct effects of sex hormones and other possibly confounding factors (e.g. childhood maltreatment or disease duration). Furthermore, collecting a genetic database, in line with the Psychiatric Genomics Consortium (PGC), may elucidate the role of common genetic variation in shaping the cortisol stress response, as well as gene-environment interactions.

Genetic vulnerability and trauma exposure

Finally, the absence of explained variance in the development of post-deployment depressive and PTSD symptoms by common genetic variation, trauma exposure and their interaction (chapter 7) gives an indication of the etiological heterogeneity of MDD and PTSD, and of how well we target these disorders with our current genetic and environmental parameters.
Heritability estimates for MDD and PTSD based on twin studies range from 0.31 to 0.42 (Sullivan et al., 2000) and from 0.28 to 0.46, (Stein et al., 2016) respectively. This genetic basis is mostly attributable to common genetic variation. Whereas rare and structural variation

are important in autism and schizophrenia, their role in stress-related psychopathology is still obscure (Smoller, 2016). Despite the importance of common genetic variation, even the latest GWASs for MDD (Hyde et al., 2016) and PTSD (Stein et al., 2016) explain only 6-7% of the variance in the phenotype, and this finding does not reach statistical significance in the PTSD GWAS. Of note, nominally significant single nucleotide polymorphisms (SNPs) from the latest schizophrenia GWAS also explained 7% of the variance, while it was estimated that current GWAS arrays index one third to half of the genetic risk for schizophrenia (Ripke et al., 2014). Increasing sample sizes and more specific phenotyping are essential to explain more of the variance in stress-related psychopathology.

Environmental factors, however, also play a crucial role in the etiology of stress-related psychopathology, especially in PTSD and MDD. The single most important environmental contributor to these disorders is (traumatic) stress (Smoller, 2016). Interestingly, exposure to traumatic events with a high risk for subsequent development of PTSD is itself a moderately heritable trait (heritability estimate: 0.3-0.6) (Smoller, 2016), indicating that genetic vulnerability and environmental exposure are intertwined. For a better understanding of genetic and environmental contributions to MDD and PTSD, a detailed dissection of the additive or interaction effects of genetic vulnerability with different types of (traumatic) stress over the life-span is warranted.

Accordingly, previous studies have focused on the interaction of childhood trauma or stressful life events with PRS (based on the MDD GWAS from 2013 (Ripke et al., 2013)) on depression. Childhood trauma showed a significant additive and interaction effect with PRS on MDD risk (higher risk if both childhood trauma and PRS were high). The explained variance of the interaction effect was similar to the total PRS effect in this study (Peyrot et al., 2014). In contrast, stressful life events only showed an additive effect with PRS on depressive symptoms in an elderly population (Musliner et al., 2015). Our study adds to this evidence by using a more refined PRS, based on the largest GWASs for MDD and PTSD to date (Hyde et al., 2016; Stein et al., 2016), and focusing on military deployment-related trauma in a prospective cohort. We found no additive or interaction effects of the PRSs with trauma exposure during military deployment or during childhood on depressive or PTSD symptoms. These findings further emphasize the polygenicity and high etiological heterogeneity of stress-related disorders.

METHODOLOGICAL CONSIDERATIONS

The polygenicity and high comorbidity of stress-related disorders warrants a better understanding of psychiatric phenotypes, both how they overlap and how they are related to our biological measures. Both are discussed below.

Specificity of (stress-related) psychiatric disorders

Distinct disease entities as presupposed by the Diagnostic and Statistical Manual of Mental Disorders (DSM) classification are not in keeping with the etiological and phenotypic complexity of psychopathology. Correspondingly, most of the clinical phenomenological work that formed the basis of the DSM assumed less distinguishable phenotypes. In support, anxiety was a widely described feature of depression prior to the DSM-III (published in 1980) (Kendler, 2016a). Furthermore, Kraepelin's classical dichotomy between manic-depressive illness (now bipolar

disorder) and dementia praecox (now schizophrenia) was not empirically based (Kendler, 2016b), and continuity from schizophrenic to affective syndromes has long been described (Angst, 2002). It is therefore hardly surprising that family-based epidemiological (Lichtenstein et al., 2009) and molecular genetic studies (Purcell et al., 2009) stumbled upon etiological overlap between these disorders. The genetic overlap among stress-related psychiatric disorders is even higher. The common genetic basis of PTSD, panic disorder, generalized anxiety disorder, phobias and obsessive-compulsive disorder is substantially more important than the disorder-specific genetic basis (Tambs et al., 2009). In addition, twin studies have shown major overlap in the genetic basis for depression and generalized anxiety disorder (Roy et al., 1995). Intriguingly, depression and migraine also overlap (Schur et al., 2009), supporting observations of clinicians who have long emphasized the importance of other physical symptoms in depression (Kendler, 2016a). The recent cross-disorder GWAS by the PGC even found a high genetic correlation of SNP heritability between ADHD and MDD (0.37) indicating that pleiotropic effects (e.g. genetic variants affecting more than one phenotype (Solovieff et al., 2013)) are wider than previously assumed based on phenomenology (Lee et al., 2013). The PGC also identified several SNPs that were associated with all five investigated psychiatric disorders (MDD, schizophrenia, bipolar disorder, autism and attention-deficit/hyperactivity disorder (ADHD)) (Lee et al., 2013). Subsequent work by the PGC showed that bipolar disorder, schizophrenia and MDD share biological pathways, related to synapses, histone methylation and neurotrophic and immune functions (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015). Either these pathways (i.e. intermediate phenotypes, such as calcium channel dysfunction) result in specific symptoms across psychiatric disorders, or symptoms do not reflect specific biological underpinnings. In the last case the question arises on what ground we would expect symptoms to reflect specific pathophysiology.

The mind-brain problem (philosophy of mind)

The above-described issue of the relationship between psychiatric symptoms and pathophysiological processes is central to psychiatric research in general. DSM diagnostic criteria are indices of disease, instead of the disease itself (Kendler, 2016a). The relation between the indices for PTSD and MDD we have investigated in this thesis and neural (or in a broader sense physiological) processes is a philosophically complicated issue that should be interpreted in the context of a philosophy of mind (e.g. functionalism or central-state identity theory, described below). A brief introduction in the predominating theories of mind over time may be helpful to understand this complicated relation (for a more comprehensive overview I refer to chapter 8 in 'Handboek psychiatrie en filosofie' (Denys and Meynen, 2012)). Descartes' dualism separated the body from the mind and placed the mind outside of the physical substances. The most evident shortcoming of this theory was that it could not explain that the mind (which is isolated in another dimension than the body) could influence the physical (and result in behavior). To deal with this problem, behaviorism aimed to explain all behavior as a reaction to a stimulus, thereby canceling out the troublesome mental aspects (Watson, 1913). This became the predominant philosophy of mind in the first half of the twentieth century. The only use of psychology to adepts of this theory was that it could catalyze establishing stimulus-response associations. Although this theory was fruitful in some domains, such as fear conditioning, it soon revealed to be a far from exhaustive theory of mind. Behaviorism had

no explanation for mental processes leading to other mental processes or mental processes not leading to behavior, and soon an attenuated form accounting for these difficulties, called central-state identity theory (Smart, 1960), became predominant. This theory claimed that neurophysiological events are equal to, and necessary for, understanding mental processes. To avoid being limited to brain theories, functionalism not only applied to neurons, but to functional roles in general. Functionalism can best be understood as an analogy to the functioning of computers. The hardware (or brain) is necessary for, but not identical to, the software (or functional role) (Fodor, 1981). This theory of mind, that is still predominant, enabled psychologists to focus on mental processes instead of on neurons and for artificial intelligence to use the same theory of mind. However, the major failure of functionalism is its inadequacy to explain consciousness. There are functional aspects of consciousness, for example: to realize that one exhibits certain behavior may lead to thoughts and/or actions of this person. However, Chalmers (Chalmers, 1996) states that there are qualitative aspects of consciousness as well, that cannot be explained by functionalism. Although these qualitative aspects may be related to the functional one, they are not identical. In this thesis, and in most psychiatric research, we probe qualitative aspects of consciousness (e.g. feelings of sadness), but we are part of a scientific field that is focused on explanations by structure and function. The fact that we try to relate qualitative aspects of consciousness to functional aspects of consciousness or even neurophysiology is important in the evaluation of our results, as the strength of these relationships is not fully understood and probably varies per individual (e.g. in person A blunting of the cortisol stress response is closely related to depressed mood, whereas this relationship is weak in person B). The variation in strength of the relationship between qualitative aspects of consciousness and neurophysiology poses a challenge that is similar to the issue of penetrance in genetics. A phenotype can be strongly related to a specific genetic variant if the finding is replicated in many patients. However, the contribution of the specific variant in the development of the phenotype is variable per patient and depends on the genetic background and environmental context of the individual. Therefore, gaining knowledge about important parameters of disease may be achieved in large studies, but moving towards personalized medicine in psychiatry may be accomplished by collecting longitudinal data relating neurobiological mechanisms to psychiatric symptoms within the same person, thereby revealing parameters most valuable for disease prediction, monitoring and treatment in a specific individual.

Biological measures studied in this thesis

It is of note that the array of techniques we used in this thesis to assess GABA system and HPA axis functionality is far from exhaustive. Our approaches to probe the GABA system were plasma GABA measurement using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), and brain GABA measurement using ^{1}H-MRS. Other methods include measurement of $GABA_A$ receptor binding (using positron emission tomography (PET) or single-photon emission computed tomography (SPECT)), GABA quantification in cerebrospinal fluid, GABA receptor measurement in resected brain tissue, and (epi)genetic evaluation of the GABA system. To probe the HPA axis, we used a targeted genetic and epigenetic approach, assessment of GR binding and transcription in peripheral mononuclear blood cells, and cortisol measurement following a psychosocial stress test. Apart from these approaches a wide range of possibilities exist to assess the HPA

axis: the cortisol awakening response, the dexamethasone suppression test (often combined with corticotropin-releasing hormone (CRH)), measurement of CRH, adrenocorticotropic hormone (ACTH), or their receptors, to name a few. All these different approaches may yield complementary information about the functioning of these two systems and some may be of value in future personalized medicine.

RECOMMENDATIONS FOR FUTURE STUDIES

The messages from this thesis in combination with the methodological considerations provide some general recommendations for future studies to successfully extend our knowledge about the nature of stress-related psychiatric disorders, as discussed below.

Longitudinal studies investigating the impact of stress

The longitudinal studies in this thesis (chapters 3 and 5) established divergent trajectories of HPA axis and GABA system parameters depending on the degree of psychopathology development. In contrast, our meta-analyses (chapters 2 and 4), based on cross-sectional data, suggest, but cannot prove state-dependent neurophysiological changes in stress-related psychopathology. These conclusions show the crucial added value of longitudinal studies in associating neurophysiological parameters with stress and psychopathology. Longitudinal studies can elucidate which parameters are causal, compensatory or a by-product to psychopathology and whether and how these parameters are influenced by stress. Moreover, the influence of confounders (e.g. genetic variation) on within-subject (longitudinal) analyses is greatly reduced compared with between-subject (cross-sectional) analyses. As such, some parameters may be very valuable at an individual level, in line with our conclusion of the paragraph on the mind-body problem. Future longitudinal studies focusing on childhood and periods later in life can enhance our understanding of stress-sensitive periods and of the interaction between multiple stressful life events on neurophysiological parameters and vulnerability to develop stress-related psychopathology. In addition, current day smart phone technology enables more elaborate and detailed longitudinal studies, yielding a wealth of clinical data around subjectively experienced daily life stressors. Of note, subjective stress has been found to better predict psychopathology than objectively determined stress (Grieger et al., 2006). In combination with genetic and neurophysiological data, such studies may ultimately improve our prediction of individual propensities to develop stress-related psychopathology.

More focus on women in stress-related psychopathology

Our meta-analysis on the cortisol stress response (chapter 2) demonstrates the importance of sex differences in relation to HPA axis functionality across psychiatric disorders. Most preclinical and clinical stress research does not include females, as stage in the menstrual cycle increases sample heterogeneity and reduces power to find significant effects. Considering the much higher prevalence of MDD and anxiety disorder in women compared to men, research efforts should be undertaken to study both sexes. For this purpose, the National Institute of Mental Health (NIMH) has started the Women's Mental Health Program, promoting the examination of sex differences in psychopathology.

Extensive phenotyping and large-scale collaborations

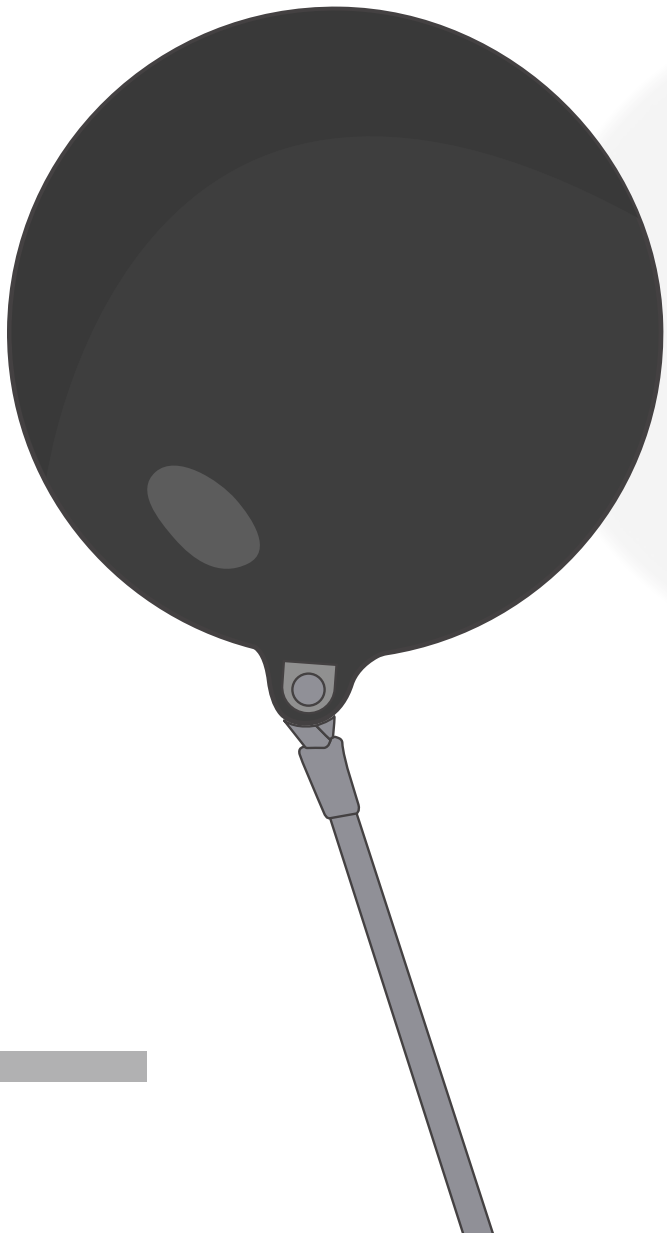
Both meta-analyses (chapters 2 and 4) highlight the need for more extensive phenotypic information in individual studies and the importance of large-scale collaborations. Considering the major problems of power failure in neuroscience (Button et al., 2013) the best approach to robust findings is to standardize data ascertainment and to share data and start large collaborations. Collecting massive sample sizes with detailed phenotypic (e.g. sex hormones, traumatic events, medication use), intermediate phenotypic (e.g. the cortisol stress response) and genetic information is probably our best bet at tackling the high level of phenotypic and etiological heterogeneity in (stress-related) psychopathology.

Cross-disorder phenotypes

Such large-scale collaborations with detailed individual information should include patients with a wide range of DSM based disorders, as these classifications do not delineate biological entities. This allows for a bias-free investigation of the relationship between biological, psychological and environmental parameters and (stress-related) psychopathology. Such efforts may yield latent, cross-disorder disease entities, but will more likely support a dimensional approach, with symptoms or symptom clusters that cross DSM boundaries but share fundamental mechanisms, as proposed in the Research Domain Criteria (RDoC) of the NIMH (Cuthbert and Insel, 2013). A great advantage of such narrowly defined intermediate phenotypes is that they allow for better translation to animal models, in which therapeutics can be developed. For example, a seminal study by Jovasevic et al. (Jovasevic et al., 2015) indicated that state-dependent contextual fear, which is key in stress-related psychopathology, depends on the state of the extrasynaptic GABA system. Alternatively, genetic variants or endophenotypes may be linked to a wide phenotypic range, as phenome-wide association studies (PheWASs) are now trying to uncover (Denny et al., 2010). Where mapping of biological measures on our phenotypes fails, we may need to try a more heuristic approach integrating genetic, clinical and environmental (e.g. stress exposure) information to guide prediction of treatment response (e.g. using further refined polygenic risk scores in addition to information on traumatic stress). Importantly, a full understanding of pathophysiology is not necessary to guide treatment development. Despite the high polygenicity of (stress-related) psychopathology, with small contributions of single alleles, the impact of moderating a specific target may be substantially higher than can be deducted from odds ratios based on GWASs. In support, the dopamine D2 receptor (the main target of antipsychotic drugs) was associated with schizophrenia in the latest GWAS (Ripke et al., 2014), showing that genetic findings are relevant from a therapeutic perspective.

CONCLUDING REMARKS

In this thesis we employed targeted approaches to explore the roles of the GABA system and the HPA axis in stress-related psychopathology. We demonstrated that changes in GR-1_F methylation and plasma GABA are associated with the development of post-deployment psychopathology. However, technological advances in the field of genetics enable increasingly unbiased approaches to elucidate the biological systems implicated in psychiatric disorders. Recent GWASs have provided a glimpse into the solid biological backbone of psychiatric disorders, but at the same time into their great etiological complexity. Establishing distinct diseases based on symptomatology and course, in analogy to infectious diseases, has long been deemed the holy grail for psychiatry, but is too simple a model for these complex diseases. Consequently, the DSM should be used as initially proposed, providing mental disease indices (Kendler, 2016a) that are preliminary, to some extent fallible and definitely incomplete. To improve our understanding and management of psychiatric diseases, two approaches emerge in parallel. We can both pursue more phenotypic refinement and focus on individual level data. Phenotypic refinement may be attained by going from biological models to clinical phenotypes in a refining iterative process (Kendler and Engstrom, 2016). A promising venue for improving biological models is the combination of (epi)genomics with environmental risk factors, such as stress (Smoller, 2016). This, in turn, can yield specific parameters that can be prospectively linked to psychiatric symptoms at an individual level, ultimately providing a heuristic approach to prognostics and personalized care.



CHAPTER 9

Nederlandse samenvatting

In de nasleep van trauma: sporen van stress-gerelateerde psychopathologie

Stress en psychopathologie

Stress is het proces dat je helpt om je staande te houden in een veranderende omgeving. Formeel gezegd: het is de ervaring van een geanticipeerde of werkelijke bedreiging van iemands evenwicht. Stress is een zeer belangrijke risicofactor voor het ontwikkelen van bijna alle psychiatrische stoornissen, waaronder posttraumatische stress stoornis (PTSS), depressie, schizofrenie, bipolaire stoornis en angststoornissen. Een beperkte of verminderde capaciteit om met stressvolle situaties om te gaan (inadequate coping) lijkt de grondslag te zijn voor dit verband tussen stress en psychopathologie. Om stressvolle situaties goed het hoofd te kunnen bieden, zijn fysiologische processen nodig die stabiliteit bewerkstelligen in een veranderde omgeving. Deze processen worden gezamenlijk ook wel allostase genoemd. De hoofdrolspelers of 'mediatoren' van allostase zijn steroïdhormonen (bijvoorbeeld cortisol), neuropeptiden, neurotransmitters (zoals gamma-aminoboterzuur of GABA) en cytokines. Deze mediatoren interacteren en hun effecten zijn afhankelijk van het weefsel of hersengebied en van de tijd die verlopen is sinds de aanvang van de stressor. Inadequate coping in stressvolle situaties kan leiden tot psychopathologie, met name in genetisch gevoelige personen, en dat kan te maken hebben met specifieke problemen van de hierboven genoemde mediatoren, zoals 1) onvoldoende productie, 2) te hoge productie, of 3) niet tijdig stopzetten van de productie. Dit proefschrift bestudeert twee systemen die een belangrijke rol spelen om in balans te blijven na stress en die elkaar direct beïnvloeden: de hypothalamus-hypofyse-bijnier-as (in het Engels: hypothalamus-pituitary-adrenal (HPA) axis) met het eindproduct cortisol, en het remmende GABA-systeem. Veranderingen in beide systemen als gevolg van traumatische en acute stress zijn herhaaldelijk aangetoond in dierstudies en, in mindere mate, ook in humane studies. Hoewel traumatische stress in de kindertijd de meest nadelige invloed heeft, kan ook stress in de volwassenheid leiden tot blijvende veranderingen van deze twee systemen. Er zijn sterke aanwijzingen dat dit soort veranderingen in de HPA-as en het GABA-systeem kunnen leiden tot het ontwikkelen van psychopathologie.

Het doel van dit proefschrift is om de rollen van de HPA-as en het GABA-systeem te onderzoeken in relatie tot het ontwikkelen van psychopathologie na traumatische stress. Daarnaast kijkt het verder dan deze systemen door te onderzoeken wat de bijdrage is van veelvoorkomende genetische risicovarianten aan het ontwikkelen van stress-gerelateerde psychopathologie.

Cortisol stressreactiviteit in psychiatrische stoornissen

Een systematische evaluatie van de wetenschappelijk literatuur in hoofdstuk 2 laat zien dat iemands geslacht en symptomen (of iemand wel of niet depressief is) essentiële factoren zijn die bepalen hoe de cortisolrespons is na psychosociale stress in patiënten met een psychiatrische stoornis vergeleken met gezonde mensen. Daar waar vrouwen met een depressieve stoornis of een angststoornis een lagere cortisolrespons lieten zien vergeleken met controles, vertoonden mannen met een depressieve stoornis of een sociale angststoornis juist een versterkte cortisolreactie na stress. Deze afwijkingen waren afwezig (in mannen) of verminderd aanwezig (in vrouwen) in mensen die in het verleden een depressieve stoornis hadden doorgemaakt en ten tijde van de meting hersteld waren. Deze bevindingen laten zien dat er nog veel winst te behalen is bij het

onderzoeken van de cortisol stressrespons in patiënten met een psychiatrische stoornis als alle factoren waarvan bekend is dat ze de cortisolrespons kunnen beïnvloeden worden meegenomen in de analyses (zoals geslacht, anticonceptiegebruik, ernst van symptomen, leeftijd, ziekteduur en aantal episodes, comorbiditeit, jeugdtrauma, geslachtshormonen en medicatiegebruik) en de data van verschillende onderzoeksgroepen gestandaardiseerd en gedeeld worden. Ook suggereren de data dat een afwijkende cortisol stressrespons een kenmerk zou kunnen zijn dat psychiatrische DSM-classificaties overstijgt.

Longitudinale veranderingen in glucocorticoïd receptor exon 1_F methylatie en psychopathologie na militaire uitzending

In hoofdstuk 3 onderzochten we methylatieveranderingen in de 1_F regio van het gen voor de glucocorticoïd receptor (GR) rondom uitzending naar Afghanistan. Methylatie op het DNA kan wijzen op veranderde productie van eiwitten, in dit geval van de GR. Eerdere studies hadden weliswaar een verband tussen GR-1_F methylatie met zowel traumatische stress als met psychopathologie laten zien, maar er waren nog geen longitudinale data rondom een stressvolle gebeurtenis beschikbaar, hetgeen conclusies over causaliteit sterk beperkte. Wij vonden dat blootstelling aan potentieel traumatische gebeurtenissen tijdens uitzending geassocieerd was met een toename van GR-1_F methylatie over de tijd en dat een specifieke toename op functioneel relevante plaatsen geassocieerd was met een toename in algemene psychopathologie. GR-1_F methylatie niveaus voor uitzending waren niet geassocieerd met jeugdtrauma en voorspelden de ontwikkeling van algemene psychopathologie of PTSS niet. Deze resultaten suggereren dat methylatieveranderingen van de GR belangrijk zijn in de ontwikkeling van psychopathologie na blootstelling aan potentieel traumatische stress.

GABA-niveaus in de hersenen van patiënten met een psychiatrische stoornis

In hoofdstuk 4 onderzochten we in een systematische evaluatie van de literatuur en meta-analyse GABA-niveaus in de hersenen (gemeten met *proton magnetic resonance imaging* (^{1}H-MRS)) van patiënten met een psychiatrische stoornis vergeleken met gezonde controles. Patiënten met een depressieve stoornis hadden lagere GABA-niveaus dan controles, maar mensen die hersteld waren van een depressieve stoornis lieten geen afwijkende GABA-niveaus zien. Ook bij autisme was er sprake van een verlaagd GABA-niveau, terwijl patiënten met schizofrenie net geen significant verlaagd GABA-niveau hadden vergeleken met gezonde controles. Er werden geen veranderde GABA-niveaus gevonden in patiënten met bipolaire stoornis, PTSS, paniekstoornis of ADHD vergeleken met gezonde controles. Net als in hoofdstuk 2 geldt dat het rapporteren van zoveel mogelijk factoren die mogelijk GABA-niveaus beïnvloeden en het delen van data tussen onderzoeksgroepen onontbeerlijk zijn voor vooruitgang in het veld. Daarnaast is het voor onderzoek naar GABA met ^{1}H-MRS cruciaal dat er meer bekend wordt over de fysiologische fluctuaties van GABA, dat er consensus wordt bereikt over de beste meet- en verwerkingsmethodes en dat er technische vooruitgang komt die het mogelijk maakt om meer hersenregio's hypothesegedreven te bestuderen (bijvoorbeeld GABA-niveaus in de hippocampus, de amygdala en subregio's van de prefrontale cortex). Dergelijke ontwikkelingen zouden in de toekomst van waarde kunnen zijn bij het verbeteren van personalized medicine.

Ontwikkeling van psychopathologie na militaire uitzending in relatie tot plasma GABA-niveaus

Vervolgens bestudeerden we in hoofdstuk 5 in een groot militair cohort dat uitgezonden was geweest naar Afghanistan de GABA-niveaus over de tijd in plasma, aangezien recente literatuur een correlatie liet zien tussen plasma GABA en GABA-binding in de hersenen. Terwijl er in de literatuur nog geen verband was beschreven tussen verandering in plasma GABA-niveaus in samenhang met verandering in psychopathologie, toonden wij aan dat een stijging in plasma GABA-niveaus samenhing met het ontwikkelen van algemene psychopathologie, PTSS-symptomen en met name depressieve symptomen. Plasma GABA-veranderingen waren niet geassocieerd met blootstelling aan potentieel traumatische gebeurtenissen. Aangezien plasma GABA pas steeg na het ontwikkelen van psychopathologie suggereert deze studie dat GABA stijgt als compenserend mechanisme voor symptomen. In overeenstemming met deze conclusie bleek dat GABA-niveaus voor uitzending niet de ontwikkeling van psychopathologie voorspellen. Deze studie pleit voor verdere exploratie van het GABA-systeem als mogelijk aangrijpingspunt voor behandeling.

Effecten van acute stress op GABA- en glutamaatniveaus in de prefrontale cortex

In deze ^{1}H-MRS-studie op 7 Tesla in hoofdstuk 6 bestudeerden we veranderingen in mediaal-prefrontale GABA- en glutamaatniveaus in gezonde mensen voor en na een psychosociale stress test vergeleken met een controleconditie, en in samenhang met verandering van speekselcortisol over de tijd. We vonden geen bewijs voor een effect van stress of cortisol over de tijd op GABA- en glutamaatniveaus 30 minuten na aanvang van de stress. Deze bevindingen suggereren dat er ofwel geen stress- of cortisol-effect op GABA- en glutamaatniveaus in de mediaal-prefrontale cortex is na 30 minuten, ofwel dat deze veranderingen niet detecteerbaar zijn met huidige ^{1}H-MRS-technieken.

De interactie tussen genetische kwetsbaarheid en militaire uitzendinggerelateerde stress op de ontwikkeling van PTSS en depressie

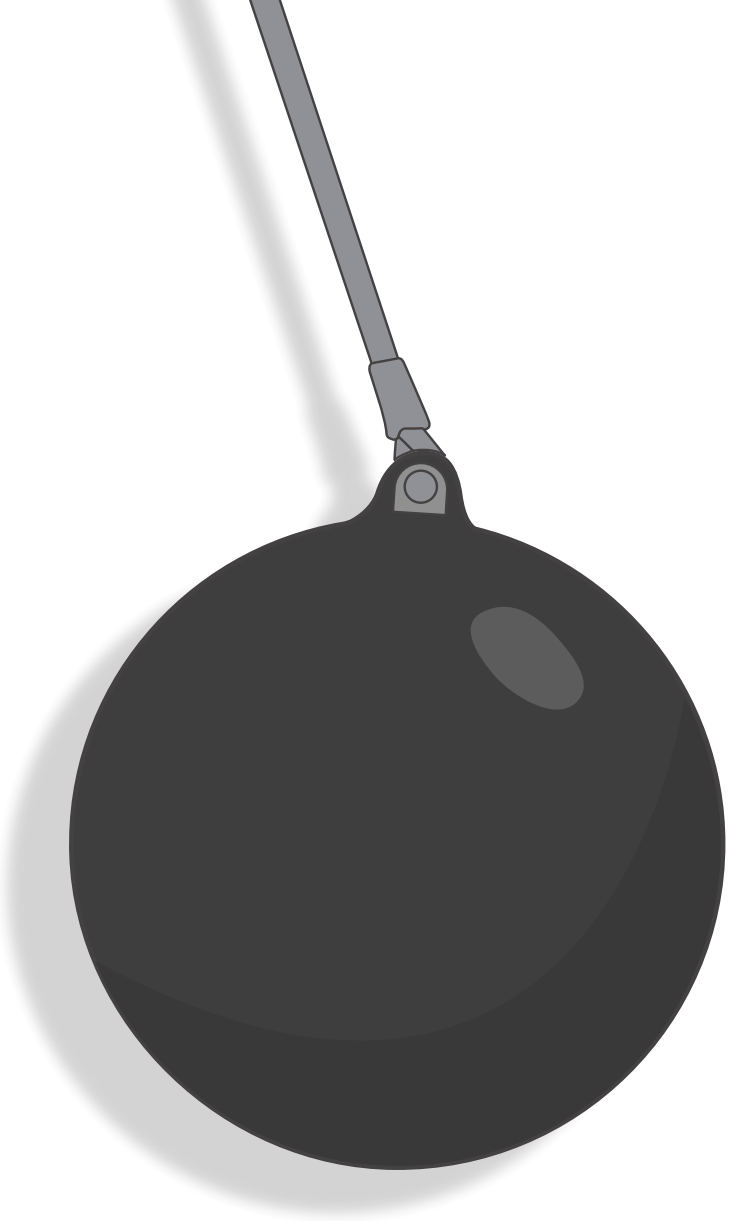
Tenslotte maakten we in hoofdstuk 7 van de militairen die uitgezonden waren geweest naar Afghanistan polygenetische risicoscores (PRS), gebaseerd op de grootste genoom-wijde associatiestudies voor PTSS en depressieve stoornis tot nu toe. Een PRS wordt berekend aan de hand van heel veel plekken op het DNA die een lichte associatie hebben laten zien met de specifieke stoornis in een genoom-wijde associatiestudie. Een PRS kan dus worden gezien als een indicatie van het genetisch risico om een stoornis te ontwikkelen. We bekeken in dit hoofdstuk of het genetische risico op depressie en PTSS, al dan niet door traumatische gebeurtenissen tijdens uitzending of in de kindertijd, geassocieerd was met het ontwikkelen van PTSS- en depressieve symptomen na uitzending. We vonden geen enkele significante associatie tussen het genetische risico en het ontwikkelen van symptomen na uitzending. Deze studie, die recht doet aan de heterogeniteit van de ontstaanswijze van depressieve en PTSS-symptomen, geeft aan dat de genetische risicoscores nog verder ontwikkeld moeten worden om in de toekomst een voorspelling te kunnen doen over wie er psychopathologie ontwikkelt na (traumatische) stress en wie niet.

Toekomst van onderzoek naar stress-gerelateerde psychopathologie

Op basis van de onderzoeken uit dit proefschrift doen we enkele aanbevelingen voor toekomstige studies naar stress-gerelateerde psychopathologie. Allereerst benadrukken de hoofdstukken 3 en 5 dat longitudinale studies onmisbaar zijn om vast te stellen welke parameters causaal, compensatoir of een bijproduct zijn van psychopathologie, hoe deze parameters beïnvloed worden door stress en hoe veranderlijk ze zijn. Toekomstige longitudinale studies kunnen de invloed van specifieke stressoren tijdens afgebakende periodes in de levensloop (zoals de vroege kindertijd) blootleggen en daarmee onze kennis over de ontstaanswijze van stress-gerelateerde psychopathologie verder vergroten. Daarnaast kunnen longitudinale data aan het licht brengen welke biologische parameters in een specifiek individu bruikbaar zijn bij het voorspellen van ziektebeloop en behandelingsuitkomst, iets wat we nu in de psychiatrische praktijk erg missen. Ten tweede suggereert hoofdstuk 2 dat geslacht meer aandacht moet hebben in onderzoek naar stress-gerelateerde psychopathologie dan algemeen wordt toegepast in de wetenschappelijke literatuur en dat onderzoek in zowel mannen als vrouwen dient plaats te vinden. Ten derde benadrukken de meta-analyses (hoofdstukken 2 en 4) dat deelnemers aan onderzoek veel uitgebreider dienen te worden beschreven dan nu gebruikelijk is (door bijvoorbeeld altijd geslacht, anticonceptiegebruik, ernst van symptomen, leeftijd, ziekteduur en aantal episodes, comorbiditeit, jeugdtrauma, geslachtshormonen en medicatiegebruik te rapporteren), en dat er een grote behoefte is aan grootschalige samenwerkingen om de aantallen in het onderzoek te vergroten en zo tegemoet te komen aan de grote fenotypische en etiologische heterogeniteit van stress-gerelateerde psychopathologie en hardere conclusies uit het onderzoek te kunnen trekken. Tenslotte is het cruciaal dat de aandacht voor stress in psychopathologie verder gaat dan de vaak gebruikte indeling van DSM-categorieën, aangezien er tussen deze categorieën veel overlap is qua symptomen en neurobiologische achtergrond, zoals overlappende genen en overeenkomende afwijkingen in het GABA-systeem en in de HPA-as. Mogelijk brengt het bestuderen van stress buiten de gebaande DSM-paden meer biologisch bepaalde fenotypes aan het licht en leidt dit tot het aannemen van een meer dimensionele benadering van (stress-gerelateerde) psychopathologie. Daar waar biologische maten niet goed te koppelen zijn aan symptomen kan het wellicht vruchten afwerpen om een 'heuristische methode' toe te passen die genetische, klinische en omgevingsinformatie combineert om voorspellingen te doen over wie wel en wie niet goed op behandeling reageert.

Conclusie

In dit proefschrift hebben we ons specifiek gericht op de rol van de HPA-as en van het GABA-systeem in stress-gerelateerde psychopathologie. We toonden aan dat veranderingen in GR-1_F methylatie en plasma GABA geassocieerd zijn met het ontwikkelen van psychopathologie na militaire uitzending. Echter, technologische vooruitgang in de genetica maakt het steeds beter mogelijk om hypothese-vrije methodes te gebruiken bij het ophelderen van de biologische systemen die een rol spelen in psychiatrische stoornissen. Recente genoom-wijde associatiestudies hebben we een kijkje gegeven in de aanzienlijke genetische achtergrond van psychiatrische stoornissen, maar ze hebben ook aangetoond hoe complex en heterogeen de ontstaanswijze van deze stoornissen is. Het vaststellen van specifieke ziektes op basis van symptomatologie en beloop, naar het voorbeeld van de infectieziekten, blijkt niet mogelijk te zijn voor deze complex-genetische aandoeningen. Nieuwe benaderingen zijn in opkomst om het begrip van de biologische achtergrond van psychiatrische stoornissen te vergroten: we moeten verder gaan met fenotypische verfijning en we moeten ons concentreren op individuele data. Voor fenotypische verfijning zal het belangrijk zijn om heen en weer te gaan tussen klinische fenotypes en nieuwe biologische modellen. Voor de ontwikkeling van nieuwe biologische modellen is het combineren van (epi)genetica met stress uitkomsten veelbelovend. Dit soort onderzoek kan, op zijn beurt, specifieke maten opleveren die prospectief gerelateerd kunnen worden aan psychiatrische symptomen op individueel niveau, uiteindelijk leidend tot betere en meer op het individu gerichte zorg.



CHAPTER 10

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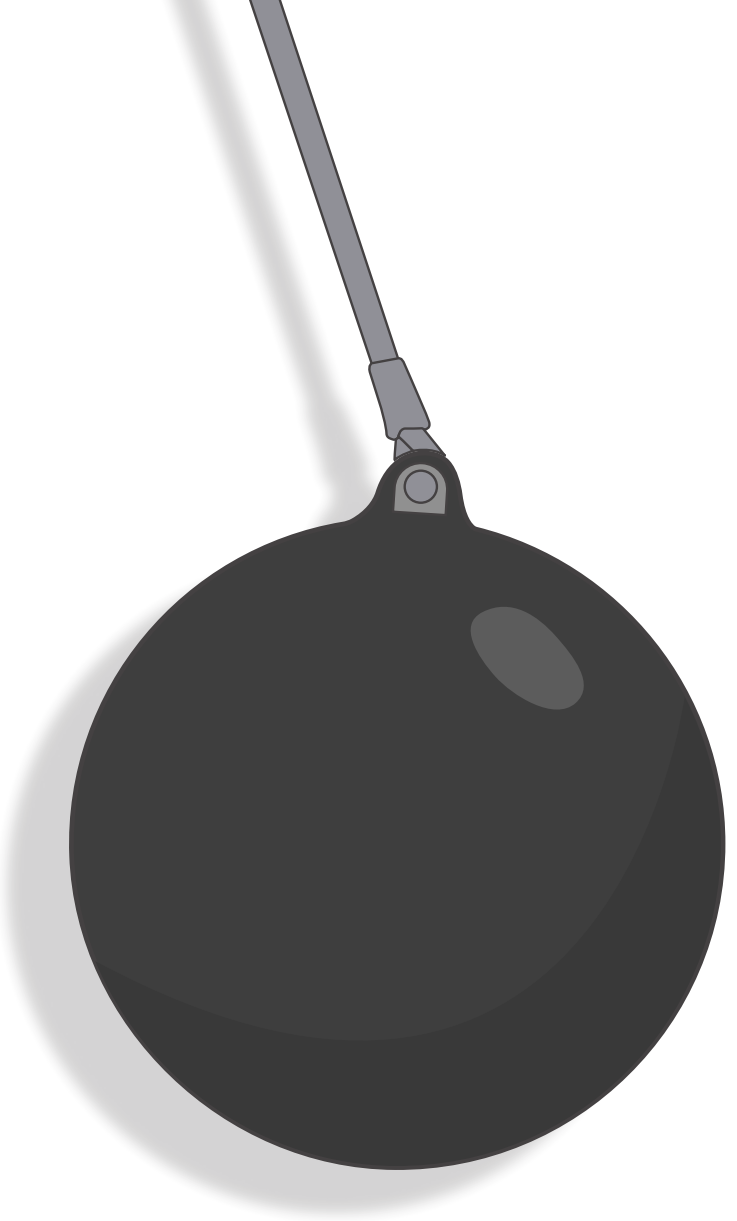

CHAPTER 11

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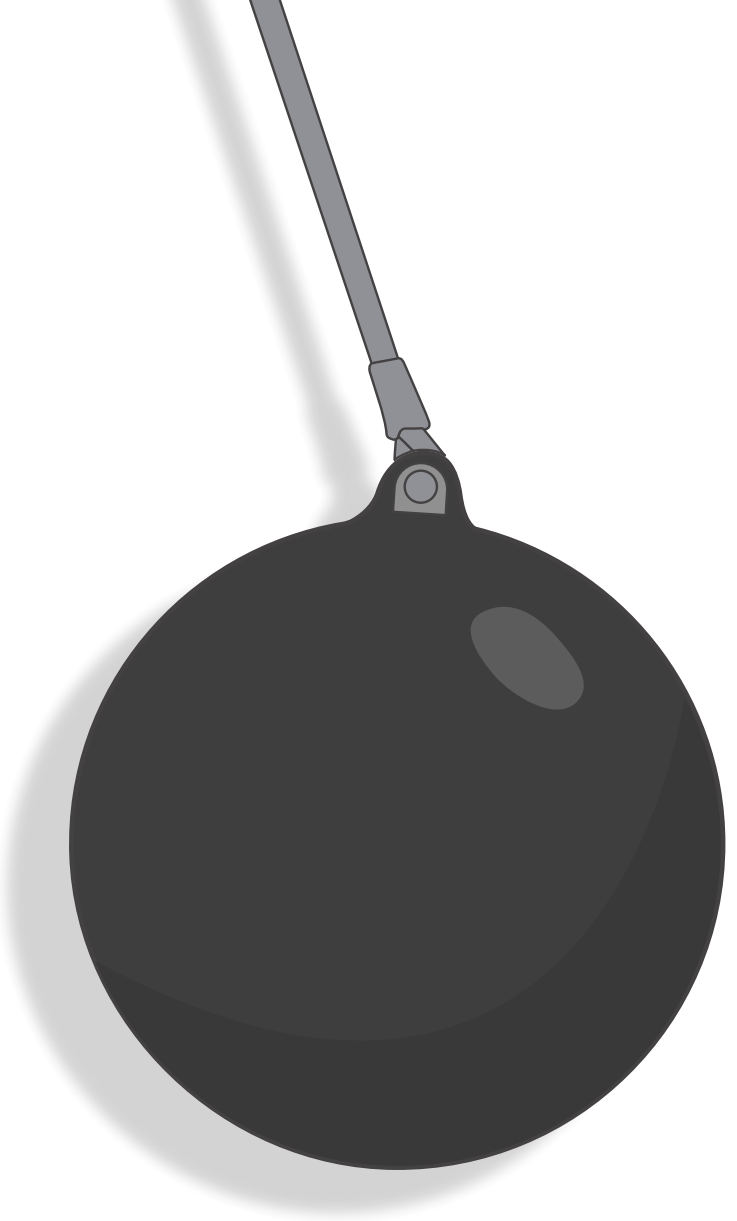
Marijn, volgens professor Koerselman heeft een mens behoefte aan geborgenheid, autonomie en bewondering. Met jou kan ik rekenen op een perfecte mix van deze drie zaken. Je bent niet alleen mijn klankbord, maar we delen ook veel voorkeuren voor humor, kunst, politiek, muziek en eten. Daarnaast vind ik het heel mooi dat we soortgelijke trajecten doorlopen waardoor we onze ervaringen goed met elkaar kunnen delen. Heel erg bedankt voor je steun, geduld, vertrouwen en motiverende invloed, die van groot belang zijn geweest bij het voltooien van dit proefschrift.
Rafaël, je was mijn grootste motivatie om het proefschrift vlot af te krijgen. Ik ben heel blij dat we alvast tijd hebben vrijgemaakt om je te laten opgroeien zonder al te veel stress door aandacht deprivatie.



CHAPTER 12

Curriculum vitae

Remmelt Rudolf Schür was born on February 4 1988 in Haren, the Netherlands. He spent his childhood in nearby Glimmen and a year in Vizille, France in 1993-1994. After completing gymnasium at Maartenscollege Haren in 2006, he started Medical School at Utrecht University (UU), driven by a fascination for Psychiatry. He combined Medicine with studying French language and culture at the UU, where he finished the first year and followed language-oriented second and third year courses. The last years of his medical studies were marked by interesting neurology (under dr. Marc Engelen in the Academic Medical Center Amsterdam) and psychiatry (under dr. Saskia Palmen in the University Medical Center Utrecht (UMCU)) internships, a clinical gynecology internship in Paramaribo, Suriname and a research internship on structural brain alterations in bipolar disorder at the University of California, Los Angeles. It was during this last internship, in the inspiring labs of his supervisor dr. Scott Fears and his girlfriend's supervisor professor Roel Ophoff, that he became motivated to pursue a PhD. In the months between obtaining his MD in 2012 and starting his training in Psychiatry in the UMCU in 2013, he worked as a research assistant in dr. Neeltje van Haren's lab. During his first year as a resident in Psychiatry, he oriented on biological research topics extending beyond DSM categories and got the great opportunity to be involved in stress research at the Brain Center Rudolf Magnus under dr. Christiaan Vinkers, dr. Marco Boks, professor René Kahn and professor Marian Joëls, the product of which is presented here. He will finish his residency in Psychiatry in the upcoming three years, in combination with family life and hopefully some time for research on stress and (epi)genetics.

Remmelt Rudolf Schür werd geboren op 4 februari 1988 te Haren. Hij bracht zijn jeugd door in het nabijgelegen Glimmen en een jaar in Vizille, Frankrijk in 1993-1994. Na het gymnasium aan het Maartenscollege te Haren, begon hij in 2006 met de studie geneeskunde aan de Universiteit Utrecht (UU), gedreven door een fascinatie voor psychiatrie. Naast geneeskunde studeerde hij Franse taal en cultuur aan de UU, waarvan hij zijn eerste jaar voltooide en tweede- en derdejaars vakken volgde gericht op taalverwerving. Het laatste jaar van zijn studie geneeskunde werd gekenmerkt door interessante coschappen in de neurologie (onder dr. Marc Engelen in het Academisch Medisch Centrum Amsterdam) en de psychiatrie (onder dr. Saskia Palmen in het Universitair Medisch Centrum Utrecht (UMCU)), een coschap gynaecologie in Paramaribo, Suriname en een wetenschappelijke stage over structurele hersenveranderingen in bipolaire stoornis aan de Universiteit van Californië te Los Angeles. Tijdens deze laatste stage, in de inspirerende labs van zijn supervisor dr. Scott Fears en zijn vriendins supervisor professor Roel Ophoff, raakte hij gemotiveerd om een promotietraject in te gaan. In de maanden tussen het behalen van zijn artsexamen in 2012 en het beginnen aan zijn specialisatie tot psychiater in 2013 werkte hij als onderzoeksassistent in het lab van dr. Neeltje van Haren. Gedurende het eerste jaar van zijn specialisatie oriënteerde hij zich op mogelijkheden om biologisch onderzoek te doen losstaand van DSM-categorieën. Hij kreeg de mooie mogelijkheid om betrokken te raken bij stress onderzoek aan het Brain Center Rudolf Magnus onder dr. Christiaan Vinkers, dr. Marco Boks, professor René Kahn en professor Marian Joëls, waarvan dit proefschrift het product is. Hij zal in de komende drie jaar zijn specialisatie tot psychiater voltooien, in combinatie met het gezinsleven en hopelijk wat tijd voor onderzoek naar stress en (epi)genetica.

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