Stressed-out stress systems: dysregulated stress-systems in the pathophysiology of stress-related disorders

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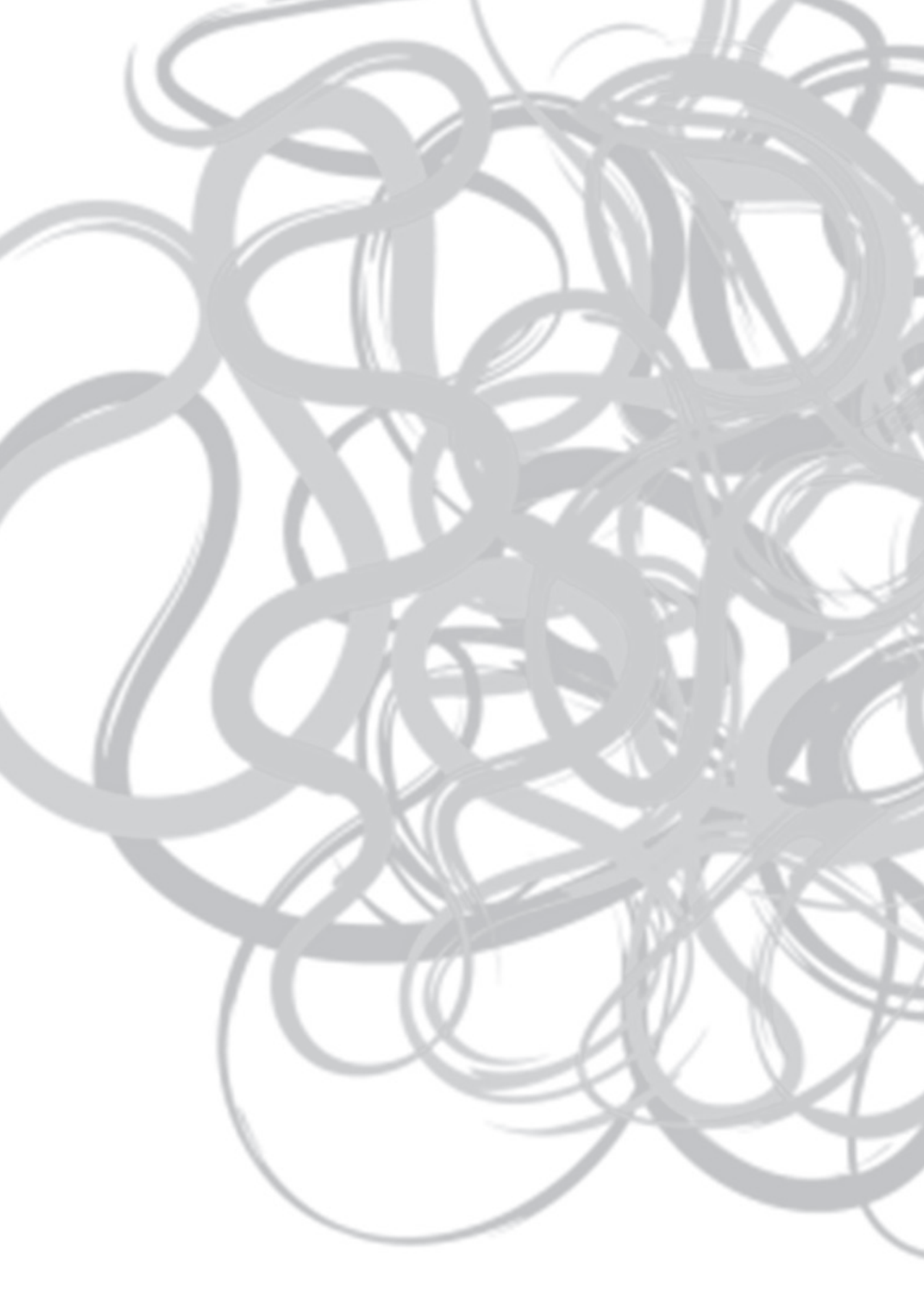

Chapter 1

Stressed-out stress systems

Dysregulated stress-systems in the pathophysiology of stress-related disorders

"It's not stress that kills us, it is our reaction to it."
– Hans Selye

General Introduction

What is stress?
The term 'stress' was first coined by endocrinologist Hans Selye in order to describe the "nonspecific response of the body to any demand"[1]. The first study with regard to this phenomenon that Selye published in 1936 under the title: "A Syndrome Produced by Diverse Nocuous Agents" identified a "typical syndrome", of which the symptoms "are independent of the nature of the damaging agent of the pharmacological type of drug employed, and represent rather a response to damage as such". This syndrome presented if the organism was severely damaged by diverse, nonspecific, nocuous agents such as surgical injury, trauma to the spinal cord leading to spinal shock, exposure to cold, excessive muscular exercise, or intoxications with sublethal doses of various drugs (for example, formaldehyde, morphine, adrenaline, and atropine). Since then the term 'stress' has been further elaborated upon, and differentiation has been made between somatic stress as described by Selye on the one hand, and psychological stress on the other hand. It is on psychological stress where focus of this thesis will lie. Psychological stress has been defined in a variety of ways. One of the more globally accepted definitions is "a particular relationship between the person and the environment that is appraised by the person as taxing or exceeding his or her resources and endangering his or her well-being"[2], referring to the processes that are thought to contribute to the onset and maintenance of several stress-related disorders.

By defining 'stress' as such, the term seems to carry a negative connotation. However, the stress response is essential as it allows for adaption and survival. Throughout our lives, we are presented with a variety of challenges, ranging from daily hassles to severe traumatic events. Our stress-response enables us to respond to a stressor as quickly and efficiently as possible in order to speedily return our bodies to a homeostatic state, although the intra-individual variation in this response is large.

The American Psychological Association (APA) divides stress into two different major types or forms, namely acute stress and chronic stress. These stress types are characterized in a different way in terms of characteristics, duration, symptoms, and treatment approaches. Acute stress is the most common and frequent form of stress. This form of stress usually characterizes itself as brief, and is often caused by reactive thinking, stemming from "the demands and pressures of the recent past

and anticipated demands and pressures of the near future"[3]. Chronic stress is a long-term form of stress. This form of stress is characterized by feelings of hopelessness and despair3.

In the case of chronic stress, where stress systems are no longer only stressed, but stressed-out[4], the repeated exposure to a stressor, numerous stressors, or exposure to a severe acute stressor can result in alterations in psychological and neurobiological processes. Upon persistence of these reactive psychoneurobiological processes, a variety of stress-related psychiatric disorders, such as mood, anxiety, and/or somatic symptom disorders, but also somatic disorders, may develop in vulnerable individuals. As a result, poor coping strategies can develop such as substance abuse, maladaptive avoidance techniques, or social withdrawal[5].

Stress-systems

When an individual experiences stress, whether it be acute or long-term, stress-systems are activated, setting a cascade of biological and psychological processes in motion. The hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS), specifically the sympathetic nervous system that triggers the acute flight, fight, or freeze response, are the two major systems that respond to stress in humans and most mammals[6].

The HPA axis

In an uncontrollable, challenging, or threatening situation, the HPA axis, the primary driver of the endocrine stress response, is activated. The hypothalamus synthesizes and secretes corticotropin-releasing factor (CRF), causing the pituitary gland to release adrenocorticotropic hormone (ACTH) into the blood stream, stimulating the adrenal cortex to produce and secrete the glucocorticoid (GC) cortisol (also known as one of the main stress hormones). This leads to increased concentrations of free cortisol circulating in the body. As these levels rise, CRF release is blocked, leading to a decline in ACTH levels, and in turn, a decline in cortisol levels, extinguishing the stress response and returning the body back to its homeostatic state. This process is referred to as the negative feedback loop[7,8]. In reaction to acute stress, cortisol enables a person to react by suppressing the immune function, mobilizing stored energy, and facilitating several processes of the central nervous system, such as learning and memory. However, chronically increased levels of CRF or cortisol also come paired with numerous deleterious effects, such as cognitive disturbances, depressed mood, anxiety, immune destabilization[9,10], and increased risk of cardiovascular disease, diabetes, and stroke[11].

GCs affect both the nervous and immune systems. GCs bind to intracellular,

mineralocorticoid (MR-), and glucocorticoid (GR-) receptors. GC action in the brain is mediated by the two types of corticosteroid receptors, the MR[12] and the GR[13,14]. However, they differ in their distribution throughout the brain GRs are located widely throughout the brain, whereas MRs are located predominately in the limbic brain areas, specifically in the hippocampus and the amygdala[15]. MR affinity to cortisol is 10-fold higher than GR affinity, and MRs have been found to mediate the effect of cortisol on the regulation of initial stress reactions[16-22]. Imbalances in the MR-/GR-mediated signaling pathways that develop under conditions of chronic stress can increase susceptibility to stress-related disorder and diseases[23,24]. Loss of functioning and expression of the MR in the limbic brain reduces the MR-mediated inhibition of the HPA-axis, leading to higher levels of cortisol in the brain[24]. Therefore, the hypothesis has been offered that MR activation in the limbic brain could have potential as an anti-depressant strategy[25-27].

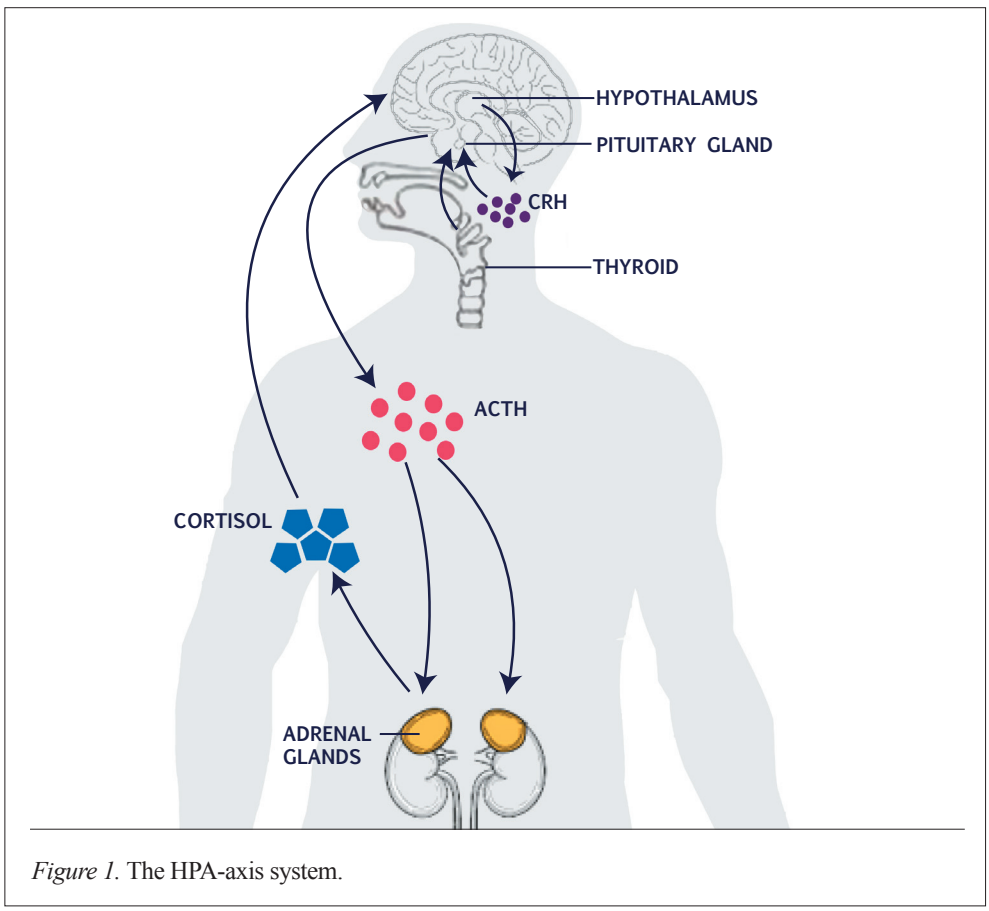

Figure 1. The HPA-axis system.

Under normal circumstances, cortisol follows a distinct diurnal pattern that has been well documented. Typically, cortisol concentration increases upon awakening and slowly declines throughout the day. Upon awakening, a peak in cortisol production is typical, which is referred to as the cortisol awakening response (CAR; the variation in cortisol concentration occurring in the first hour after awakening, with cortisol typically peaking approximately 30 minutes after awakening)[28,29]. Generally, cortisol levels are low at the end of the day towards night, which is indicative of basal HPA axis activity (see Figure 2).

As cortisol is a low weight lipophile molecule, it can diffuse passively across the blood brain barrier. Cortisol can be measured in bodily fluids such as blood, urine, and saliva. Measuring cortisol in saliva is one of the most preferred methods to date, as it is able to measure the amount unbound free cortisol (i.e. the biologically active form), in contrast to the bound form in, for example, blood. Furthermore, saliva sampling is non-invasive and can be obtained by respondents themselves at home, and under normal, stress-free conditions. Additionally, this saliva sampling method makes it possible for respondents to measure their cortisol levels over the course of the day, including the CAR, therefore enabling respondents to capture the dynamics of their HPA axis activity more accurately.

A number of decades ago, salivary cortisol was thought to be a promising biomarker specific to depression. However, ample research has found salivary cortisol levels to be altered in many stress-related disorders. To begin, studies investigating basal cortisol levels in psychiatric disorders have rendered equivocal results. Many studies have found evidence of hypersecretion of cortisol in depressed and anxious patients[30,32], although long-term stress has also been found to lead to a downregulation or exhaustion of the HPA-axis, and less hypercortisolemia[31]. Studies specifically investigating the salivary CAR in this patient population have shown a high level of variability. A number of studies have found similar morning salivary cortisol concentrations in depressed patients and healthy controls [(e.g.32)], although other studies found a blunted cortisol response in patients with major depressive disorder (MDD) in comparison to healthy controls[33,34]. Again, other studies have found an increased CAR for both area under the curve with respect to the ground (AUCg) and area under the curve with respect to the increase (AUCi) variables in MDD patients in comparison to healthy controls [(e.g.31,35-37)]. Two earlier studies conducted with MDD patients and healthy controls found (partially) increased evening cortisol levels in depressed subjects[37,38]. Finally, several studies have identified diminished negative feedback in depressed patients compared to healthy controls after a low-dose dexamethasone-suppression test (DST)[31,33-37]. Studies examining HPA activity in patients with generalized anxiety disorder (GAD)

have reported normal[39-41] to increased cortisol levels[42] and normal suppression[43] to more non-suppression following a DST[44]. In sum, these findings provide evidence for differences in HPA stress-axis regulation in patients with mood- and/or anxiety disorders on the one hand and healthy controls on the other, however highlight the inability of salivary cortisol to distinguish between these disorders. With regard to the research conducted concerning the HPA-axis in this thesis, depression and anxiety will be the stress-related disorders predominately focused on. Further research with regard to HPA-axis activity will be conducted using Cushing's disease as a naturalistic model for the effects of long-term exposure to high amounts of endogenous cortisol (see section: The HPA axis: prolonged exposure to endogenous cortisol for further detail).

The ANS

The ANS is a component of the peripheral nervous system. This system plays a crucial role in both the manifestation and maintenance of stress-related symptoms and biological stress processes45. The ANS consists of two systems, the parasympathetic nervous system (PNS), responsible for the body's rest and digest response, and the sympathetic nervous system (SNS), responsible for the fight or flight response. When the SNS is activated, it signals the adrenal glands to release hormones (i.e. adrenalin (epinephrine) and cortisol). As with HPA-axis activation, the SNS response is fairly sudden in order to prepare the body to respond to an emergency situation, an acute stress situation, or short-term stressors.

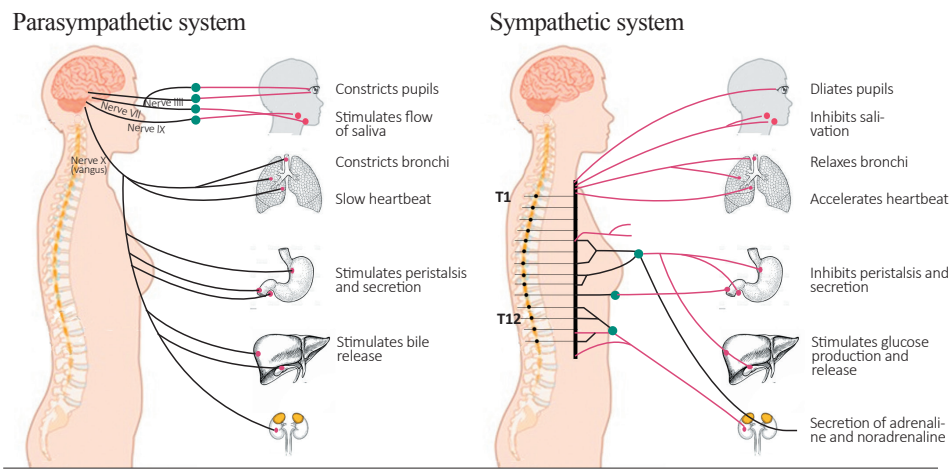

Figure 2. The autonomic nervous system.

Once the crisis is over, the body typically returns to a homeostatic state. This recovery is facilitated by the PNS, which generally has opposing effects to the SNS. Several parameters serve as indices for ANS activity, such as plasma levels of catecholamines, heart rate, heart rate variability, and blood pressure.

A lesser known parameter for ANS activation: salivary alpha amylase

The enzyme sAA has been studied less extensively than salivary cortisol. In contrast to the small cortisol molecule, sAA is a long-chain macromolecule protein, that is secreted in the oral cavity by the salivary gland upon beta-adrenergic stimulation, is a relatively new candidate marker of autonomic nervous system (ANS) functioning and reactivity, accounting for 40 to 50% of salivary protein[46-48]. It is strongly conserved in evolutionary history and can be found in other animals, insects, plants, and bacteria[49].

Research into the oral microbiome has found that particular strains of oral bacteria are adapted in order to break down starch, and have a unique ability to capture sAA, with which they feed themselves[49]. This mechanism is only activated when the regular diet includes starch, that has been found to be essential to survival[50]. There is evidence that carbohydrate intake increases sAA response in individuals whose ancestors consumed starch-rich diets. For this reason, people of Southern European ancestry experience larger sAA increases in sAA levels in comparison to people of Northern European ancestry[51].

sAA plays an important role in the first step of starch digestion in the oral cavity as it catalyzes (breaks down) large long-chain carbohydrate starch molecules into dextrin, glucose and maltose by cleaving alpha-1,4-glucosidic bond[52,53]. Starch is further digested in the small intestine by pancreatic alpha amylase, an enzyme similar to sAA that is produced by the exocrine pancreas and released into the duodenum. Both pancreatic and salivary amylase isoforms are also present in serum with an approximately even proportion[54]. In contrast to the diurnal profile of salivary cortisol, studies with healthy controls have demonstrated that sAA presents an opposing, distinct diurnal profile (see Figure 3), with lower levels in the morning and higher levels in the early evening[45,46].

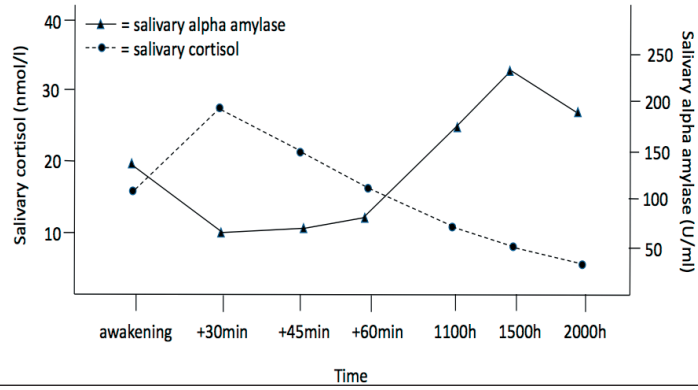

Figure 3. An example of the diurnal profiles of sAA and salivary cortisol for illustration purposes.

This thesis aims to further unravel the role of the stress systems in the pathophysiology of stress-related psychiatric disorders, by exploring elements of regulation and dysregulation of the two major stress systems (i.e. the ANS and the HPA-axis), and their relation with psychological and psychiatric symptoms.

Salivary alpha-amylase in stress-related disorders

Studies with healthy controls have shown that sAA is highly sensitive to acute stress-related changes, increasing under psychosocial stress tasks administered in the afternoon (i.e. Trier Social Stress Test) [(e.g.47,55,56)]. These elevations of sAA levels have been found to be indicative of increased autonomic activity and have been found to occur in response to neurotransmitter stimulation[57,58]. The enzymatic activity and quantity of sAA have been found to vary between individuals under environmental factors, such as stress levels, but also circadian rhythms[59,60]. Within the mood-, anxiety-, and symptom somatic disorder (MAS)-patient population, interventional studies using psychosocial stress tasks in the afternoon (between 12:00 and 5:00 p.m.) have provided evidence that sAA levels in patients with MAS-disorders increase more than those of healthy controls [(e.g. 61-64)].

Five previous observational studies have been conducted with the MAS-patient group. Two were conducted in a laboratory setting and found low baseline sAA levels in MDD patients in comparison to healthy controls in the morning[65], and elevated sAA levels in the afternoon in current MDD patients in comparison to remitted MDD patients and healthy controls[66]. Three studies were conducted in a naturalistic setting. One study found that patients with generalized social anxiety disorder (gSAD) had increased sAA levels in the area under the curve with respect

to the ground (AUCg) on day 1 and at 4:00 p.m. on day 2[67]. The two other studies were conducted with (remitted) MDD patients and found (i) elevated levels of sAA in MDD patients using tricyclic antidepressants (but not SSRIs), in comparison to the controls and remitted MDD patients in the late evening (between 10:00 and 11:00 p.m.)[68], and (ii) higher salivary cortisol and sAA levels in the morning, afternoon, and evening sample times in MDD patients in comparison to healthy controls, although this study was largely underpowered[69].

Based on these findings, it seemed that sAA levels in the late evening do not differentiate between MDD patients who do not use tricyclic antidepressants, remitted MDD patients, and healthy controls. However, sAA levels may be able to differentiate between MDD patients and healthy controls more effectively at other time points throughout the day (i.e. in the morning, afternoon, and in the evening based on the Booij et al.[69] study). Still, as sAA levels were also found to be elevated in gSAD patients at certain time points, sAA may not be able to adequately differentiate between MDD patients and patients with other MAS-disorders at every time point. Furthermore, certain factors have been found to influence sAA levels and should be considered as potential confounders in epidemiological studies. For example, there is evidence that carbohydrate intake increases sAA response in individuals whose ancestors consumed starch-rich diets. Thus, those of Southern European ancestry experienced a larger increase in sAA levels and vice versa[70]. Furthermore, a study conducted in a sample of healthy participants (N = 487), found that sAA levels were also influenced by age and alcohol use[68].

In sum, several gaps in the literature remain with regard to sAA research within psychiatric patient populations. One important aspect that is currently lacking is the mapping of naturalistic diurnal sAA levels in patients with MAS-disorders in comparison to healthy controls. Chapter 2 of this thesis will explore this, taking important characteristics as mentioned above (i.e. ancestry, age, and alcohol use) into account.

Salivary alpha amylase and social withdrawal

Social withdrawal (SW) has recently been defined as "an umbrella term referring to an individual's voluntary self-isolation from familiar and/or unfamiliar others through the consistent display of solitary behaviors such as shyness, spending excessive time alone, and avoiding peer interation"[71]. SW has been identified an early symptom of several stress-related psychiatric disorders [(e.g. 72,73)], and hypothesized to be partially due to long-term activation of biological stress systems. As such, and in line with the National Institute of Mental Health Research Domain Criteria (RDoC) project aimed at identifying new ways of classifying psychiatric disorders

based on dimensions of neurobiological measures and observable behavior, SW has been posited to be related to a more stable endophenotype that is more closely connected to biological pathways than psychiatric disorders are[74,75]. Increased SW can lead to poor social functioning and social isolation, and can in turn cause feelings of loneliness. Loneliness prevalence in European countries has been found to range from 10% in the West and North to 55% in the East[76].

Low levels of SW have been found to be positively related to longevity, physical-, psychological-, and emotional well-being[77,78], whereas high SW has been associated with severe detrimental health outcomes, such as depression[79,80], adverse coronary condition rates[81-84], alcoholism[83], increased mortality rates[81-83], increased suicidality[85,86], and Alzheimer's disease[87]. Furthermore, associations between SW and alterations in hypothalamic-pituitary-adrenocortical (HPA) axis activity have been found[88,89]. Thus, it seems that the dimension of SW may aid in linking overlapping biological underpinnings across several conditions[75,76]. The identification of dimensional behavioral phenotypes across disorders may help to deepen our understanding of the neurobiology involved and complements the approach to incorporate dimensional measures as in the DSM-5 system.

As mentioned above, previous research has found associations between SW and HPA-axis activation[89-94], although certain findings were not consistent with this[76,94]. Evidence has also been found supporting the likelihood of a temporal relationship starting with SW and leading to subsequent depression[79]. Furthermore, increased SW has been found to be a mediating variable in the relationship between salivary cortisol and depression, although this study did not adjust for numerous influential covariates (i.e. adjusted only for gender, age, and cortisol concentration)[95]. However, the interrelationships between sAA, SW, and depression have not yet been explored, and based on the previous research regarding HPA-axis activation and SW, it is reasonable to hypothesize there may be an association with SNS activity and SW. These interrelationships will be further explored transdiagnostically in a population of patients with MAS-disorders and healthy controls in Chapter 3 of this thesis, using a dimensional approach in line with the RDoC guidelines in order to determine whether SW could offer a new way of classifying certain stress-related psychiatric disorders. Also, the interrelationships between sAA, social withdrawal, and depression will be investigated in this Chapter.

The HPA axis: prolonged exposure to endogenous cortisol

As mentioned previously, chronic stress, the repeated exposure to a stressor, numerous stressors, or exposure to a severe acute stressor can result in alterations in psychological and neurobiological processes. However, these processes can also be set in motion by Cushing's syndrome (CS). CS is caused by excess cortisol in the body, regardless of the cause. In Cushing's disease (CD), the stressor causing the

activation of these reactive processes has an endogenous origin. Specifically, CD is an endocrine disorder caused by a benign tumor (i.e., adenoma) located on the pituitary that produces adrenocorticotropic hormone (ACTH), in turn stimulating the release of GCs by the adrenal cortex[96]. In individuals without CD, an increase in GCs will trigger a negative feedback loop, inhibiting the release of ACTH. However, the ACTH-producing tumor in CD is insensitive to this inhibition, therefore, the system is unable to regulate itself, resulting in increased levels of GCs or hypercortisolism. CD typically displays several physical manifestations, including hypertension, abnormal fat distribution, thin skin sensitive to bruising, muscle weakness, osteoporosis, hirsutism, and gonadal dysfunction. Alongside these physical symptoms, patients can also display a wide variety of psychiatric symptoms, including emotional instability, cognitive impairments, psychosis, apathy, anxiety, and depression[96-98]. These symptoms are indicative of the effects of Cushing's disease on the central nervous system (CNS). CD provides a unique human model with which to investigate the effects of prolonged exposure to vast amounts of endogenous cortisol on the brain, and also to investigate the associations between these effects, and psychiatric- and clinical symptomatology.

The association between hypercortisolism and CNS damage was first described in 1952 by Trethowan and Cobb[99]. Their findings were based on autopsy reports in which they found enlarged ventricles and a decrease in brain weight in patients with Cushing's syndrome. These findings were further validated by the first in-vivo study conducted by Momose et al.[100], using pneumoencephalography. They found high incidences of atrophy in both cerebral and cerebellar regions in patients with Cushing's disease. Since then, several studies have investigated the effects of long-term exposure to endogenous cortisol on the brain in patients with (remitted) CD and in 2015, Andela et al.[101] wrote an elaborate systematic review of the findings of studies on structural and functional abnormalities. The main findings reported that the large amounts of endogenous GCs seemed to lead to profound effects on the brain, specifically on grey matter volumes of the ACC and the cerebellum, widespread reductions of white matter integrity, and alterations in specific neuronal metabolites in the bilateral hippocampus. However, since that time, there have been several relevant publications within this area of research, indicating that a review of the newest findings may be helpful in identifying current knowledge gaps. Chapter 4 of this thesis will explore these newest findings in more detail.

Cortical thickness and surface area of the brain

The cerebral cortex is the outer covering of the surfaces of the cerebral hemispheres. It is folded into peaks (i.e. gyri) and grooves (i.e. sulci). Cortical thickness represents the combined thickness of all cerebral cortex layers with the average human cortical thickness over the whole brain being approximately 2.5 to 3 mm[102]. Although

interpersonal variation of cortical thickness is present, an abnormally thin or thick cortex could be associated with changes in gray matter that correlate with specific neurological conditions and neuropathologies[103]. Cortical surface area likely reflects folding and gyrification, which both depend on division of progenitor cells in the periventricular area during embryogenesis[104].

Several studies have reported a reduction in cortical thickness in patients with stress-related disorders (for example, generalized and social anxiety disorder[105,106], bipolar disorder[107,108], and major depressive disorder[109,110]). Two studies investigated cortical thickness in Cushing's syndrome (CS) patients and healthy controls: the first found no differences in cortical thickness[111], and the second reported increased cortical thickness in the lateral orbitofrontal and superior frontal cortex in children with CS in compared to HCs, however this study did not adjust for multiple comparisons[112]. Furthermore, studies have found loss of brain volume in CS patients (for example, in the hippocampus, bicaudate, and third ventricle), which were found to be partially reversible upon biochemical remission[113-115].

Previous analyses conducted in a cohort of long-term remitted Cushing's disease (CD) patients have revealed reductions in white matter integrity throughout the brain in addition to altered resting-state connectivity between the limbic system and the subgenual anterior cingulate cortex (ACC) in comparison to healthy controls[116,117]. Furthermore, in this same patient population a voxel-based morphology study found reductions of ACC volumes[118]. As subregions of the ACC are considered critical in cognitive control, emotional functioning and reward-based decision making; damage to this region may lead to reductions in motivation, spontaneity, and problem-solving capacity, as well as increased apathy and verbalization[119-121]. These findings suggest that alterations in structure and connectivity in the brain, and in particular the ACC, may explain part of the cognitive and psychiatric symptoms commonly observed both in active and remitted CD patients.

There are two frequently used measures for gray matter analysis. The first is cortical thickness, which is indicative of neuron and glia size, number, and arrangement in specific cortical regions[122-124]. The second is cortical surface area, which is related to the number of columns in a region of interest[123,125]. These measures together constitute gray matter volume, however separately, they provide more detailed information on changes in cortical structures. For this reason, cortical thickness and surface area have been suggested to be of more etiological relevance than gray matter volume alone[126,127]. Examining the gray matter volume by means of cortical thickness and cortical surface area in the remitted CD patient population separately may therefore yield more specific insights into the seemingly lasting impairments of

CD on the brain. This will be explored in Chapter 5 of this thesis. In this study, the ACC will be identified as the region of interest (ROI), as previous research has identified this region as altered in both structure and resting-state connectivity. This will then be followed by an exploratory whole-brain analysis, after which the associations with psychiatric- and clinical symptomatology will be explored if appropriate.

As mentioned previously, research has shown that patients with (remitted) Cushing's disease often suffer from stress-related psychopathology, such as depression and anxiety. In Chapter 6 we would like to explore whether we can identify possible resilience-specific correlates in cortical thickness and cortical surface area, and their correlations with psychometric measures. We will explore this by means of a three-group design consisting of one group of 'resilient' participants (i.e. Dutch police officers that have been exposed to trauma and do not present any psychopathology), one group of 'vulnerable' participants (i.e. Dutch police officers that have been exposed to trauma and present with psychopathology), and a control group (i.e. Dutch recruits from the police academy with no trauma exposure and no psychopathology). Previous studies have found (parts of) the ACC to be altered in patients with trauma-related psychopathology[e.g. 128-130]. For this reason, the ACC will be identified as the ROI in this study, followed by an exploratory whole-brain analysis. If appropriate, associations between brain regions with psychiatric- and clinical symptomatology will be explored.

Cognitive planning and executive functioning in remitted CD patients

As denoted earlier, several studies have found (persistent) impairments of cognitive function in the (remitted) CD patient population. Previous studies have examined cognitive functioning by means of standard neuropsychological testing in active CD patients[131,132], as well as in remitted patients after a follow-up period of up to 18 months[133]. These studies found that cognitive and executive functioning (i.e., psychomotor functioning, visuoconceptual tracking, processing speed, auditory attention, auditory working memory, verbal fluency, reading speed, and brief attention) remains impaired in remitted CD patients.

An important cognitive function necessary to lead a functional life is the cognitive skill of planning. Cognitive planning encompasses the neurological processes that are involved with the strategy formulation, coordination, evaluation, and selection of a thought sequence, and the necessary actions that are needed in order to achieve that goal[134]. Reductions of these cognitive abilities in patients with remitted CD may lead to lasting effects on planning abilities, in turn effecting one's daily functionality, psychological state, and quality of life. Examining possible neurobiological alterations in brain activity patterns regarding cognitive planning and executive functioning

within the remitted CD patient population could provide further objective insights into these possibly lasting impairments within this cognitive domain. A task that is often used to detect alterations in brain activation with regard to cognitive planning and executive function is the Tower of London (ToL) task[135]. In Chapter 7 of this thesis we will explore the cognitive planning and executive functioning of remitted CD patients using the visuo-spatial ToL task, again using the ACC as ROI, followed by a whole brain analysis, and, if appropriate, further investigating associations with psychiatric- and clinical symptomatology.

Further exploration of the anterior cingulate cortex in remitted Cushing's disease patients
Although current treatment strategies abrogate excessive cortisol signaling and offer substantial alleviation of several associated symptoms, as mentioned earlier, certain debilitating psychological symptoms often persist in remitted CD patients, even after long-term remission. (f)MRI studies conducted with this patient population have found alterations in both brain structure and connectivity, in which the ACC has often been implicated. This region has therefore has been pinpointed as a region where further exploration could lead to considerable insights with regard to these observed alterations.

Previously, the hypothesis has been posed that the intrinsic impairments and alterations in connectivity and/or biochemistry of certain brain regions may have caused the structural differences observed in remitted CD patients, specifically in the ACC[101]. Unfortunately, MRI studies alone cannot offer sufficient insight into these underlying biological processes. However, such underlying biological processes could be further explored by combining information obtained from high resolution MRI scans with whole genome mRNA expression data. This data is openly available in the Allen Human Brain Atlas (AHBA), a multi-modal atlas mapping gene expression across the healthy human brain[135]. Exploring the potential mechanisms through which the structure of the ACC changes when exposed to prolonged endogenous cortisol excess by linking information derived from high resolution MRI scans with gene expression data derived from the AHBA, could offer more insights into the potential mechanisms through which these persistent alterations occur. This will be explored in Chapter 8 of this thesis.

Finally, Chapter 9 will summarize the empirical findings reported in this thesis. These findings will then be discussed and integrated into our current knowledge, focusing not only on how these findings can help us advance, but also on their implications.

In sum, this thesis aims to further unravel the role of the stress systems in the pathophysiology of stress-related psychiatric disorders, by exploring elements of

regulation and dysregulation of the two major stress systems (i.e. the ANS and the HPA-axis), and their relation with psychological and psychiatric symptoms. The two main hypotheses are that (i) sAA can differentiate between certain stress-related disorders, and (ii) that brain abnormalities in patients with remitted Cushing's disease will partially overlap with the brain abnormalities found in patients with stress-related disorders further increasing the validity of using Cushing's disease as a naturalistic model for the effects of long-term exposure of cortisol on the brain.

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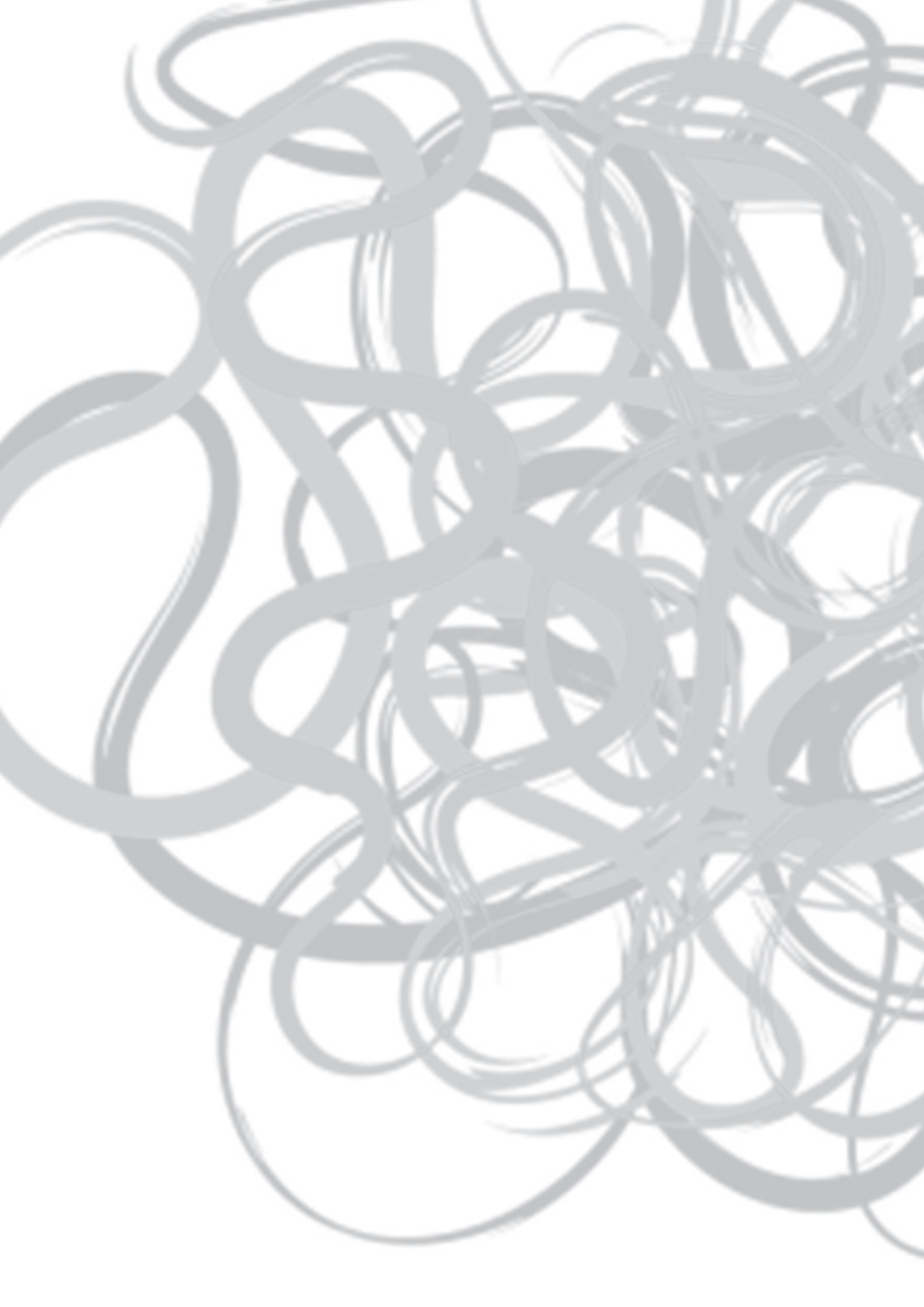

Chapter 2

Elevated salivary alpha-amylase levels at awakening in patients with depression

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Abstract

Introduction

Specific Major Depressive Disorder (MDD) biomarkers could help improve our understanding of MDD pathophysiology and aid in the refinement of current MDD criteria. While salivary cortisol (SC) can differentiate between healthy controls and patients with psychiatric disorders, salivary alpha amylase (sAA), may be a putative candidate biomarker for MDD specifically.

Methods

In a naturalistic cohort of consecutive out-patients and healthy controls, sAA and SC were determined in 833 participants (97 MDD patients, 142 patients with other mood, anxiety, and/or somatoform (MAS-) disorders, and 594 healthy controls). Samples were collected at 7 different time points (at awakening, after 30, 45, and 60 min, at 10:00 p.m., at 11:00 p.m., and at awakening on day 2).

Results

The mean age of the sample was 43.8 years (SD=12.9; 63.9% female). Concerning sAA, MDD patients had higher sAA levels upon awakening on two consecutive days ($p=0.04$, $p=0.01$ respectively), as well as a higher area under the curve with respect to the increase (AUCi; $p=0.04$) in comparison to both controls and the other MAS-disorders group. Regarding SC, mean levels of evening SC were elevated in MDD patients ($p=0.049$) in comparison to both controls and the other MAS-disorders group. SC values on day 2 after ingestion of dexamethasone were elevated in both MDD patients and the other MAS-disorders group ($p=0.04$, $p=0.047$ respectively).

Conclusions

sAA at awakening and not cortisol differentiates MDD from other psychiatric disorders in outpatients. This suggests that sAA may be a valuable candidate biomarker specifically for MDD.

1. Background

Major depressive disorder (MDD) is one of the most common psychiatric disorders and the leading cause of disability worldwide (WHO, 2017). Despite extensive research, no specific biomarkers for MDD have been identified to date (Fried and Nesse, 2015). Although the mean level of salivary cortisol (SC; a hormone reflecting hypothalamus-pituitary-adrenal (HPA) axis activity) has often been found to be elevated in patients with mood, anxiety, and somatic disorders (MAS-disorders), this possible biomarker lacks the potential to distinguish among these disorders (Rief et al., 1998; Hellhammer et al., 2009). Recent studies suggest that salivary alpha amylase (sAA), a newer candidate biomarker that involves the autonomic nervous system (ANS) axis, may differentiate MDD from other MAS-disorders more specifically than SC (van Veen et al., 2008; Ishitobi et al., 2010; Booij et al., 2015).

sAA is an enzyme that is secreted under autonomic regulation in the oral cavity and accounts for 40–50% of salivary protein content (Rohleder et al., 2004; Schumacher et al., 2013). Studies with healthy controls have demonstrated that sAA presents a distinct diurnal cycle opposing that of SC (Nater et al., 2007; Rohleder et al., 2004), with lower levels in the morning and higher levels in the early evening. The enzymatic activity and quantity of sAA have been found to vary among individuals in relation to certain environmental factors, such as stress levels and circadian rhythms (Granger et al., 2007; Chatterton et al., 1996). Elevations of sAA levels are indicative of increased autonomic activity and have been found to occur in response to neurotransmitter (i.e. norepinephrine) stimulation (Garrett, 1999; Nater et al., 2005). Studies with healthy controls have shown that sAA is highly sensitive to acute stress-related changes, with increasing levels during psychosocial stress tasks (i.e. Trier Social Stress Test, Kirschbaum et al., 1993; Nater et al., 2005, 2006; Rohleder et al., 2004). Within the MAS-patient population, interventional studies using psychosocial stress tasks in the afternoon (between 12:00 and 5:00 p.m.) have provided evidence that sAA levels in patients with anxiety disorders and MDD increase more than those of healthy controls (Tamura et al., 2013; Tanaka et al., 2012a, 2012b).

To our knowledge, five previous observational studies on sAA have been conducted in patients with mood and anxiety disorders: two in a laboratory setting and three in a naturalistic setting. The laboratory studies found low baseline sAA levels in MDD patients in the morning (Cubala and Landowski, 2014), and elevated sAA levels in the afternoon (Ishitobi et al., 2010) in comparison to healthy controls. Two naturalistic studies were conducted with MDD patients and found higher sAA levels in the morning (no time specified), afternoon (six hours later), and early evening (six hours

after the afternoon sample) in comparison to healthy controls (Booij et al., 2015), as well as elevated levels of sAA in MDD patients using tricyclic antidepressants (but not SSRIs) in the late evening (between 10:00 and 11:00 p.m; Veen et al., 2013). In a naturalistic study with generalized anxiety disorder (gSAD) patients, elevated sAA levels were found in the area under the curve with respect to the ground (AUCg) on day 1 and at 4:00 p.m. on day 2 in comparison to healthy controls (van Veen et al., 2008). In sum, based on the small amount of naturalistic observational research carried out to date, it seems morning, afternoon, and early evening sAA levels may differentiate between MDD and healthy controls, whereas late evening sAA levels are less successful in doing so. Furthermore, gSAD patients seem to have elevated AUCg and afternoon sAA values in comparison to healthy controls. However, no studies have investigated sAA levels in patients with MAS-disorders at awakening to date.

In this study, we investigated awakening and diurnal levels of basal sAA and SC in patients with MDD, other MAS-disorders, and healthy controls. Our main aim was to determine whether sAA may differentiate MDD from other MAS-disorders at certain time points. Due to the naturalistic nature of our study, SC levels were determined to compare our samples to the distinct diurnal pattern of SC found in earlier studies. Replicating these SC diurnal patterns indicate adherence to the sampling protocol to a certain extent, and thus increase the validity of the saliva samples collected. We hypothesized that MDD patients would have the same diurnal sAA patterns as those found in healthy controls. However, based on the existing literature, we expected that the morning levels of sAA would be higher in the MDD patient group than in the other MAS-disorders patient group and the healthy controls group. Furthermore, we expected to find an elevation of sAA in the AUCg in both the MDD and other MAS-disorders patient groups in comparison to healthy controls. Finally, as previous studies have found the differences in SC levels to be most pronounced after a low-dose dexamethasone-suppression test (DST; Pruessner et al., 2003a; Stetler and Miller, 2005; Vreeburg et al., 2009, 2010; Wardenaar et al., 2011), we expected both the MDD group and other MAS-disorders group to have higher SC levels in comparison to the healthy controls upon the DST.

2. Methods

2.1. Participants

Participants were recruited from two different sources: The Routine Outcome Monitoring (ROM) and NormQuest cohorts. Patients who were recruited participated in ROM interviews between 2007 and 2011. A detailed description of the ROM protocol has previously been published elsewhere (De Beurs et al., 2011).

These were ambulatory patients who were referred to one of three Riverduinen Mental Health Clinic locations (GGZ Leiden RijnVeste, GGZ Lisse, or GGZ Katwijk mental health clinics), or the Department of Psychiatry in the Leiden University Medical Center (LUMC). These patients all underwent extensive ROM interviews as part of routine patient care and the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM IV) diagnoses were assessed by means of the Mini International Neuropsychiatric Interview (MINI-Plus; van Vliet and De Beurs, 2007). Healthy controls were recruited from the NormQuest cohort, a study that was initiated to obtain a reference group for the ROM patients (Schulte-van Maaren et al., 2013). NormQuest participants were recruited from the general population via medical practitioners. The NormQuest sample was stratified for gender, age, and urbanization to reflect the ROM patient group. The majority of the complete Norm-Quest sample (> 90%) did not have a psychiatric diagnosis according to the Mini International Neuropsychiatric Interview (MINI-Plus; van Vliet and De Beurs, 2007).

All of the recruited participants were asked to collect seven saliva samples on a regular working day for our biobank for Mood, Anxiety, and Somatoform disorders and the HPA-axis (MASHbank). In line with the protocol used in the Netherlands Study of Depression and Anxiety (NESDA, Vreeburg et al., 2010), participants were instructed to refrain from eating, drinking, smoking, or brushing teeth within the preceding 15 min of sampling. The protocol was approved by the LUMC Ethical Review Board. Of the 949 saliva samples collected from the patient and reference groups, 44 forms were returned without a date or lacked a ROM interview date. Cases that sampled>=60 days after the ROM interview were also removed from the dataset (n=23) as were cases missing Brief Symptom Inventory (BSI; Derogatis, 1975) total scores (n=15). Furthermore, we excluded NormQuest controls using psychotropic medication (n=26), participants from all groups whose medication use was unknown (n=7), as well as all participants lacking demographic information (n=6), resulting in a total of 39 excluded subjects. The final sample (N=828) was then categorized into three groups, namely 594 controls, 95 MDD patients, and 139 “other MASdisorders” patients with predominately current anxiety and/or somatoform disorders (43 patients in this group either did not receive a diagnosis on the MINI-Plus or were diagnosed with other psychiatric disorders such as remitted MDD with comorbid alcohol and/or drug abuse).

2.2. Saliva sampling

To determine the diurnal patterns, seven samples were obtained by means of Salivettes® (Sarstedt, Germany). A Salivette is a cotton wad in a plastic tube.

Participants were asked to remove the wad from the tube, chew on it gently for at least 2 min, place the wad back into the tube, and store it in the refrigerator. The instructions stated to collect morning saliva prior to breakfast at four time points: at awakening (T1), after 30 min (T2), after 45 min (T3), and after 60 min (T4). The fifth (T5) and sixth (T6) samples were to be collected at 10:00 p.m. and 11:00 p.m. respectively. After the sixth sample, participants were asked to prepare for a DST by ingesting 0.5 mg of dexamethasone. At awakening the next morning, participants were asked to collect the final saliva sample (DST/ T7). At each time point, participants were instructed to write down the exact time that saliva was collected on the sampling form. After return by mail, Salivettes were centrifuged, and the saliva was aliquoted and stored at −80 °C.

2.3. Laboratory analysis of salivary cortisol and sAA

Cortisol analysis was performed by the Cortisol (gen 1) competitive electrochemiluminescence immunoassay (ECLIA, Modular E170 immunoanalyser, Roche Diagnostics, Basel, Switzerland), as described in van Aken et al. (2003). The functional detection limit was 2.0 nmol/l and the intra- and interassay variability coefficients in the measuring range were less than 10%. Assays were repeated if cortisol levels were very high (> 80 nmol/l) or very low (< 1 nmol/l). All very high samples remained high and the mean of both values was used. In 80% of the very low samples, the repeated cortisol value was within the normal range and used for analysis, otherwise the mean was used. Three cortisol indicators were used: 1-hour awakening cortisol; evening cortisol; and DST.

After overnight thawing of the saliva at 4 °C, samples for alphaamylase analysis were diluted 50-fold with a Hamilton Microlab 500 B/ C dilutor in physiological saline solution (Versylene® Fresenius, Cat. Nr. B230551). Analyses were performed using a kinetic colorimetric assay for total amylase activity (Cat Nr. 03183742, Roche Diagnostics, Mannheim, Germany) on a routine clinical chemistry analyzer. The total amylase assay is standardized to the IFCC reference measurement procedure (Lorentz, 1998), guaranteeing worldwide comparability of the data. Amylase activities are measured and expressed in IU/L at 37 °C. Within-run imprecision for the control pool ranged from 0.7% to 2.7% for the combined predilution step and the analysis across the entire study period. Between-run analytical imprecision was lower than 5% throughout this study.

2.4. Data cleaning

SC and sAA samples with values higher than 3 SD from the mean were denoted

as missing (SC n=66; sAA n=114) as they exceed the realistic range for SC and sAA and are likely to be the result of measurement issues (e.g. gingivitis or bleeding gums). Missing data was imputed for cases that remained in the dataset by means of linear regression for both missing SC values (n=170; 2.9%), and for sAA values (n=129; 2.2%). Participants missing>=2 morning saliva samples and/or>=1 evening saliva sample were excluded (cortisol n=27; sAA n=20). Participants that did not note a sampling time and those that sampled outside of the sampling time ranges (for details of sampling times, see Appendix) were also excluded (morning SC and sAA n=29; evening SC and sAA n=34). Participants that did not ingest the 0.5 mg of dexamethasone (n=87) were excluded from the SC DST analyses. No cases were removed from the sAA samples in this regard, as no negative feedback effect is to be expected upon DST.

2.5. Saliva sample indicators

Three sAA indicators were used: the AUCg, and with respect to the increase (AUCi), and evening sAA levels. The AUCg and the AUCi were calculated in accordance with Pruessner's et al. formulas (2003b), and evening sAA levels were calculated using the T5 and T6 samples (T5+T6/2). Five salivary cortisol indicators were used: two measures reflecting the cortisol awakening response (CAR), the evening cortisol value, DST, and the cortisol suppression ratio (CSR). The CAR measures the increase in cortisol over four fixed time points within the first hour after awakening (e.g. T1-T4; Fries et al., 2009). These morning cortisol values allow for the calculation of the AUCg, and AUCi, which are used to reflect the dynamics of the CAR (Pruessner et al., 2003b; Fekedulegn et al., 2007). The low-dose DST assesses the (dis-)inhibition of the HPAaxis (Carroll et al., 1976), and the CSR indicates the cortisol suppression ratio (T1/T7).

2.6. Other measurements and covariates

The BSI (Derogatis, 1975), is a 53-item self-report questionnaire that was designed as a rapid screening method for symptoms of psychological disorders. In this study, the BSI total score was used as an index of general psychopathology. The total score is the mean score of all of the 53 items, whereby each item can range between 0–4 (a higher score indicates more general psychopathology). The BSI has been found to be both a reliable and valid measurement instrument (Croog et al., 1986; Conoley and Kramer, 1989).

Certain specific factors have been found to influence sAA levels and should be taken into account as potential confounders in epidemiological studies. There is evidence that carbohydrate intake increases sAA response in individuals whose ancestors

consumed starch-rich diets. Thus, those of Southern European ancestry experience a larger increase in sAA levels in comparison to those of Northern European ancestry (Perry et al., 2007; Rigaud et al., 2015). Furthermore, a study conducted in a sample of healthy participants (N=487), found that sAA levels are influenced by age and alcohol use (Veen et al., 2012). In addition to ancestry status, age was entered as a continuous variable and alcohol use the day of testing as a dichotomous variable. Additionally, we adjusted for gender and education level (no/low education versus middle/high education). Analysis of covariance (ANCOVA) was used to adjust for these sociodemographic factors.

Regarding SC, the following covariates were entered into the model: gender, age, Northern European (NE) ancestry, education level (to adjust for social economic status; no/low education versus middle/high education), season (more daylight hours versus less daylight hours), time of awakening (continuous), hours of sleep (< 6 h of sleep,>6 h of sleep),weekday versus weekend, and alcohol use the day of testing in adherence, where possible, to the expert consensus guidelines denoted by Stalder et al. (2016) to yield adjusted means.

2.7. Statistical analysis

Both the cortisol values and the sAA values showed positively skewed distributions and were therefore naturally loge transformed before analyses. Back-transformed geometric means and 95% confidence intervals (CI) of the mean are presented in the tables. The baseline characteristics of the three groups (MDD, other MAS-disorders, and healthy controls) were compared by means of $\chi 2$–tests for categorical variables and analyses of variance statistics (ANOVA) for continuous variables. These variables were expressed by means, percentages, and standard deviations (see Table 1 for further details). A twosided p-value<0.05 was considered statistically significant. No adjustments were made for multiple comparisons. IBM SPSS Statistics for Windows version 23 (IBM Corp. Armonk, N.Y., USA) was used for dataanalysis.

3. Results

3.1. Demographic characteristics

Characteristics of the three groups, (i) MDD patients (N=95, of whom 6 patients were diagnosed with dysthymia), (ii) patients with other MAS-disorders (N=139), and (iii) healthy controls (N=594), are presented in Table 1. The mean age of this sample was 43.8 years (SD=12.9), of which 63.9% was female. Gender distribution was similar amongst the three groups. MDD patients and patients with the other MAS-disorders

were more often less educated, less often of Northern European ancestry, less likely to have a partner, less likely to have a job, more likely to smoke, sleep fewer hours at night, and collect samples more often during the weekend in comparison to healthy controls. Use of alcohol on sampling days, sampling season, and time of awakening did not differ significantly between the groups. MDD patients had higher mean BSI total scores in comparison to both of the other groups. Of the patients in the MDD group, 34 patients had a comorbid anxiety disorder (35.1%), 8 patients had a comorbid somatic disorder (8.2%), and 11 patients had both disorders (11.3%). Regarding the time-interval between the interview and saliva sampling, patients in the other MAS-disorders group had the most days between the interview and saliva sample return, followed by the MDD group. The median time between the ROM interview and the sampling was 7.0 days (25th-75th percentile: 3–14). Regarding medication use, patients in the MDD group used more medication than those in the other MAS-disorders group (see Table 1).

3.2. Salivary alpha-amylase (sAA)

Comparisons of sAA levels between groups are presented in Table 2. sAA levels were found to be significantly elevated in the MDD group at T1, AUCi, and T7 ($p= 0.01$; $p= 0.04$; $p < 0.001$), in comparison to the sAA levels in healthy controls (see Fig. 1A). After multivariate adjustment (i.e. adjusted for gender, age, Northern European ancestry, education level, and alcohol use), these sAA levels remained significantly elevated in comparison to the control group (T1: $p= 0.04$ AUCi: $p= 0.04$; T7: $p= 0.01$ respectively). sAA levels in the other MAS-disorder group were lower than those of healthy controls at all time points, although none of these differences were statistically significant. These differences remained statistically insignificant after adjusting for confounders (see Table 2). Pearson correlations between mean sAA levels at awakening were found to be significantly associated with BSI depression subscale scores ($p=0.015$), indicating that there is a relationship between sAA levels and depression symptom severity. Finally, sAA levels were able to differentiate between the MDD group and the other MAS-disorders group at these sampling points after multivariate adjustment (T1: $p= 0.005$; AUCi: $p= 0.009$; T7: $p= 0.01$).

Table 1. Comparison of demographic and clinical characteristics between controls, patients with MDD, and patients with other MAS-disorders.

Variables	Controls (n = 594)	MDD[a] (n = 95)	Other[a] (n = 139)	p-value[c]
Sociodemographic characteristics:				
Female (%)	64.1	66.3	61.2	0.70
Age in years (mean, SD)	42.8 ± 12.5	47.2 ± 13.0	45.4 ± 13.6	0.50
Higher educational (%)	75.6	57.9	66.2	< 0.001
Northern European ancestry (%)	86.9	74.7	81.3	0.005
Marital status with partner (%)	73.7	55.8	67.6	0.001
Employment status (%):				
Employed (full-/part-time)	83.3	38.9	54.0	< 0.001
Unemployed/Sick leave	5.4	38.9	21.6	
Retired/stay-at-home parent	11.3	22.1	24.5	
Smokers n (%)[d]	80 (13.7)	27 (30.3)	29 (21.5)	< 0.001
Alcohol use on sampling days (%)				
Yes	69.3	79.8	71.6	0.13
No	30.7	20.2	28.4	
Sampling Factors:				
Time of awaking (mean, SD) [d]	6:57 ± 0:58	7:10 ± 1:03	7:08 ± 0:52	0.36
Hours of sleep (%)[d]				
< 6 hours	16.1	36.3	26.1	< 0.001
> 6hours	83.2	61.5	72.5	
Workday (%)[d]	77.0	44.0	54.8	< 0.001
Light season (April-September)	46.3	41.1	47.5	0.58
Clinical characteristics n (%):				
current comorbid anxiety disorder	–	35 (36.8)	6 (4.3%)	
current comorbid somatic disorder	–	8 (8.4)	6 (4.3%)	
current comorbid anxiety and somatic disorder	–	10 (10.5)	6 (4.3%)	
BSI total score (mean, SD)	0.18 ± 0.19	1.37 ± 0.67	0.59 ± 0.47	< 0.001
Assessment characteristics:				
Time interval between assessment and saliva sampling (days, SD)	9.2 ± 9.1	10.6 ± 11.5	12.4 ± 12.7	0.03
Medication use n (%)[b]:				
Using any psychotropic medication	–	49 (51.6)	31 (22.3)	< 0 .001
TCAs	–	5 (5.3)	3 (2.2)	< 0.001
SSRIs	–	25 (26.3)	14 (10.1)	< 0.001
Benzodiazepines	–	15 (15.8)	11 (7.9)	< 0.001
Stimulants	–	0 (0.0)	0 (0.0)	–
Antipsychotics	–	5 (5.3)	3 (2.2)	< 0.001
Stabilizers	–	3 (3.2)	3 (2.2)	< 0.001
Other ADs	–	16 (16.8)	3 (2.2)	< 0.001

[a] MDD denotes the patient group with current Major Depressive Disorder and/or current Dysthymia (n = 89 and n = 6 respectively). Other denotes the patient group with remitted mood disorders and/or other (comorbid) MAS-disorders.

[b] TCA denotes Tricyclic Antidepressant; SSRI denotes Selective Serotonin Reuptake Inhibitor; Other AD denotes other Antidepressants.

[c] P-values were achieved by using χ^2-tests for categorical variables and analyses of variance statistics (ANOVA) for continuous variables.

[d] smoking information was missing for 2.2% of the sample; alcohol intake information was missing for 3.4% of the sample; Time of awakening was missing for 2.4% of the sample; hours of sleep information was missing for 1.0% of the sample; workday information was missing for 2.2% of the sample.

Table 2. Comparison of morning and evening salivary alpha amylase (sAA) variables between controls and MDD patients and controls and other MAS-disorders patients.

Variables	n	Controls	n	MDD patients	p-value	n	Other MAS-disorders	p-value
Crude values								
Morning sAA variables								
T1 (U/ml) at awakening	554	63.8 (57.8; 70.4)	83	90.9 (71.8; 115.1)	**0.01**	129	55.2 (45.3; 67.4)	0.23
T2 (U/ml) 30' after awakening	554	37.3 (34.4; 40.6)	83	46.0 (37.6; 56.2)	0.07	129	34.9 (30.3; 40.3)	0.48
T3 (U/ml) 45' after awakening	554	40.1 (37.1; 43.5)	83	43.8 (36.3; 52.9)	0.43	129	36.5 (31.6; 42.2)	0.30
T4 (U/ml) 60' after awakening	554	44.4 (41.0; 48.0)	83	48.5 (38.0; 62.0)	0.44	129	40.2 (34.4; 47.0)	0.28
AUC_g (U/ml)	554	44.4 (41.2; 47.9)	83	54.3 (45.3; 64.9)	0.06	129	40.4 (35.5; 46.0)	0.21
AUC_i (U/ml) [a]	554	−38.3 (-44.4; -32.2)	83	−60.0 (-80.3; -39.7)	**0.04**	129	−30.9 (-41.3; -34.2)	0.28
Evening sAA variables								
T5 (U/ml) at 10 PM	553	101.7 (94.0; 109.9)	85	108.5 (86.4; 136.3)	0.56	124	92.6 (79.2; 108.1)	0.31
T6 (U/ml) at 11 PM	553	79.3 (72.9; 86.4)	85	98.2 (79.0; 122.1)	0.07	124	75.5 (63.3; 89.9)	0.62
Evening cortisol (U/ml)	553	89.8 (83.1; 97.1)	85	103.2 (83.2; 128.0)	0.21	124	83.6 (71.9; 97.2)	0.43
T7 (U/ml), at awakening	549	63.7 (57.6; 70.5)	83	97.7 (79.7; 119.7)	**< 0.001**	127	61.8 (50.2; 76.1)	0.80
Adjusted values [b]								
Morning sAA variables								
T1 (U/ml) at awakening	542	66.1 (60.1; 72.7)	82	86.9 (67.9; 111.4)	**0.04**	128	55.4 (45.5; 67.4)	0.11
T2 (U/ml) 30' after awakening	542	38.3 (35.4; 41.5)	82	43.9 (35.7; 54.0)	0.23	128	34.6 (29.3; 40.7)	0.27
T3 (U/ml) 45' after awakening	542	41.6 (38.5; 44.8)	82	41.9 (34.3; 51.0)	0.95	128	36.1 (30.9; 42.2)	0.11
T4 (U/ml) 60' after awakening	542	45.2 (41.7; 49.0)	82	46.4 (37.7; 57.2)	0.81	128	40.0 (33.9; 47.1)	0.19
AUC_g (U/ml)	542	45.8 (42.6; 49.2)	82	51.8 (43.0; 62.4)	0.22	128	40.0 (34.6; 46.4)	0.11
AUC_i (U/ml) [a]	542	−39.5 (-45.6; -33.4)	82	−57.1 (-72.9; -41.3)	**0.04**	128	−30.5 (-43.0; -17.9)	0.20
Evening sAA variables								
T5 (U/ml) at 10 PM	543	103.5 (95.6; 111.9)	85	107.0 (87.6; 130.9)	0.75	123	92.9 (78.8; 109.4)	0.25
T6 (U/ml) at 11 PM	543	81.5 (74.8; 88.7)	85	94.1 (75.8; 117.0)	0.23	123	74.9 (62.7; 89.5)	0.41
Evening sAA (U/ml)	543	91.8 (85.0; 99.2)	85	100.4 (82.4; 122.4)	0.41	123	83.4 (70.9; 98.1)	0.30
T7 (U/ml) at awakening	537	66.0 (59.9; 72.8)	82	92.7 (72.0; 119.4)	**0.01**	126	61.0 (49.8; 74.6)	0.48

Table shows sample sizes, back-transformed geometric means and 95% confidence intervals (CI) of the mean.

[a] These geometric means were not back-transformed as the variable was normally distributed.

[b] Adjusted for gender, age, Northern European ancestry, education level, and alcohol use.

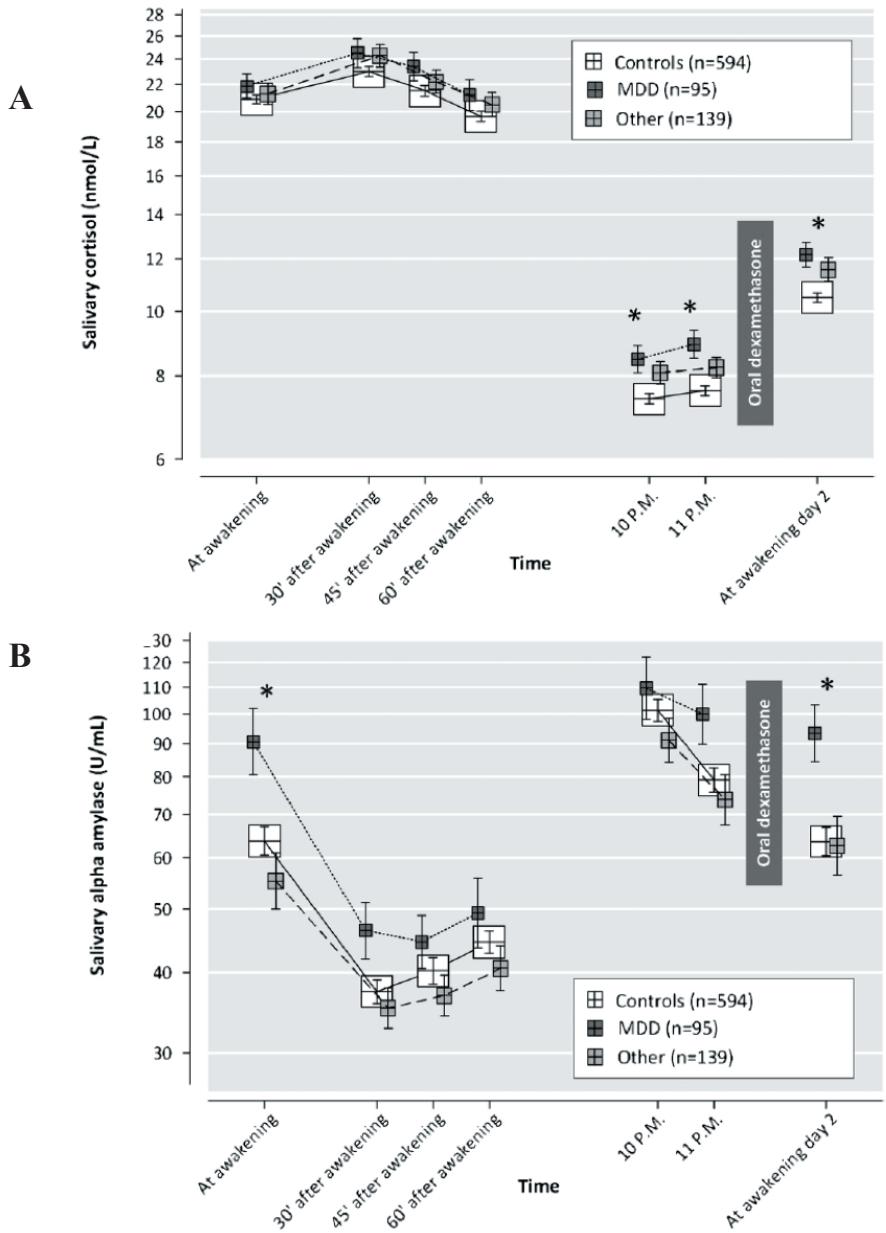

Figure 1. Unadjusted mean values of salivary alpha amylase [A] and salivary cortisol [B] levels according to psychiatric diagnoses presented in Table 1. Figure A and B show awakening, diurnal, and awakening day 2 sAA and SC levels (± S.E.M.) in MDD patients, patients with other MAS-disorders, and healthy controls. Error bars represent standard errors. * = $p < 0.05$.

3.3. Salivary cortisol

Comparisons of SC levels between groups are presented in Table 3. We found no significant differences with regard to the CAR between the three groups in the unadjusted model. Comparisons of unadjusted evening SC levels between the three groups showed that patients in both the MDD group and other MAS-disorders group presented significantly higher T5 ($p= 0.004$; $p= 0.02$), T6 ($p < 0.001$; $p= 0.03$), and mean evening cortisol levels ($p < 0.001$; $p= 0.01$). DST levels were also elevated in both patient groups ($p= 0.001$; $p= 0.02$ respectively) in comparison to the control group (see Table 3 and Fig. 1B). Patients in the MDD group also had a significantly elevated CSR in comparison to the other MAS-disorders group and healthy controls ($p= 0.03$). After multivariate adjustment (i.e. adjusted for gender, age, Northern European ancestry, education level, season, time of awakening, hours of sleep, weekday versus weekend, and alcohol use), SC levels at T6 and mean evening cortisol levels remained significantly higher in the MDD group (T6: $p= 0.04$; mean evening cortisol: $p= 0.049$) in comparison to the other MAS-disorders group and the healthy controls group.

DST remained significantly elevated in both the current MDD group and the other MAS-disorders group in comparison to the control group ($p= 0.04$ and $p= 0.047$; see adjusted values in Table 3). There were no significant differences in SC levels between the MDD group and the other MAS-disorders group at any of the sampling times.

Table 3. Comparison of morning and evening cortisol variables between controls and MDD patients and controls and other MAS-disorders patients.

Variables	n	Controls	n	MDD/DYS	p–value	n	Other MAS-disorders	p–value
Crude values								
Morning cortisol variables								
T1 (nmol/L) at awakening	555	20.9 (20.3; 21.5)	84	21.8 (20.0; 23.7)	0.33	132	21.3 (19.8; 22.9)	0.61
T2 (nmol/L) 30' after awakening	555	22.8 (22.1; 23.6)	84	24.1 (22.0; 26.5)	0.24	132	24.4 (22.5; 26.3)	0.09
T3 (nmol/L) 45' after awakening	555	21.4 (20.7; 22.1)	84	23.4 (21.2; 25.7)	0.07	132	22.4 (20.7; 24.2)	0.27
T4 (nmol/L) 60' after awakening	555	19.5 (18.9; 20.2)	84	20.9 (18.9; 23.1)	0.17	132	20.6 (19.0; 22.4)	0.20
AUC_g (nmol/L/h)	555	21.5 (21.0; 22.1)	84	22.9 (21.2; 24.7)	0.11	132	22.6 (21.1; 24.2)	0.20
AUC_i (nmol/L/h)[a]	555	1.0 (0.4; 1.6)	84	1.7 (-0.5; 3.8)	0.42	132	1.8 (0.6; 3.0)	0.22
Evening cortisol and DST variables								
T5 (nmol/L) at 10 PM	555	7.4 (7.1; 7.6)	87	8.5 (7.7; 9.3)	**0.004**	125	8.1 (7.5; 8.8)	**0.02**
T6 (nmol/L) at 11 PM	555	7.6 (7.3; 7.8)	87	9.0 (8.1; 9.9)	**< 0.001**	125	8.3 (7.7; 8.9)	**0.03**
Evening cortisol (nmol/L)	555	7.5 (7.3; 7.7)	87	8.7 (8.0; 9.5)	**< 0.001**	125	8.2 (7.6; 8.8)	**0.01**
DST (nmol/L), at awakening	522	10.5 (10.2; 10.8)	85	12.2 (11.2; 13.2)	**0.001**	125	11.6 (10.7; 12.6)	**0.02**
CSR (nmol/L)	566	3.8 (3.7; 3.9)	86	3.6 (3.5; 3.8)	**0.03**	135	3.7 (3.5; 3.9)	0.18
Adjusted values[b]								
Morning cortisol variables								
T1 (nmol/L) at awakening	540	21.1 (20.4; 21.7)	82	21.3 (19.6; 23.2)	0.79	129	20.9 (19.6; 22.3)	0.82
T2 (nmol/L) 30' after awakening	540	29.9 (22.1; 23.7)	82	23.9 (21.8; 26.2)	0.50	129	24.5 (22.8; 26.3)	0.14
T3 (nmol/L) 45' after awakening	540	21.4 (20.6; 22.2)	82	23.3 (21.2; 25.6)	0.10	129	22.6 (21.0; 24.3)	0.19
T4 (nmol/L) 60' after awakening	540	19.5 (18.8; 20.2)	82	21.1 (19.2; 23.2)	0.13	129	21.0 (19.5; 22.6)	0.07
AUC_g (nmol/L/h)	540	21.6 (21.0; 22.3)	82	22.7 (21.0; 24.5)	0.26	129	22.6 (21.3; 24.0)	0.20
AUC_i (nmol/L/h)[a]	540	0.9 (0.3; 1.5)	82	1.9 (0.3; 3.5)	0.27	129	2.2 (1.0; 3.5)	0.06
Evening cortisol and DST variables								
T5 (nmol/L) at 10 PM	540	7.5 (7.2; 7.8)	86	8.1 (7.4; 8.9)	0.12	123	8.0 (7.4; 8.6)	0.13
T6 (nmol/L) at 11 PM	540	7.7 (7.5; 8.0)	86	8.5 (7.8; 9.3)	**0.04**	123	8.1 (7.6; 8.7)	0.22
Evening cortisol (nmol/L)	540	7.6 (7.4; 7.8)	86	8.3 (7.7; 9.0)	**0.049**	123	8.0 (7.5; 8.6)	0.14
DST (nmol/L) at awakening	504	10.6 (10.2; 10.9)	78	11.7 (10.7; 12.7)	**0.04**	118	11.4 (10.7; 12.2)	**0.047**
CSR (nmol/L)	551	3.8 (3.7; 3.9)	84	3.6 (3.4; 3.8)	0.07	132	3.7 (3.5; 3.8)	0.18

Table shows sample sizes, back-transformed geometric means and 95% confidence intervals (CI) of the mean.

[a] These geometric means were not back-transformed as the variable was normally distributed.

[b] Adjusted for gender, age, Northern European ancestry, education level, season, time of awakening, hours of sleep, weekday versus weekend, and alcohol use.

4. Discussion

The current study examined the characteristics of diurnal sAA and SC in a naturalistic sample of psychiatric out-patients and healthy controls. We found consistent elevated sAA levels at awakening over two days in MDD patients in comparison to healthy controls and patients with other MAS-disorders, indicating increased early morning sympathetic nervous system activation in this patient group. Evening SC at 11.00 p.m. and mean evening SC levels were found to be significantly increased in MDD patients. Elevated levels of SC after dexamethasone ingestion on day two were found in both the MDD patient group and in the other MAS-disorder group. Our findings suggest that sAA levels at awakening may be a putative new candidate biomarker for MDD specifically.

Research has indicated that the parotid glands contribute most to sAA, reflecting ANS, and in particular sympathetic nervous system (SNS) activation via synergistic sympathetic-parasympathetic interactions (Bosch et al., 2011). Increases of sAA in MAS-patients have been recorded in response to both psychological and physical stress (Schumacher et al., 2013), suggesting that sAA may be a promising marker of SNS dysregulations in MAS-disorders in general. However, previous studies investigating sAA levels in the MAS-disorder patient population have been few, whereof none have determined the diurnal profile of sAA (Tamura et al., 2013; Tanaka et al., 2012a, 2012b; van Veen et al., 2008; Cubala and Landowski, 2014; Booij et al., 2015; Ishitobi et al., 2010; Veen et al., 2013). Moreover, the study designs employed have been heterogeneous. For example, sample sizes ranged from 30 (Booij et al., 2015) to 1683 participants (Veen et al., 2013), sampling settings differed (i.e. naturalistic setting versus laboratory setting), as did sampling times (morning, afternoon, early evening, late evening), numbers of samples collected (1–90), and patient populations (MDD, remitted MDD, gSAD patients, panic disorder, and healthy controls).

Our study is the first to investigate sAA levels at awakening in a larger sample of MDD patients and patients with other MAS-disorders. This, along with the multiple sampling points in the morning and in the evening, makes it possible to extend our current knowledge regarding naturalistic sAA levels in patients with MAS-disorders. To date, three previously conducted naturalistic studies investigating sAA levels in MDD patients found elevated sAA levels in MDD patients in comparison to healthy controls, which is partially in line with the results of this study. Specifically, significantly elevated morning, afternoon, and early evening sAA levels were found

in MDD patients in comparison to healthy controls (Booij et al., 2015), significantly elevated afternoon sAA levels in MDD patients in comparison to healthy controls (Ishitobi et al., 2010), and significantly elevated levels of late evening sAA in MDD patients using tricyclic antidepressants in comparison to MDD patients not using this medication and healthy controls (Veen et al., 2013). We were unable to replicate these results with regard to potential differences in sAA levels between participants using TCAs and those who were antidepressant medication free, as our sample size consisted of only 8 participants using TCAs (n=5 in the MDD group and n=3 in the other MAS disorders group). A sensitivity analysis excluding these participants did not alter our results significantly. We were able to replicate the absence of differences in sAA levels between MDD patients and healthy controls in the late evening (Veen et al., 2013). One study found significantly lower morning sAA levels in MDD patients in comparison to healthy controls (Cubala and Landowski, 2014). We did not find any significant differences between the groups in morning sAA levels, with the exception of the novel finding of differences between the groups directly after awakening.

With regard to SC, we did not find any differences in morning SC levels between MDD patients, patients with other MAS-disorders, and healthy controls. This is not surprising as study findings have shown a high level of variability regarding CAR activity. On the one hand, studies have found similar morning SC concentrations in depressed patients and healthy controls (e.g. Strickland et al., 2002), as was the case in the current study. On the other hand, studies have also found a blunted cortisol response in patients with MDD in comparison to healthy controls (Burke et al., 2005; Stetler and Miller, 2005). Again, other studies have found an increased CAR for both AUCg and AUCi variables in MDD patients in comparison to healthy controls (e.g. Bhagwagar et al., 2005; Pruessner et al., 2003a; Vreeburg et al., 2009; Wardenaar et al., 2011). Our study did find significantly elevated levels of evening cortisol in both the MDD patient group as well as other MAS-disorders patient groups in comparison to healthy controls before adjustment. This difference remained significant for the MDD group after adjusting for influential covariates (Stalder et al., 2016). These results support the findings from two earlier studies conducted with MDD patients and healthy controls in which evening cortisol levels were found to be (partially) increased in depressed subjects (Young et al., 1994; Vreeburg et al., 2009). We also found that SC levels post-dexamethasone intake remained elevated in both the MDD patient group and other MAS-disorders group after adjustment for covariates. These findings provide further evidence for differences in HPA stress-axis regulation among patients with MAS-disorders and healthy controls, however again highlight

the inability of SC to clearly distinguish between MAS disorders.

Our findings are relevant to the ongoing search for possible biomarkers differentiating between MAS-disorders as it seems that basal sAA levels at awakening may distinguish MDD patients from patients with other MAS-disorders. The identification of a putative biomarker that might distinguish MDD from other psychiatric disorders has both scientific and clinical implications in that it may improve our understanding of MDD pathophysiology, may function as an aid in predicting or interpreting response to treatment, and may help in improving current MDD criteria.

We believe that a strength of our study was that we included a MDD and other MAS-disorders, therefore likely reflecting the general MAS-disorder patient population to a reasonable extent and thus increasing the external validity of the findings. Secondly, we were also able to compare our clinical sample to a large sample of healthy controls that were specifically recruited as a reference group for the ROM participants, from which the patient sample in this study was derived. Thirdly, the two saliva samples collected directly after awakening on day 1 and day 2 allowed us to verify our initial finding of sAA differentiating between MDD patients and patients with other MAS-disorders and healthy controls by means of our day 2 sample. Finally, we collected saliva samples using a non-invasive and inexpensive sampling method, which is suitable for clinical use (Nater and Rohleder, 2009).

Our study has a number of potential limitations that should be acknowledged. Firstly, due to the cross-sectional nature of this study we cannot determine the directionality of the relationship between sAA and MDD. However, findings of a prior study within remitted MDD patients (Bagley et al., 2011) seem to suggest that increased sAA levels reflect a state of current depression rather than a vulnerability factor. Secondly, in an ambulatory and naturalistic setting, compliance is more difficult to monitor than in a systematic clinical trial. Although strict instructions were given to the participants regarding protocol adherence (i.e. to refrain from eating, drinking, smoking, or brushing teeth within the preceding 15 min of sampling alongside the importance of adhering to the sampling time frames), we did not ask our participants to refrain from other factors such as the consuming of antioxidants and napping before sampling, as the potential bias through these factors had not yet been well established at the time of data collection. However, these factors are unlikely to influence sAA and SC concentrations at awakening. Additionally, and in accordance with the Salivette® instructions for use manual, participants were instructed to gently chew on the swab for at least two minutes.

This may have affected the levels of sAA recorded as chewing has been found to increase salivary flow rate and in turn sAA concentrations (Bosch et al., 2011). As we did not measure salivary flow rate we were unable to take this into account. Passive drool methods for saliva sampling (DeCaro, 2008) may have yielded more accurate values, although outcomes with regard to whether or not salivary flow rate is independent of stress-induced sAA increase have been inconsistent and evidence has been found supporting both theories (Rohleder et al., 2006; Bosch et al., 2003; Beltzer et al., 2010). Therefore, salivary flow rates should be taken into account in future studies. It should be noted that we instructed our participants to keep the swab in their mouths for two minutes, and research has indicated decreases in sAA concentration and output within the first several minutes of chewing-induced secretion (Proctor and Carpenter, 2001). Moreover, an objective verification method of participants' awakening time (e.g. by means of wrist actigraphy), as well as electronic monitoring devices may have provided more accuracy in the cortisol assessments after awakening (e.g. Broderick et al., 2004; Stalder et al., 2016). However, this was not feasible in the current study, and may therefore have led to some (random) measurement error. A possible explanation for not finding any significant group differences in morning SC parameters could be due to errors in timing. However, a sensitivity analysis excluding samples with less strict timeframes (Stalder et al., 2016) was conducted and the AUCg and AUCi findings remained non-significant. Furthermore, although the samples were collected in a naturalistic setting, we found the distinct diurnal patterns of sAA and SC found in earlier studies (e.g. Nater et al., 2007; Rohleder et al., 2004; Kirschbaum and Hellhammer, 1989), supporting the validity of our results. A further potential limitation may be that we were unable to adequately adjust for psychotropic medication use in our analyses as there were no participants who used psychotropic medication in the healthy control group. Also, information with regard to (childhood and adulthood) trauma exposure was lacking for a large portion of the sample and we could therefore not take this into account. However, although several studies have found that trauma-exposed individuals experience an increase in cortisol levels (e.g. Pico-Alfonso et al., 2004; Klaassens et al., 2010a, 2010b), other studies did not find this effect (e.g. de Kloet et al., 2007; Rohleder et al., 2004; Gill et al., 2008). Moreover, a meta-analysis found that neither adulthood trauma nor PTSD were associated with differences in HPA-axis functioning (Klaassens et al., 2012). Still, this meta-analysis did not look into the effect of childhood trauma exposure on HPA-axis functioning. It is therefore of importance to include information on trauma exposure in future studies to further explore the possible relationship between trauma and HPA-axis and ANS-axis activation. A further limitation is that we did not record the day that

saliva samples were posted by participants and were therefore unable to correct for possible warming effects during postal delivery. However, if this were to have an effect it is likely to have added random measurement error, and thus result in findings approaching the null hypothesis. Finally, collecting samples at more time points over a number of days would have reflected the diurnal patterns of SC and sAA more accurately and further increased the reliability of the SC and sAA levels measured at each time point (Hellhammer et al., 2007).

In conclusion, our findings support earlier findings that sAA levels are increased in patients with current MDD and offer further evidence towards the recently emerging hypothesis that sAA may be a potential biomarker for MDD. Although this study indicates that sAA levels are higher at awakening only in patients with MDD, there is a possibility that these levels are also higher in patients with other psychiatric disorders. Further research is necessary in this regard. Future studies should replicate the elevated awakening sAA levels found in MDD patients in comparison to other MAS-disorders using larger sample sizes to further substantiate the validity of these findings. Furthermore, creating expert consensus guidelines with regard to assessing sAA, as has been done for the CAR (Stalder et al., 2016), may be supportive in obtaining consistent data in future studies. Also, samples should be collected at more time points throughout the day (i.e. during the course of the afternoon and the early evening), and ideally also over more days (Hellhammer et al., 2007).

Appendix

Sampling time frames morning:
Time between T1 and T2< =60 and>=15 min, time between T2 and T3< =45 min and>=5 min, time between T3 and T4< =45and>=5 min, and time between T1 and T4< =90 and>=44 min.

Sampling time frames evening:
Time between T5 and T6< =120 min and>=0 min.

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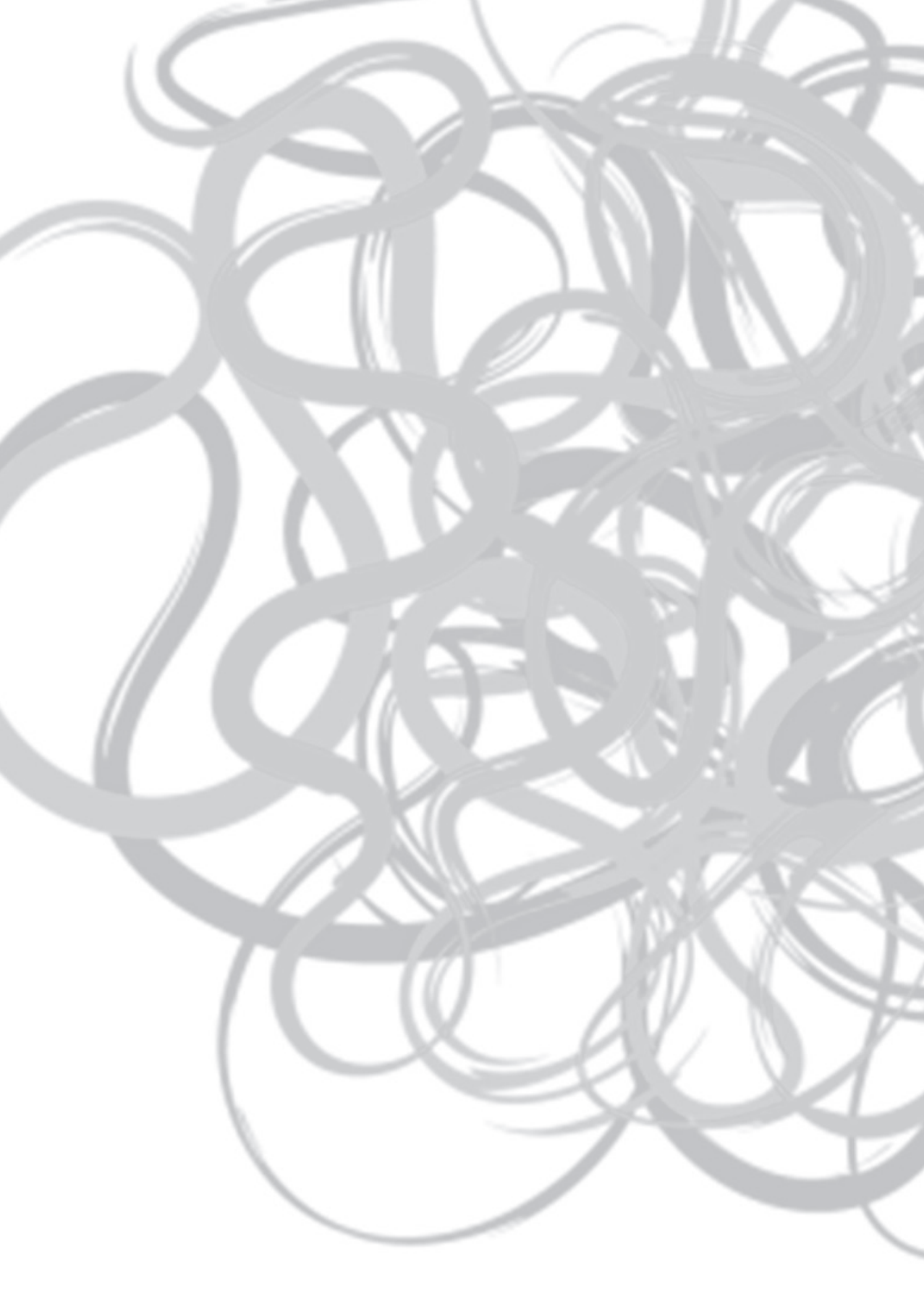

Chapter 3

Salivary markers of stress system activation and social withdrawal in humans

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Abstract

Social withdrawal is an early and common feature of psychiatric disorders. Hypothalamic-pituitary-adrenal (HPA)-axis activation through increased salivary cortisol (sC) and sympathetic activation through increased salivary alpha-amylase (sAA) may play a role. We aimed to study whether the link between increased sC and sAA on the one hand and depression on the other hand is mediated by social withdrawal. In this cross-sectional, observational study, sC and sAA measures were measured in seven saliva samples in 843 participants (231 psychiatric patients and 612 healthy controls). Social withdrawal was assessed through the Brief Symptom Inventory (BSI)-, the Short Form 36-, and the Dutch Dimensional Assessment of Personality Pathology social withdrawal subscales, and analyzed using linear regression and mediation analyses. On average, participants were 44.0 years old (SD=12.8; 64.1% female). Basal and diurnal sAA were unrelated to any social withdrawal scale and depression. Certain sC measures were positively associated with the BSI social withdrawal subscale (i.e., area under the curve with respect to the increase, beta=0.082, p=0.02; evening sC value: beta=0.110, p=0.003; and mean sC value: beta=0.097; p=0.01). We found limited support for statistical mediation by social withdrawal (measured using a composite social withdrawal score) on the relationship between evening sC and depression. Thus, although we found no support for a role of basal and diurnal sAA in social withdrawal, HPA-axis activation may partly aggravate social withdrawal in depressive disorders.

1. Introduction

Social withdrawal, or withdrawal from family, friends, and colleagues, is a common feature of several psychiatric disorders, such as major depressive disorder (Saris et al., 2017; Bora & Berk, 2016; Kupferberg, Bicks, & Hasler, 2016), anxiety disorders (Saris et al., 2017) , and schizophrenia (Galderisi, Mucci, Buchanan, & Arango, 2018), and is also one of the earliest signs of these disorders (e.g. Cross, Scott, & Hickie, 2017; Nelis et al., 2011). In line with the National Institute of Mental Health Research Domain Criteria (RDoC) project aimed at identifying new ways of classifying psychiatric disorders based on dimensions of (neuro-)biological measures and observable behavior, social withdrawal has been posited to be related to a more stable endophenotype that is more closely connected to biological pathways than psychiatric disorders are (Porcelli et al., 2018; van der Wee et al., 2018). Increased social withdrawal can lead to poor social functioning and social isolation, and can in turn cause feelings of loneliness. Loneliness prevalence of people reporting frequent feelings of loneliness in European countries has been found to range from approximately 5-7% in the West and North to approximately 11% in the East, with an overall European average of 7.9% (d'Hombres, Schnepf, Barjakovà, & Teizeira, 2018). Constructs such as social withdrawal, social isolation, poor social functioning, and loneliness partially overlap. However, for ease of interpretation, social withdrawal will be used as an umbrella term throughout this paper.

Several psychiatric disorders, such as MDD, have been associated with heightened inflammation (Najjar et al., 2013). Inflammatory activity has been found to enhance amygdala activity (a region involved in social withdrawal in animals and associated with social avoidance in humans) to socially threatening images, implicating amygdala activity involvement in sickness-induced social withdrawal (Inagaki et al., 2012). Brain-derived neurotrophic factor (BDNF), dopamine and their receptors likely play important roles, as is supported by experimental studies in rodents investigating the biology of social withdrawal (Campi et al., 2014; Greenberg et al., 2014).

Whereas low levels of social withdrawal have been found to be positively related to longevity, physical-, psychological-, and emotional well-being (Holt-Lunstad, Smith, & Layton, 2010; Uchino, 2006), high social withdrawal has been associated with severe detrimental health outcomes, such as depression (Cacioppo, Hawkley, & Thisted, 2010; Cacioppo, Hughes, Waite, Hawkley, & Thisted, 2006), adverse coronary condition rates (Holt-Lunstad, Smith, Baker, Harris, & Stephenson, 2015; Patterson & Veenstra, 2010; Sorkin, Rook, & Lu, 2002), alcoholism (Qualter et al., 2015), increased mortality rates (Holt-Lunstad et al., 2015; Patterson & Veenstra,

2010; Sorkin, Rook, & Lu., 2002), increased suicidality (Conroy & Smith, 1983; Peck, 1983), and Alzheimer's disease (Wilson et al., 2007). Additionally, associations between social withdrawal and alterations in hypothalamic-pituitary-adrenocortical (HPA) axis activity have been found (Adam, Hawkley, Kudielka, & Cacioppo, 2006; Steptoe, Owen, Kunz- Ebrecht, & Brydon, 2004). Thus, it seems that the dimension of social withdrawal may aid in linking overlapping biological underpinnings across several conditions (van der Wee et al., 2018; Porcelli et al., 2018). The identification of dimensional behavioral phenotypes across disorders may help to deepen our understanding of the biology involved and complements the approach to incorporate dimensional measures as in the DSM-5 system.

Social withdrawal has often been associated with HPA-axis activation (Adam, Hawkley, Kudielka, & Cacioppo, 2006; Arnetz, Theorell, Levi, Kallner, & Eneroth, 1983; Doane & Adam, 2010; Grant, Hamer, & Steptoe, 2009; Hawkley, Cole, Capitanio, Norman, & Cacioppo, 2012; Pressman et al., 2005), although certain findings were not consistent with this (Cacioppo et al., 2002; Steptoe et al., 2004). Activation of the HPA-axis leads to increased circulatingconcentrations of free cortisol in the body. St udies investigating basal cortisol levels in psychiatric disorders have rendered equivocal results. Many studies have found evidence of hypersecretion of cortisol in depressed and anxious patients (Gil et al., 2008; Goodyer, Herbert, Tamplin, & Altham, 2000; Lenze et al., 2011), although long-term stress may lead to a downregulation or exhaustion of the HPA-axis, and less hypercortisolemia (Wardenaar et al., 2011). Extensive literature suggests that hypercortisolemia may be a biological risk factor for depression, and a diminished negative feedback in depressed patients compared to healthy controls after a low-dose dexamethasone-suppression test has been well established (DST; Goodyer et al., 2000; Pruessner et al., 2003a; Stetler & Miller, 2005; Vreeburg et al., 2009, 2010; Wardenaar et al., 2011). Evidence for a temporal relationship starting with social withdrawal and subsequent depression has also been found (Cacioppo, Hawkley, & Thisted, 2010; Engeland et al., 2006).

In contrast to data on sC, salivary alpha amylase (sAA) is a relatively new candidate marker of autonomic nervous system (ANS) functioning and reactivity. The ANS consists of two systems, the parasympathetic nervous system (PNS), that is responsible for the body's rest and digest response, and the sympathetic nervous system (SNS), responsible for the fight or flight response. sAA is secreted in response to neurotransmitter stimulation from salivary glands that are innervated by sympathetic as well as parasympathetic nerves (Vester & Garrett, 1987). Studies have provided evidence that stress increases SNS activity and decreases PNS activity, leading to an increase in epinephrine and norepinephrine levels and a decrease in the

level of acetylcholine. This can lead to an increase in the level of pro-inflammatory cytokines (e.g. TNF-alpha, IL-1, IL-6) and interferons, and decrease the level of anti-inflammatory cytokines (e.g. IL-10), possibly initiating a state of enhancing low-grade inflammation, which in turn may induce indoleamine 2,3-dioxygenase activity, increase the kynurenine/tryptophan ratio. This can lead to an imbalance between neurotoxic and neuroprotective kynurenine metabolites. Ultimately, this can lead to neurodegenerative changes of the brain, that could leave the brain more susceptible to, for example, depression (Won & Kim, 2016). Associations have also been reported between inflammatory cytokines, the kynurenine pathway, and the neurotoxicity of the metabolites in the pathway in depression (Hazari & Bhad, 2015; Jo, Zhang, Emrich, & Dietrich, 2015).

Recently, a study conducted by our group within the same sample population found higher levels of sAA at awakening in MDD patients in comparison to both healthy controls and patients with other common psychiatric disorders (i.e. predominately anxiety and somatoform disorders; Bauduin et al., 2018). Another study found elevated afternoon sAA levels in patients with generalized social anxiety disorder (van Veen et al., 2008). This indicates that elevations of sAA within disorders seem to be time-point specific, suggesting the importance of diurnal patterns. Importantly, these sAA elevations in MDD and social anxiety also suggest a potential role of sAA in social withdrawal. Moreover, a recent study found that social withdrawal was related to heightened sympathetic nervous system activation in response to social stress (Duijndam, Karreman, Johan, & Kupper, 2020). Furthermore, increased social withdrawal has been found to be a mediating variable in the relationship between salivary cortisol (sC) and depression (Tse & Bond, 2004). No studies have explored the interrelationships between sAA, social withdrawal, and depression to date.

In this study, we examine the relationship between sC and sAA with social withdrawal using salivary samples collected in a naturalistic setting. We used a mixed sample of psychiatric out-patients and controls to analyze associations along the dimension of social withdrawal. Moreover, we explored whether the putative relationship between biological stress system activation and depression is mediated through social withdrawal. Based on previous literature, we hypothesized that high sC and sAA levels would be positively associated with social withdrawal. Furthermore, we expected that social withdrawal would mediate the relationship with both salivary markers of stress system activation and depression.

2. Methods

2.1 Participants

The population recruited for this study came from the Routine Outcome Monitoring (ROM) and NormQuest cohorts. The ROM participants were ambulatory outpatients that participated in an extensive ROM interview between 2007 and 2011 as part of routine patient care. ROM is restricted to patients referred for treatment of mood, anxiety, and somatoform disorders. In order to partake in the ROM, participants must have sufficient command of the Dutch language. Detailed ROM protocol information has previously been published elsewhere (de Beurs et al., 2011). NormQuest participants were recruited from the general population via the registration at medical practices as a reference group for the ROM patients (Schulte-van Maaren et al., 2013). These participants were stratified for age, gender, and urbanization in order to reflect the ROM patient population as accurately as possible within this naturalistic study design. According to the Mini International Psychiatric Interview (MINI-Plus; van Vliet & de Beurs, 2007), more than 90% of the NormQuest population were not diagnosed with a psychiatric disorder. Exclusion criteria for the NormQuest controls were: (i) treatment in a secondary psychiatric care center in the last six months and/or dependence on drugs or alcohol; ii) a hearing impairment or limited cognitive abilities (i.e. aphasia, severe dyslexia or dementia); iii) illiteracy or insufficient mastery of the Dutch language, and; iv) a terminal disease.

A total of 949 participants consisting of ROM participants with either a (comorbid) mood, anxiety, or somatoform (MAS-) diagnosis and healthy controls collected saliva samples for our Mood, Anxiety, and Somatoform disorders and HPA-axis biobank (MASHbank), using a protocol that was similar to the protocol used in the Netherlands Study of Depression and Anxiety (NESDA, Vreeburg et al., 2010). The protocol was approved by the LUMC Ethical Review Board, participants gave written consent, and the study was carried out in accordance with the principles of the declaration of Helsinki. After explanation of the protocol, informed consent was obtained from all of the participants. Sample forms and ROM interviews without a recorded date were excluded (n = 44). Participants that returned samples more than 60 days after the ROM interview were also excluded (n = 23), as well as NormQuest participants using psychotropic medication, patients whose medication use was not specified, and all participants lacking demographic information (n = 26, n = 7, and n = 6 respectively). Thus, our final sample consisted of 843 (88.8%) of 949 participants.

2.2 Saliva sampling

Participants were given detailed written instructions in which they were asked to collect saliva samples on a regular working day using Salivettes® (Sarstedt, Germany) at awakening, after 30, 45, and 60 minutes (in order to be able to calculate the area under the curve with respect to the ground (AUCg; which measures total cortisol output), and the area under the curve with respect to the increase (AUCi, which measures change in cortisol over repeated measures; Khoury et al., 2015). Participants were also asked to collect saliva samples at 10:00 PM and 11:00 PM, as earlier studies have found elevated evening cortisol in MDD patients (Young et al., 1994; Vreebrug et al., 2009; Bauduin et al., 2018), and at awakening the following day (to determine the effect of a dexamethasone suppression test (DST) on sC levels), which is in accordance with the NESDA protocol for the MASHbank. Participants were therefore asked to ingest 0.5 mg of dexamethasone after collecting the 11:00 PM sample on day 1.

Participants were asked to record the sampling times on a sampling form and store the samples in the refrigerator. When all samples were collected, they were returned by regular postal mail after which the Salivettes were centrifuged, the saliva aliquoted, and subsequently stored at -80°C.

2.3 Laboratory analysis of salivary cortisol and sAA

Cortisol analysis was performed by the Cortisol (gen 1) competitive electrochemiluminescence immunoassay (ECLIA, Modular E170 immunoanalyzer, Roche Diagnostics, Basel, Switzerland), as previously described in van Aken et al. (2003). The functional detection limit was 2.0 nmol/l and the intra- and inter-assay variability coefficients in the measuring range were less than 10%. Assays were repeated if cortisol levels were very high (>80 nmol/l) or very low (<1 nmol/l). All very high samples remained high and the mean of both values was used. In 80% of the very low samples, the repeated cortisol value was within the normal range and used for analysis, otherwise the mean was used. Three cortisol indicators were used: 1-hour awakening cortisol, evening cortisol, and DST.

After overnight thawing of the saliva at 4°C, samples for alpha-amylase analysis were diluted 50-fold with a Hamilton Microlab 500 B/C dilutor in physiological saline solution (Versylene® Fresenius, Cat. Nr. B230551). Analyses were performed using a kinetic colorimetric assay for total amylase activity (Cat Nr. 03183742, Roche Diagnostics, Mannheim, Germany) on a routine clinical chemistry analyzer. The total amylase assay is standardized to the IFCC reference measurement procedure

(Lorentz, 1998), guaranteeing worldwide comparability of the data. Amylase activities are measured and expressed in IU/L at 37 °C. Intra assay coefficient of variation ranged from 0.7% to 2.7% for the combined predilution step and the analysis across the entire study period. Inter assay coefficient of variation was lower than 5% throughout this study.

2.4 Data cleaning

Both sC and sAA samples 3 SD higher than the mean were excluded (sC n = 66 of the 5901 samples (1%); sAA n = 114 of the 5901 samples (< 2%)), as these values exceed the range that is realistic for sC and sAA samples and are likely to be caused by measurement issues (e.g., bleeding gums or gingivitis). However, for the sake of completeness we also ran the analyses using all of the original values, which resulted in largely comparable findings. Missing values were then imputed using linear regression for cases remaining in the dataset (n = 170; 2.9% values for sC and n = 129; 2.2% values for sAA).

2.5 Psychometric rating scales for social withdrawal

Currently, there is no broadly accepted gold-standard with which to measure social withdrawal. In this study, three subscales were used in order to measure social withdrawal. The first is a subscale is part of the Brief Symptom Inventory (BSI; Derogatis & Melisaratos, 1983). The BSI is a 53-item self- report inventory which uses a 5-point Likert scale (range 0-4) and evaluates the psychological symptom status of individuals on 9 primary symptom dimensions. It has been found to be a valid and reliable measurement instrument (Aroian & Patsdaughter, 1989; Croog et al., 1986; Derogatis & Melisaratos, 1983). Regarding our focus on social withdrawal, we used the Dutch version of the BSI social withdrawal subscale consisting of five items. These items are related to the withdrawn lifestyle that is commonly experienced in patients with schizophrenia or depression. A higher score on this subscale indicates a higher level of social withdrawal. An example of an item on this subscale is “Feeling lonely even when you are with people”. Cronbach’s alpha of this subscale in this study is 0.782 (N = 843).

The second subscale used to measure social withdrawal is part of the Short Form 36 (SF-36; Ware & Sherbourne, 1992). The SF-36 is a 36 item self-report survey used to determine an individual’s general health status over eight domains. The SF-36 is a valid and reliable measurement instrument (Razavi & Gandek, 1998). In this study we used the scores given on the social functioning subscale of this survey, which consists of two items. One of the two items in this subscale is “During the past 4

weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?". Cronbach's alpha of this subscale is 0.872 (N = 843).

The third social withdrawal subscale is part of the Dutch Dimensional Assessment of Personality Pathology – Short Form (DAPP-SF; van Kampen, de Beurs, & Andrea, 2008). The DAPP-SF comprises 136 of the original 290 items of the Dimensional Assessment of Personality Pathology – Basic Questionnaire (DAPP-BQ; Livesley & Jackson, 2001). Scores range from 1 (very unlike me) to 5 (very like me). The DAPP-SF has been found to be both a valid and reliable questionnaire (de Beurs, Rinne, van Kampen, Verheul, & Andrea, 2009). The DAPP- SF social avoidance subscale in this questionnaire consists of 6 items geared towards establishing whether one has a low level of affiliation, is fearful of interpersonal hurt, has defective social skills, has a desire for improved affiliative relationships, and for the level of social apprehensiveness. A translation of one of the items on the subscale is "At parties or meetings I tend to avoid people". Cronbach's alpha for this subscale in this study is 0.897 (n = 420).

A composite social withdrawal scale was created using the items from the BSI, SF36, and DAPP social withdrawal subscales. This scale was created by computing the standardized z-scores of each subscale and averaging these scores into one composite. Subscales missing <=1 observation were included in the composition of the social withdrawal composite scale. This scale was used for means of visual interpretation of sC and sAA values at each time point (see Figures 1A and B), and as the scale reflecting social withdrawal in the statistical mediation analysis. Cronbach's alpha for this subscale in this study is 0.780 (n = 420).

2.6 Psychometric depression rating scales

The Montgomery-Åsberg Depression Rating Scale (MADRS; Montgomery & Asberg, 1979) is a 10-item observer-rated instrument assessing the range and severity of depressive symptoms. Items are rated on a 7-point Likert scale anchored at 4 points (i.e. 0 indicating the no abnormality, 6 indicating severe abnormality). Summed total scores lie between 0 and 60. The MADRS has a good internal consistency and reliability (Goekoop & Zwinderman, 1994; Montgomery & Asberg, 1979). The time frame for the MADRS encompasses the previous seven days.

2.7 Statistical analysis

As published in a previous paper by our group (Bauduin et al, 2018), sC and sAA were

positively skewed and therefore naturally loge transformed before analyses. Baseline characteristics were compared across the mixed group consisting of patients with mood-, anxiety-, and/or somatoform diagnoses and healthy controls using analysis of variance for continuous variables and chi-squared tests for categorical variables. These variables were summarized using means, percentages, and standard deviations. Associations between the three social withdrawal subscales and the seven sC and sAA samples were determined by means of Pearson's correlations.
The measures computed for both the sAA and sC samples were the AUCg, the AUCi, mean evening sAA levels, and mean sAA levels at awakening. The relationship between sAA AUCg levels, AUCi levels, mean evening sAA levels, and sAA levels at awakening with the BSI, the SF-36, and the DAPP social withdrawal subscales were determined by means of both unadjusted and adjusted linear regression. In the adjusted model, NE ancestry, age (continuous), and alcohol use (dichotomous) were included. These are variables that earlier studies have reported may influence sAA concentrations (Perry et al., Rigaud et al., 2015; Veen et al., 2012). Additionally, we adjusted for gender and educational level (no/low education versus middle/high education).

The relationship between sC AUCg levels, AUCi levels, mean evening sC levels, and sC levels at awakening after the DST with the BSI, the SF-36, and the DAPP social withdrawal subscales were determined by means of both unadjusted and adjusted linear regression. The model was adjusted for gender, age, Northern European (NE) ancestry, education level (to adjust for social economic status; no/low education versus middle/high education), season (more daylight hours versus less daylight hours), time of awakening (as a continuous variable), hours of sleep (<6 hours of sleep, >6 hours of sleep), weekday versus weekend, and alcohol use the day of testing in adherence, where possible, to the expert consensus guidelines (Stalder et al., 2016).

In order to investigate whether social withdrawal is a mediating variable in the relationship between sC and sAA elevations and increased depression severity as measured on the MADRS, a statistical mediation analysis was run using the Preacher and Hayes mediation method (Preacher & Hayes, 2008). Potential mediation was determined by directly testing the significance of the indirect effect of the independent variable (IV) on the dependent variable (DV) through the mediating variable (M; the c'-path). This is quantified as the product of the effects of the IV on M (a -path) and the effect of M of DV (b-path). Analyses were performed using a multiple mediator model with a bootstrapping approach whereby the point estimate

of the indirect effect was deduced by means of 5000 estimates of the a x b path. 95% confidence intervals (CI) of the empirical distribution were estimated using cut-offs for the 2.5% highest and lowest scores. Mediating effects were considered to be significant when the CI did not include zero. A two-sided p-value <0.05 was considered statistically significant. To determine effect sizes, the completely standardized indirect effect was determined using the following formula: abcs = ßMXßYM (Preacher & Kelley, 2011). IBM SPSS Statistics for Windows version 24 (IBM Corp. Armonk, N.Y., USA) was used for data-analysis.

3. Results

The mean age of the sample was 44.0 years (64.1% female; N = 843). MADRS scores means were 2.00 (IQR 0-7), as this variable is positively skewed. Further sample characteristics can be found in Table 1. A strong correlation van found between the SF36 and the BSI social withdrawal subscales ($r = 0.64$; $p < 0.001$), the BSI and the DAPP-SF social withdrawal subscales ($r = 0.60$; $p < 0.001$), and the SF36 and the DAPP social withdrawal subscales ($r = 0.45$; $p < 0.001$).

Pearson's correlations between the seven sC measurements indicated that all measurements were significantly positively associated with one another ranging from 0.286 through 0.775. Pearson's correlations between the seven sAA measurements indicated that these were also positively associated with one another ranging from 0.480 through 0.811. sC and sAA were often only weakly correlated with correlation coefficients ranging between -0.069 and 0.209.

Table 1. Socio-demographic variables for the population sample (MAS-disorder patients and healthy controls)

Variables	N = 843
Sociodemographic characteristics:	
Gender, female (%)	540 (64.1)
Age in years, mean (SD)	44.00 (12.8)
Higher education (%)	599 (71.1)
North European ancestry (%)	715 (84.8)
Alcohol use on sampling days (Yes) %	247 (29.3)*
Sampling Factors:	
Time of awakening (mean, SD)	7:01 (0:58)
Hours of sleep (less than 6 hours) %	168 (19.9)*
Workday (%)	577 (68.4)*
Light Season (April-September)	373 (44.2)
Clinical Characteristics:	
MADRS total score (median, SD)	1.10 (1.10)
BSI social withdrawal subscale (median, SD)	0.00 (0.53)
SF-36 social functioning subscale (median, SD)	1.50 (0.93)
DAPP-SF social avoidance (N = 420; median, SD)	1.83 (0.97)
Composite subscale (mean, SD)	0.00 (1.00)
Assessment characteristics:	
Time interval between assessment and saliva sampling (median, SD)	7.0 (10.00)
Medication use n (%):	
Using any psychotropic medication	129 (15.3)
TCAs	8 (0.9)
SSRIs	55 (6.5)
Benzodiazepines	30 (3.6)
Stimulants	1 (0.1)
Antipsychotics	8 (0.9)
Stabilizers	8 (0.9)
Other ADs	19 (2.3)

*:Alcohol use n = 16 (<2%) missing; hours of sleep n=9 (%) missing; workday n = 4 missing ; MADRS n = 1 missing, SF-36 n = 19; BSI n = 5; composite subscale z-scores 72 missing.

Associations between salivary alpha amylase and social withdrawal subscales
Associations between AUCg, mean sAA levels at awakening, and diurnal sAA with the SF36 social withdrawal subscale were found to be significant ($p = 0.01$, $p = 0.01$, and $p = 0.005$ respectively). However, none of these associations remained significant after adjustment for
covariates (see Table 2). Age was found to be the covariate of most influence in the adjusted analyses ($p < 0.01$). None of the other covariates were found to be significant. A visual
representation of sAA values per time point can be found in Figure 1A. The sample is categorized in quartiles (i.e. the adjusted scores on the composite subscale divided into four groups of equal size) for visual purposes only in order to facilitate interpretability. In this Figure the moment of ingestion of the dexamethasone has been included ('oral dexamethasone'). We have denoted this in both Figures 1A and 1B for continuity purposes, as sAA and sC values were derived from the same saliva sample, although dexamethasone does not influence sAA levels (as can be seen in Figure 1A).

Associations between salivary cortisol and social withdrawal subscales
Significant associations between sC AUCg, AUCi, evening sC, diurnal sC and the BSI social withdrawal subscale were found in unadjusted models, before adjusting for covariates (see Table 3). After multivariate adjustment only associations between AUCi, evening sC, diurnal sC, and the BSI social withdrawal subscale remained significant ($p = 0.02$; $p = 0.003$; $p = 0.01$ respectively). Evening sC was found to be significantly associated with the SF36 social withdrawal subscale, however this association did not remain significant after adjustment for covariates. The DAPP social withdrawal subscale consisting of 420 observations (thus approximately half of the number of observations recorded on the SF36 and BSI social withdrawal subscales) was not significantly associated with sC at any of the time points (see Figure 1B for visual representation of sC levels per time point). The covariates of largest influence in all of the analyses were working on the day of saliva collection and sleeping less than six hours before the day of saliva collection ($p < 0.001$ respectively).

Table 2. Associations between salivary alpha-amyase (sAA) and social withdrawal subscales.

		N	BSI Social withdrawal	N	SF36 Social Functioning	N	DAPP social avoidance
AUCg (U/ml)	Crude	763	0.048 (*p*=0.18)	749	**0.033 (*p*=0.01)**	445	0.052 (*p*=0.28)
	Adjusted	749	0.029 (*p*=0.43)	735	0.021 (*p*=0.11)	433	0.044 (*p*=0.37)
AUCi (U/ml)	Crude	763	-0.013(*p*=0.72)	749	<-0.001 (*p* =0.56)	445	0.033 (*p* =0.49)
	Adjusted	749	0.005 (*p* =0.90)	735	<0.001 (*p* =0.63)	433	0.043 (*p* =0.39)
Mean T1 and T7 (nmol/ml),	Crude	767	0.054 (*p*=0.14)	753	**0.092 (*p*=0.01)**	449	0.013 (*p*=0.79)
at awakening	Adjusted	741	0.083(*p* =0.07)	739	0.049 (*p* =0.18)	437	<0.001 (*p*=0.998)
Diurnal sAA	Crude	726	0.051 (p=0.17)	712	**0.104 (p=0.005)**	420	0.043 (p=0.38)
	Adjusted	715	0.034 (p=0.37)	701	*0.071 (p=0.057)*	410	0.028 (p=0.58)

*Adjusted for gender, age, Northern European ancestry, and alcohol use.
**Values presented in Table are standardized beta-coefficients using linear regression models.

Table 3. Associations between salivary cortisol (sC) and social withdrawal subscales.

		N	BSI Social withdrawal	N	SF36 Social Functioning	N	DAPP social avoidance
AUCg (U/ml)	Crude	769	**0.076 (*p*=0.03)**	755	0.014 (*p*=0.70)	451	0.013 (*p*=0.78)
	Adjusted*	749	0.067 (*p*=0.06)	735	-0.007 (*p*=0.85)	436	0.016 (*p*=0.74)
AUCi (U/ml)	Crude	769	**0.079 (*p*=0.03)**	755	0.043 (*p*=0.24)	451	0.028 (*p*=0.55)
	Adjusted*	749	**0.082 (*p*=0.02)**	735	0.057 (*p*=0.11)	436	0.038 (p=0.44)
Evening sC	Crude	765	**0.147 (*p* < 0.001)**	749	**0.113 (*p* = 0.002)**	442	0.034 (*p* = 0.48)
(U/ml)	Adjusted*	747	**0.110 (*p* = 0.003)**	731	0.050 (*p* = 0.17)	428	0.027 (p = 0.59)
sC after DST(U/ml),	Crude	752	0.027 (*p*=0.45)	739	0.019 (*p*= 0.61)	448	-0.068 (*p* = 0.15)
at awakening	Adjusted*	720	0.007 (p=0.86)	709	-0.015 (*p*=0.69)	425	-0.069 (*p*=0.16)
Diurnal sC	Crude	726	**0.112 (p=0.002)**	712	0.064 (p=0.09)	420	0.009 (p=0.86)
	Adjusted*	710	**0.097 (p=0.01)**	696	0.020 (p=0.58)	407	0.010 (p=0.84)

*Adjusted for gender, age, Northern European ancestry, education level, season, time of awakening, hours of sleep, weekday versus weekend, and alcohol use.
**Values presented in table 1 are standardized beta-coefficients using linear regression models.

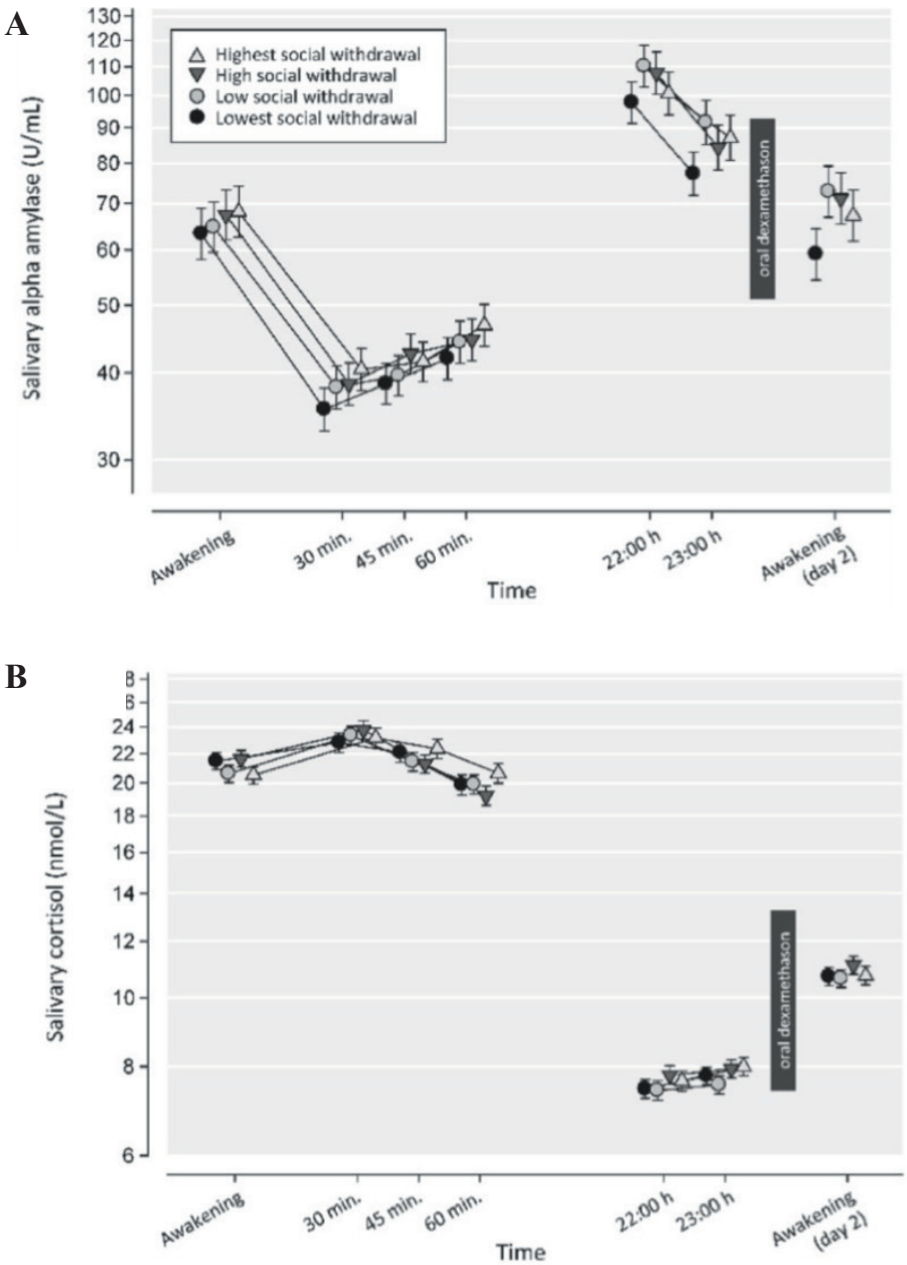

Figure 1. (A) A visual representation of sAA levels according to the composite SW scale at each time point. (B) A visual representation of sC levels according to the composite SW scale at each time point.

Mediation analyses

Using Preacher and Hayes' mediation model (2008), a statistical mediation analysis was run to determine whether social withdrawal is a mediating variable in the relationship between sAA and sC elevations on the one hand and increased depression severity as recorded on the MADRS on the other hand (see Figure 2). After adjustment for covariates, social withdrawal, which was measured using the composite scale comprised of all the three standardized subscales, was not found to have a mediating effect in the relationship between sAA and MADRS scores at any of the time points. Social withdrawal was only found to have a significant mediating effect in the relationship between evening sC and MADRS scores (see Mediating effects (M) in Table 4 and Figure 2). The effect size for this significant association was calculated using the Completely Standardized Indirect Effect (abcs = 0.06, 95% CI: 0.012-0.111), indicating a small effect size.

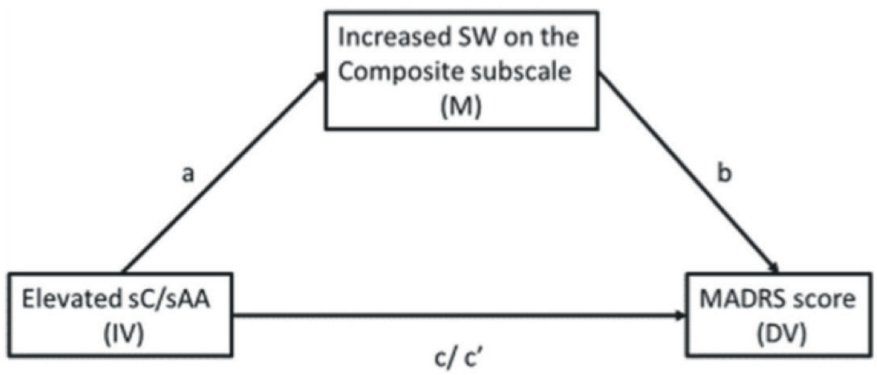

Figure 2. Model of elevated sC/sAA, increased SW as scored on the composite scale, and increased MADRS scores suggesting that increased SW is the intermediate factor between elevated sC/sAA secretion and increased depression scores.

Table 4. Potentioal mediating effects of the social withdrawal (SW) composite on the relationship between sAA and sC levels (IV) and MADRS score (DV).

	Model	n	Effect of IV on M (a)	Effect of M on DV (b)	Direct effect of IV on DV (c')	Mediating effect (a x b; 95% CI) (M)	Total effect of IV on DV (c)
sAA indicators (IV):							
AUC_g	Crude	764	0.0728*	-0.0196	-0.0196	**0.0518 (0.0032; 0.0974)**	0.0322
	Adjusted	750	0.0478	-0.0259	-0.0259	0.0338 (-0.0150; 0.0790)	0.0079
AUC_i	Crude	764	-0.0124	-0.0157	-0.0157	-0.0088 (-0.0585; 0.0387)	-0.0245
	Adjusted	750	0.0147	-0.0056	-0.0056	0.103 (-0.0434;0.0611)	-0.0047
Mean T1 and T7	Crude	768	0.0716*	0.0004	0.0004	**0.0509 (0.0072; 0.0914)**	0.0514
at awakening	Adjusted	754	0.0424	-0.0073	-0.0076	0.0312 (-0.0168; 0.0761)	0.0235
Mean sAA	Crude	727	0.0793*	-0.0267	-0.0267	**0.0565 (0.0066; 0.1046)**	0.0298
	Adjusted	716	0.0560	-0.0354	-0.0354	0.0396 (-0.0094; 0.0882)	0.0042
sC indicators (IV):							
AUC_g	Crude	770	0.0425	0.0551*	0.0551*	0.0300 (-0.0207; 0.0826)	0.851*
	Adjusted	750	0.0263	0.0537	0.0537*	0.0181 (-0.0309; 0.0695)	0.0718*
AUC_i	Crude	770	0.0033*	0.0049	0.0049	0.0445 (-0.0152; 0.1058)	0.0494
	Adjusted	750	0.0669	-0.0055	-0.0055	0.0462 (-0.0152; 0.1073)	0.0407
Evening SC	Crude	727	0.1341***	0.0683***	0.0683*	**0.0943 (0.0423; 0.1507)**	0.1626***
	Adjusted	711	0.0941*	0.0665*	0.0665*	**0.0644 (0.0118; 0.1165)**	0.1309***
sC after DST	Crude	686	0.0309	0.0845**	0.0845**	0.0217 (-0.0300; 0.0729)	0.1062**
at awakening	Adjusted	666	0.0003	0.0904**	0.0904**	0.0002 (-0.0513; 0.0519)	0.0407*
Mean sC	Crude	727	0.0846*	0.0832**	0.0832**	**0.0596 (0.0076; 0.1119)**	0.1428***
	Adjusted	711	0.0591	0.0857**	0.0857**	0.0405 (-0.0107; 0.0919)	0.1262**

MADRS, Montgomery-Åsberg Depression rating scale; BSI, Brief Symptom Inventory; Composite subscale, a composite of the BSI social withdrawal, the SF-36 social functioning, and the Dimensional Assessment of Personality Pathology – Short Form (DAPP-SF) social avoidance subscales. Mediation analyses according to Preacher & Hayes: IV denotes the Independent variables, M denotes mediating variable, DV denotes dependent variable, a denotes effect of IV on M, b denotes effect of M on DV, c' denotes direct effect, a x b denotes indirect mediating effect, c denotes total effect.

sAA adjusted for gender, age, Northern European ancestry, and alcohol use. sC adjusted for gender, age, Northern European ancestry, education level, season, hours of sleep, weekday versus weekend, and alcohol use.

*** *p-value* < .001; ** *p-value* < .01; * *p*-value < .05.

4. Discussion

The current study examined the association between sAA and sC with three social withdrawal subscales in a naturalistic sample of psychiatric out-patients and healthy controls. Although no significant associations were found between sAA and social withdrawal subscales, significant associations were found between sC AUCi, evening sC, and diurnal sC samples with social withdrawal, but only as measured on the BSI social withdrawal subscale. Furthermore, social withdrawal was not found to be a mediating factor in the relationship between sC and sAA on the one hand and the severity of depressive symptoms on the other hand. Therefore, our findings do not support the idea that increased basal and diurnal sAA are associated with social withdrawal, but suggest that HPA axis activation may be implicated in this relationship.

Recent studies have found an association between sAA, an enzyme that is increasingly secreted under the stimulation of the ANS, and certain psychiatric disorders at specific time points. More specifically, we found elevated levels of naturalistic sAA at awakening in patients with a current major depressive disorder (MDD) diagnosis in comparison to patients with (comorbid) anxiety and somatoform disorders and healthy controls, in a previous analysis of the current sample (Bauduin et al., 2018). Also, a study with gSAD patients and healthy controls found elevated sAA levels in a naturalistic patient sample (van Veen et al., 2008). These findings gave rise to the idea that elevations in sAA may be disease and time-point specific reflections of ANS activation. As more targeted research has been geared towards the understanding of social withdrawal as a common endophenotype across various psychiatric disorders and research has indicated that there may be an association between HPA-axis activation and social withdrawal, we hypothesized a role of the ANS in the relationship with social withdrawal as well. However, no significant associations were found between sAA and any of the social withdrawal subscales indicating that sAA (as measured in this study) is unlikely to be an important biomarker of social withdrawal. We also did not find evidence for a mediating role of social withdrawal in the relationship between sAA and depression severity. In sum, the findings suggest that ANS activation as assessed using the basal and diurnal sAA samples collected in this study is unlikely to be involved in the etiology of social withdrawal.

Ample research has found that the hypersecretion of the stress hormone cortisol secreted under activation of the HPA-axis is associated with a variety of psychiatric disorders (Gil et al., 2008; Goodyer et al., 2000; Lenze et al., 2011). Our findings

offer further support for this as the statistical mediation analysis replicated these findings (i.e. the effect of the independent variable on the dependent variable), and also found this positive association between the sC AUCg, evening sC, sC after the DST, and mean sC levels and the MADRS scores, increasing the validity with regard to our sAA findings. Previous studies investigating the association between social withdrawal and HPA-axis activity have found equivocal results, echoing findings from animal studies (Hawkley et al., 2012; Cacioppo, Cacioppo, Capitanio, & Cole, 2015), the majority of which have found a positive association between sC and social withdrawal (Adam et al., 2006; Arnetz, Theorell, Levi, Kallner, & Eneroth, 1983; Doane & Adam, 2010; Grant, Hamer, & Steptoe, 2009; Hawkley, Cole, Capitanio, Norman, & Cacioppo, 2012; Pressman et al., 2005; Steptoe et al., 2004). This was also partially the case with regard to the current study. We found significant positive associations between sC markers and the BSI social withdrawal subscale, but not between sC and the other social withdrawal subscales.

Regarding social withdrawal as a putative mediating variable between sC and depression, an earlier statistical mediation analysis found that social withdrawal mediates the association between sC and depression as measured on the Beck Depression Inventory (Tse & Bond, 2004). However, this study did not adjust for numerous influential covariates (Stalder et al., 2016; i.e. the model was adjusted only for gender, age, and cortisol concentration). In our study we found similar results, namely that social withdrawal mediated part of the relationship between evening and mean sC samples and depression, although this effect only remained significant with regard to the evening sC samples after covariate adjustment with a very small effect size. It is possible that we were unable to find the mediating effect of social withdrawal in the unadjusted analyses with the other sC samples due to the lack of specificity of the composite scale or the limited number of scores in the higher range of the MADRS. It may likewise be the case that we were unable to replicate the results from the previous study in all of our sC samples because we used a different and sophisticated mediation method (Preacher & Hayes, 2008). A previous study conducted by our group using the same saliva samples as in the current study found elevated evening sC, and not morning sC levels, in depressed subjects. This is likely to have influenced the significant mediation effect we found. Although other studies have found similar results (Young et al, 1994; Vreeburg et al., 2009), the most likely possibility is that social withdrawal is only a minor mediating factor in the association between sC and depression, as it is plausible that the significant but relatively small statistical mediation effect found in the earlier paper would be attenuated if all influential covariates would have been accounted for.

There were a number of limitations in this study. The first is the cross-sectional nature of the study, which does not allow for causal inferences. Second, the subscales that were used to measure social withdrawal may not have been sensitive enough in their ability to measure social withdrawal without enough precision. Also, the DAPP-SF social withdrawal subscale, the psychometric tool with the strongest face validity to measure social withdrawal, had some missing observations in comparison to the other two social withdrawal subscales. Third, changes in subjects' body position were not considered, as standing to sitting and vice versa can induce rapid changes in sAA enzyme activity (Bosch, Veerman, de Geus, & Proctor, 2010). Fourth, although participants were given strict instructions to refrain from eating, drinking, smoking, or brushing teeth 15 minutes prior to sampling, it should be noted that due to the ambulatory and naturalistic setting of this study it is more difficult to monitor compliance than in a systematic clinical trial. However, a previous study using the same saliva samples as the current study replicated the well-established diurnal curve of sC, suggesting adequate validity (Bauduin et al., 2018). A further potential limitation may be that we were unable to adjust for psychotropic medication use as there were no participants who used psychotropic medication in the healthy control group. Also, although we adjusted our analyses for several potential confounders, there may be residual confounding (e.g. menstrual cycle, gingivitis). Furthermore, only a small group of participants suffered from moderate to severe depression (i.e. n = 61 (7%) with MADRS score >19). Therefore, our findings cannot be extrapolated to patients with more severe depressive psychopathologies or patients with other psychiatric disorders. Moreover, selection bias may also be the case, as highly socially withdrawn patients may not have volunteered to participate in the study. Finally, we did not record the postage day of the saliva samples and were therefore unable to correct for possible warming effects during postal delivery. However, if this were to have an effect on the samples, it is likely to have added random error, leading to findings approaching the null hypothesis.

Strengths of this study include the dimensional approach of the construct social withdrawal as an endophenotype across psychiatric disorders using three subscales. Furthermore, we were able to include a reasonably large naturalistic sample of consecutive ROM out-patients with MAS- disorders alongside a large sample of healthy controls that were specifically recruited as a reference group for the ROM participants. Moreover, saliva samples were obtained over the course of the day using a non-invasive sampling method, which is suitable for clinical use (Nater & Rohleder, 2009).

Future research investigating social withdrawal should use dedicated psychometric assessment tools to measure social withdrawal, for example the World Health Organization Disability Assessment Schedule 2 social withdrawal scale (Üstün et al., 2010), and should also differentiate between state and trait characteristics of social withdrawal (Doane & Adam, 2010). Furthermore, the association between social withdrawal and stress-related psychiatric disorders should preferably use prospective longitudinal designs, and include a wider severity range of psychopathology. Also, although previous research has found an association between sC and social withdrawal, the effect sizes in these studies were generally rather small and these studies often did not adjust for a number of important covariates (i.e. Stalder et al., 2016). Therefore, it may be prudent to also further explore associations that have previously been found to be promising biological markers for social withdrawal, namely the cardiovascular system (Steptoe et al., 2004; Grant, Hamer, & Steptoe., 2009), and other stress systems that involve inflammation and immune activation (i.e. acute-phase responses and natural killer cell counts; Steptoe et al., 2004), as heightened inflammatory activity seems to be involved in social withdrawal, as part of sickness behavior (Inagaki et al., 2012). Furthermore, in light of findings from experimental studies, possible gender differences in the relationship between SNS activation and social withdrawal should also be further explored (Campi et al., 2014; Greenberg et al., 2014).

In conclusion, this study found evidence for the idea that a chronic HPA-axis activation may stimulate social withdrawal, however that basal and diurnal sAA levels are less likely to be involved.

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Appendix

BSI social withdrawal subscale items (items 3, 14, 34, 44, and 53 on the BSI):

- The idea that someone else can control your thoughts
- Feeling lonely even when you are with people
- The idea that you should be punished for your sins
- Never feeling close to another person
- The idea that something is wrong with your mind

SF-36 social functioning subscale items (items 20 and 32 on the SF-36):

- During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups? (Answers range between 1 (not at all) and 5 (extremely).
- During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)? (Answers range between 1 (all of the time) and 5 (none of the time).

DAPP-SF social avoidance subscale items (items 7, 15, 27, 114, 127, and 128 on the DAPP): Subtraits of the DAPP-SF social avoidance subscale are low affiliation, fearful of interpersonal hurt, defective social skills, desire for improved affiliative relationships, social apprehensiveness.

- Ik voel me niet erg zeker van mezelf in het gezelschap van anderen
- Op feestjes of bijeenkomsten neig ik ernaar om mensen te ontlopen
- In iedere groep maak ik me zorgen dat ze me buiten zullen sluiten of zullen afwijzen
- Mensen maken me zenuwachtig
- Ik weet nooit hoe ik me moet gedragen als er andere mensen bij zijn
- Ik zou willen dat ik gezelliger met andere mensen kon omgaan

Free translation to English:

- I am not very sure of myself in the company of others
- At parties or meetings I tend to avoid people
- In every group I am concerned that they will shut me out or reject me
- People make me nervous
- I never know how to behave when other people are present
- I wish I could interact with other people more socially

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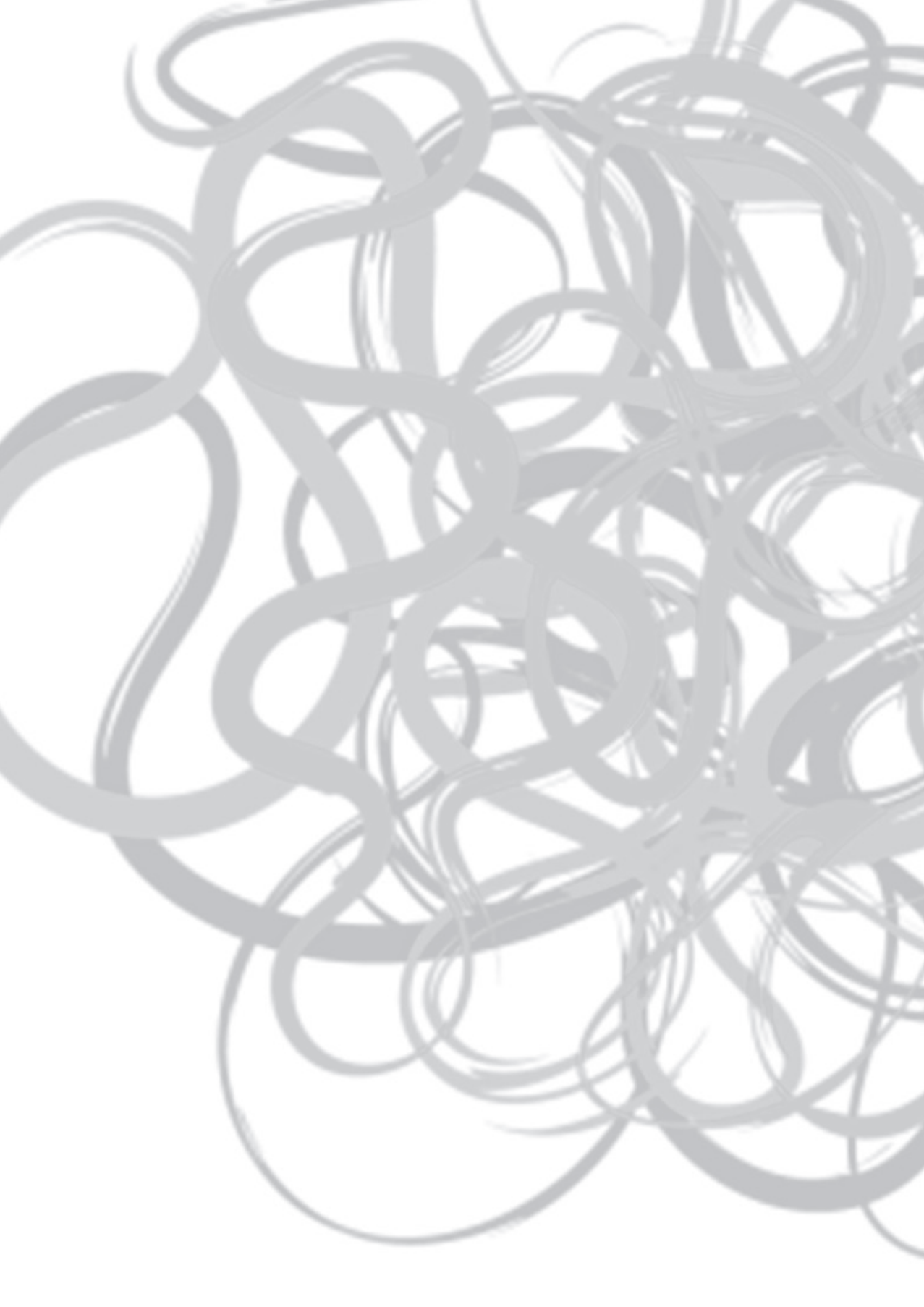

Chapter 4

Structural brain abnormalities in Cushing's syndrome

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Abstract

Purpose of review

Alongside various physical symptoms, patients with Cushing's disease and Cushing's syndrome display a wide variety of neuropsychiatric and cognitive symptoms, which are indicative of involvement of the central nervous system. The aim of this review is to provide an overview of the structural brain abnormalities that are associated with Cushing's disease and Cushing's syndrome and their relation to behavioral and cognitive symptomatology.

Recent findings

In this review, we discuss the gray matter structural abnormalities found in patients with active Cushing's disease and Cushing's syndrome, the reversibility and persistence of these changes and the white matter structural changes related to Cushing's syndrome. Recent findings are of particular interest because they provide more detailed information on localization of the structural changes as well as possible insights into the underlying biological processes.

Summary

Active Cushing's disease and Cushing's syndrome is related to volume reductions of the hippocampus and in a prefrontal region involving the anterior cingulate cortex (ACC) and medial frontal gyrus (MFG). Whilst there are indications that the reductions in hippocampal volume are partially reversible, the changes in the ACC and MFG appear to be more persistent. In contrast to the volumetric findings, changes in white matter connectivity are typically widespread involving multiple tracts.

Introduction

Cushing's disease is usually characterized by a tumor located on the pituitary that produces adrenocorticotropic hormone (ACTH), which in turn stimulates the release of glucocorticoids by the adrenal cortex. In individuals without Cushing's disease, an increase in glucocorticoids will trigger a negative feedback loop, inhibiting the release of ACTH. However, the ACTH-producing tumor in Cushing's disease is insensitive to this inhibition, therefore, the system is unable to regulate itself, resulting in increased levels of glucocorticoids or hypercortisolism. Physical manifestations of the hypercortisolism include: hypertension, abnormal fat distribution, thin skin sensitive to bruising, muscle weakness, osteoporosis, hirsutism, and gonadal dysfunction. Alongside these physical symptoms patients with Cushing's disease can also display a wide variety of psychiatric symptoms, including depression, emotional instability, cognitive impairments, apathy, anxiety and psychosis [1]. These symptoms are indicative of the effects of Cushing's disease on the central nervous system (CNS). In this review, we will summarize the existing literature available to date with regard to alterations of gray and white matter structure in the CNS related to hypercortisolism.

In 2015, Andela et al. [2] wrote a more elaborate review of the findings of studies on structural and functional abnormalities. Therefore, we will only shortly address these earlier findings to give more context to the recent findings, which we will discuss in detail. We will conclude by offering a number of suggestions for future research. As the number of studies investigating Cushing's disease is still rather limited, we expanded our review by including studies investigating excessive endogenous exposure to cortisol due to other causes (i.e. Cushing's syndrome).

Gray matter structural changes in active cushing's syndrome

The association between hypercortisolism and CNS damage was first described in 1952 by Trethowan and Cobb. Their findings were based on autopsy reports in which they found a decrease in weight of the brain and enlarged ventricles in patients with Cushing's syndrome [3]. These findings were later supported by the first in-vivo study conducted by Momose et al. [4], using a technique called pneumoencephalography. They found high incidences of atrophy in both cerebral and cerebellar regions in patients with Cushing's disease.

Starkman and colleagues in 2012 were the first to study changes related to Cushing's syndrome in a specific brain structure. Using MRI images, they manually estimated

the volume of the hippocampus in 12 patients with Cushing's syndrome. They found that 27% of the patients' hippocampal volume fell outside the 95% confidence intervals for normal individuals based on previous literature [5]. After these initial findings, several studies confirmed cerebral atrophy [6–8] and cerebellar atrophy in patients with active Cushing's disease [9]. Meanwhile, technological advancements have allowed for the acquisition of higher resolution imaging data, as well as more sophisticated automatic analysis tools. Using an automatic segmentation tool, Resmini et al. [8] were not able to replicate the finding of smaller hippocampal volumes in active Cushing's syndrome. However, they did find an impaired memory function in patients and also an association between impaired memory function and smaller hippocampal volumes [8]. In light of the high prevalence of the affective symptoms of depression and anxiety in Cushing's syndrome, a study from the same research group focused on the amygdala volumes and found smaller right amygdala volumes in Cushing's syndrome. They also found a negative correlation between the amygdala volumes and depression and anxiety scores in patients with Cushing's syndrome [10].

Manual and automatic segmentation procedures extract mean volume data from specific predetermined brain structures. These segmentation approaches are very robust measures to evaluate volumes of entire brain structures, however, they are less sensitive towards detecting smaller effects in subregions of the brain. The images derived from MRI acquisition are built up out of cubes called voxels. Voxelwise statistical tests compares the voxels of specific locations, thereby offering more information with regard to localization of effects in the brain. Burkhardt and colleagues used a voxelwise statistical approach to study structural brain changes in 19 Cushing's disease patients with a mean disease duration of 24 months compared with 40 healthy controls. They confirmed previous findings of reduced bilateral hippocampal and cerebellar volumes in Cushing's disease patients [11]. In partial agreement with these findings, Jiang et al. [12], also found reduced cerebellar volumes, although they did not find any differences in hippocampal volume in patients with Cushing's disease. In addition, they found decreased volumes in the parts of the medial frontal gyrus.

The high resolution T1-weighted MRI scans that are most commonly used to study gray matter tissue in the brain do not come without limitations. Although we can make increasingly more precise conclusions regarding localization of structural changes in the brain, we cannot draw conclusions concerning the underlying microstructural changes that are involved. Techniques that may provide more

information on this subject are Diffusion Kurtosis Imaging (DKI) and Diffusion Tensor Imaging (DTI). Both techniques rely on measuring the diffusion of water throughout the brain and its neurons. Increased diffusion has been found to be related to causes of structural changes such as the presence of an oedema and the demyelination of the white matter tracts. Jiang et al. [13&&] used DKI to investigate microstructural alterations in gray matter tissue in 15 patients with active Cushing's disease. They found increases of diffusivity parameters in the left hippocampus and parahippocampal gyrus and the left temporal lobe in Cushing's disease patients compared with healthy controls. Moreover, the diffusivity parameters in the parahippocampal gyrus correlated positively with the Cushing's syndrome severity index (CSI) scores, supporting the suggestion that hypercortisolism causes the microstructural changes [13].

Another way to obtain more information on microstructural changes associated with hypercortisolism is by measuring metabolites in the brain. Proton magnetic resonance spectroscopy (H-MRS) is a sensitive, noninvasive imaging technique that provides information on brain metabolites in vivo. Crespo et al. [14] used this technique to investigate the concentration of metabolites in the ventromedial prefrontal cortex (vmPFC) of 22 Cushing's syndrome patients, of which 15 were in remission. They found lower concentrations of glutamate and total N-acetyl-aspartate (NAA) in the vmPFC of Cushing's syndrome patients. Moreover, the duration of hypercortisolism and state anxiety were related to the decreases in NAA, suggesting a potential pathway through which hypercortisolism leads to anxiety symptoms [14]. Whilst this imaging technique sheds more light on the presence of metabolites in the brain, it does not provide information on active metabolism in the brain. Glucose metabolism in the brain can be measured using [(18)F]-fluorodeoxyglucose positron emission tomography (FDG PET), which has been used by Liu et al. [16] to acquire data regarding brain metabolism from 92 patients with Cushing's disease and 118 healthy controls. A voxelwise statistical approach revealed increased FDG uptake in the basal ganglia, anteromedial temporal lobe, thalamus, precentral cortex and cerebellum, as opposed to decreased FDG uptake within the medial and lateral frontal cortex, superior and inferior parietal lobule, medial occipital cortex and insular cortex. In most of these locations, FDG uptake was correlated with serum cortisol levels, indicating the involvement of hypercortisolism in changes of brain metabolism [15,16].

Most Cushing's disease patients are treated by means of transsphenoidal surgery, in some cases followed by postoperative radiotherapy and/or pharmacological

treatment, depending on the outcome of the surgery. Following the successful treatment of hypercortisolism, both the physical features and the psychiatric symptoms tend to improve substantially [17,18]. However, despite these improvements, a number of symptoms such as depression, anxiety, cognitive impairments and decreased quality of life persist, even in long-term remitted Cushing's disease patients [19–21]. The following two paragraphs will offer further insight into which structural changes seem to be reversible after curation, and which structural changes seem to be more persistent based on findings derived from longitudinal studies and studies conducted in remitted
Cushing's disease patients.

Reversible gray matter structural changes after remission of hypercortisolism

Reversibility of gray matter structural changes can only bemeasured using longitudinally designed studies. In 1999, Starkman et al. [22] found that the reduction of hippocampal volumes in patients with active Cushing's disease were partially reversible after curation. A follow-up study showed that these increases in hippocampal volume were associated with improvements in learning [23]. Increases in third ventricle diameter were also found to be partially reversible [7]. In children, the recovery phase after correction of hypercortisolism appears to progress at a more rapid pace. In a study conducted by Merke et al. [24], 14 patients with Cushing's syndrome, aged between 8 and 16 years, underwent an MRI scan before treatment and again 1 year after treatment. At baseline, smaller cerebral volumes, larger ventricles, and smaller amygdala were found. One year after treatment, cerebral volumes increased and ventricular size decreased to match those found in healthy age-matched controls. However, despite this reversibility, cognitive functioning remained impaired measured at follow-up [24]. In 2011, Toffaninet al. [25] manually divided the hippocampus into three subregions (i.e. the head, body and tail), and found that the reversibility of the effects of hypercortisolism were predominantly located in the head of the hippocampus. Of importance is that there are no recent studies, which have examined the reversibility of structural changes using a longitudinal design, higher resolution imaging data, and technically more advanced analyses methods such as voxelwise statistics or automatic segmentationprotocols.

Persistent changes in gray matter structure after remission of hypercortisolism

Studying patients with remitted Cushing's disease provides further insight into the persistence of the detrimental effects of hypercortisolism on the brain. Resmini et al. [8] were the first to show reduced cortical gray matter in a group constituted of both active Cushing's syndrome patients (N=11), and remitted Cushing's

syndromepatients (N=22; mean remission time: 7.3 ±2.4 years). Using voxelwise statistics, Andela et al. [26] found reduced anterior cingulate cortex (ACC) volumes in 25 patients with a mean remission duration of 11.2 years. This involvement of (areas within) the ACC was also recently demonstrated in patients with active Cushing's disease [12]. The ACC is a moderately large structure in the brain, and the reductions found in patients in comparison with healthy controls have been found to be situated almost ubiquitously throughout the entire ACC. These findings seem to explain the consistent findings with regard to reductions of cortical gray matter and provide more information on localization of the detrimental effects. Studies focusing on cerebellar volumes in remitted patients are less unidirectional. Studies have shown both increases [26] and decreases [9] in cerebellar volume in patients with Cushing's syndrome in remission. Finally, only one study using H-MRS examined metabolite levels in remitted Cushing's syndrome patients. In accordance with the results of a study conducted by the same research group in patients with active Cushing's syndrome [14], lower levels of NAA, a putative marker of neuronal vitality, were found in the left and right hippocampus and in the right hemisphere. The authors interpreted this as a sign of neuronal loss or dysfunction in these areas. They also found increased concentrations of glutamate and glutamine in both the left and right hippocampus, which could indicate glial proliferation [27].

White matter structural changes

The majority of the more recent studies conducted with (remitted) Cushing's disease and Cushing's syndrome patients have focused on examining white matter structural changes in relation to Cushing's disease. In 2014, our group investigated local white matter integrity in patients with long-term remission of Cushing's disease using DTI. We found widespread reductions of white matter integrity throughout the brain. White matter integrity in the left uncinate fasciculus (a white matter tract connecting the limbic structures with the prefrontal cortex) correlated negatively with depressive symptoms [28]. The widespread reductions in integrity were replicated by Pires et al. [29,30] in a sample of patients with both active and remitted Cushing's syndrome, providing further support for a relationship between decreased white matter integrity and depressive symptoms. The specific pattern of diffusion parameters in these studies suggest that the reductions in white matter integrity are caused by demyelination of the white matter tracts. The results of the most recent study investigating white matter tissue using DKI are in line with the previous results, showing decreases of white matter integrity throughout the brain, and the pattern of diffusion parameters indicating demyelination of the white matter tracts [13]. Finally, a study using a more conventional structural MRI

technique showed a higher degree of white matter lesions in remitted Cushing's syndrome patients, which correlated positively with DBP and hypertension, but not with cognitive performance [31].

Conclusion

Data on structural brain changes in Cushing's disease and Cushing's syndrome generally point in the same direction. The detrimental effects that have been found most consistently constitute of hippocampal volume reductions, which can partially be reversed after cure, and reduced cortical volume, which seems to be specifically driven by reduced anterior cingulate cortex volumes. Studies examining white matter tissue appear to agree that hypercortisolism affects the entire brain, and not specific locations, with indications for demyelination underlying the reductions in white matter integrity. Over recent years, advances in neuroimaging techniques and analysis methods have resulted in more specific information regarding the location of the detrimental effects of hypercortisolism on the brain. However, there is no published well-designed longitudinal study that used these state-of-the art techniques and approaches to investigate reversibility and persistence of these effects. Importantly, conclusions regarding the underlying microbiological processes cannot be drawn from MRI studies. For example, on a macroscopic level, the volume of the hippocampus appears to decrease under influence of hypercortisolism, and the reduction is at least partially reversed after correction of hypercortisolism. However, it remains unclear what microbiological processes are causing these reductions and reversibility. To develop an effective medical treatment for the detrimental effects of hypercortisolism, it is imperative that the underlying microbiological processes are uncovered. One way to get more insight into these processes is to combine the information from high resolution imaging scans with high resolution information from other sources such as the Allen Brain Atlas, which hosts information about gene expression across the human brain [32]. Combining this information could give us an indication of which genes interact with hypercortisolism to induce structural changes in the brain. Another way that we may gain more insight into the into the pathway through which hypercortisolism leads to brain structural changes are animal models. Recently, by inactivating specific gene mutations, researchers have been able to induce Cushing's syndrome resembling phenotypes in mice [33–35].

In summary, structural brain changes related to Cushing's disease have been repeatedly found and findings are generally unidirectional. With the advent of

newer imaging approaches, localization and characterization of the changes in the brain has become increasingly specific. However, state-of-the art longitudinal neuroimaging studies, which could provide on course and reversibility of the effects and their associations with symptomatology are currently lacking. In addition, more research should be conducted to uncover the underlying pathways through which hypercortisolism leads to structural changes.

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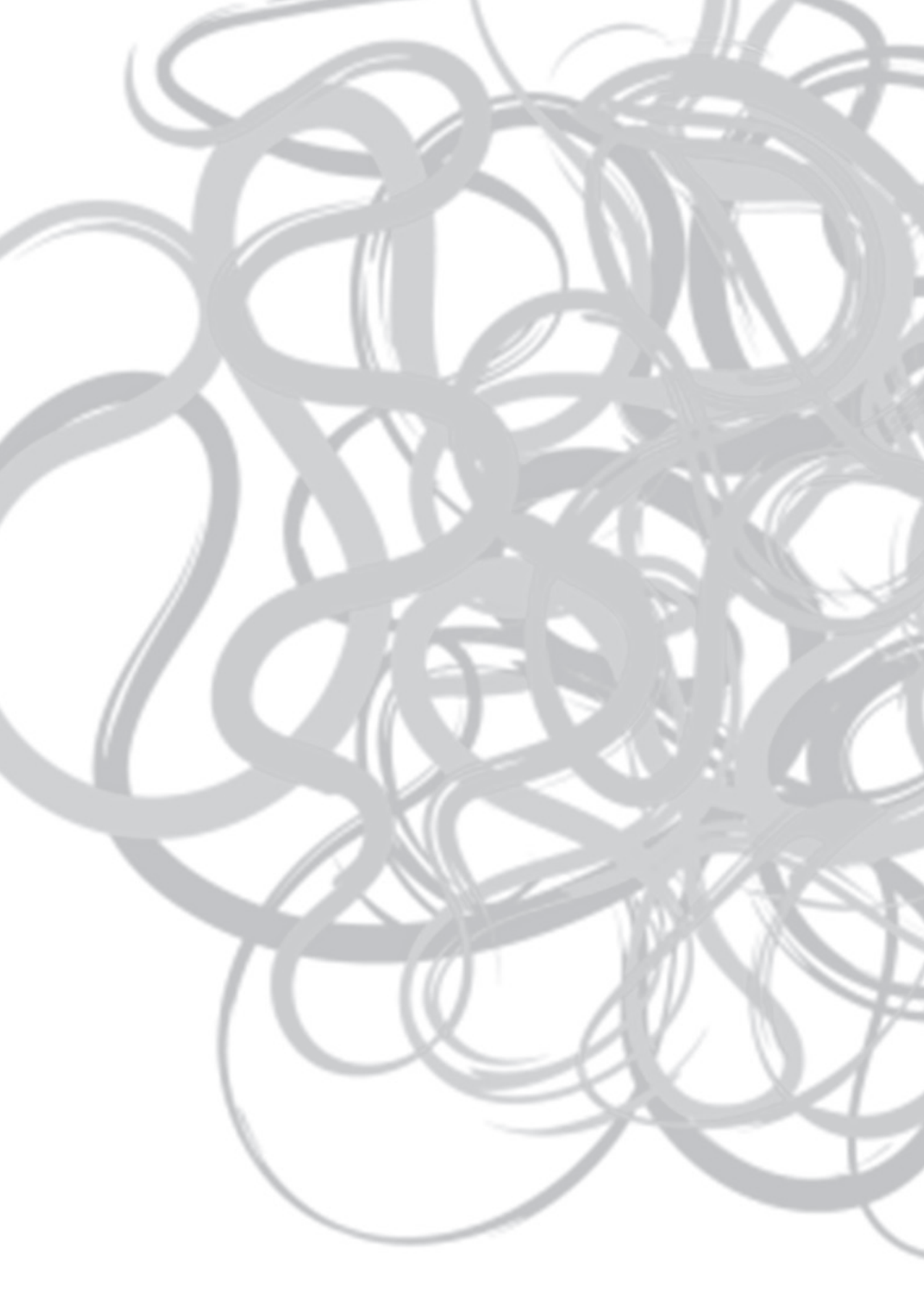

Chapter 5

Cortical thickness abnormalities in long-term remitted Cushing's disease

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Abstract

Long-term remitted Cushing's disease (LTRCD) patients commonly continue to present persistent psychological and cognitive deficits, and alterations in brain function and structure. Although previous studies have conducted gray matter volume analyses, assessing cortical thickness and surface area of LTRCD patients may offer further insight into the neuroanatomical substrates of Cushing's disease. Structural 3T magnetic resonance images were obtained from 25 LTRCD patients, and 25 age-, gender-, and education-matched healthy controls (HCs). T1-weighted images were segmented using FreeSurfer software to extract mean cortical thickness and surface area values of 68 cortical gray matter regions and two whole hemispheres. Paired sample t tests explored differences between the anterior cingulate cortex (ACC; region of interest), and the whole brain. Validated scales assessed psychiatric symptomatology, self- reported cognitive functioning, and disease severity. After correction for multiple comparisons, ROI analyses indicated that LTRCD-patients showed reduced cortical thickness of the left caudal ACC and the right rostral ACC compared to HCs. Whole-brain analyses indicated thinner cortices of the left caudal ACC, left cuneus, left posterior cingulate cortex, right rostral ACC, and bilateral precuneus compared to HCs. No cortical surface area differences were identified. Cortical thickness of the left caudal ACC and left cuneus were inversely associated with anxiety symptoms, depressive symptoms, and disease duration, although certain associations did not persist after correction for multiple testing. In six of 68 regions examined, LTRCD patients had reduced cortical thickness in comparison to HCs. Cortical thickness of the left caudal ACC was inversely associated with disease duration. This suggests that prolonged and excessive exposure to glucocorticoids may be related to cortical thinning of brain structures involved in emotional and cognitive processing.

Introduction

Cushing's disease (CD), a rare endocrine disorder that is caused by an adrenocorticotropic hormone (ACTH) producing pituitary adenoma, is the most common etiology of endogenous Cushing's syndrome (CS1). CS is characterized by chronic exposure to glucocorticoid (GC) excess, with the most common cause being exogenous CS as a consequence of pharmacological GC treatment[1]. Hypercortisolism has been associated with severe physical, psychological, and cognitive impairments, resulting in a substantial deterioration in quality of life. Physical symp- toms of CD include abdominal weight gain and abnormal fat distribution, acne, thin skin sensitive to bruising, osteoporosis, hirsutism, muscle weakness, delayed wound healing, and gonadal dysfunction[2]. Stress-related disorders such as mania, anxiety, and depression commonly present alongside CD, as does suicidality[3–5]. Cognitive deficits that are commonly experienced by CD patients include deficits in reasoning, verbal learning, language performance, difficulty in concentrating, visual and spatial information processing, and memory impairments[6–9]. These symptoms suggest that prolonged exposure to an excess of cortisol has a detrimental effect on the central nervous system. Preclinical studies have found that chronically increased GC exposure can cause psychiatric symptomology (e.g., an anxiodepressive-like phenotype in animals[10,11]), which has been linked to structural and functional changes of certain limbic structures, such as the hippocampus and the anterior cingulate cortex (ACC[12]). In line with these preclinical study findings, long-term exposure to high levels of cortisol in (remitted) CD patients has been associated with functional and structural alterations in similar limbic areas[13]. Considering the severe cortisol dysregulation in CD, changes in cortical thickness and surface area are also expected to be observed in regions affected by CD even after biochemical curation, such as the ACC. However, such analyses are lacking to date.

Pituitary corticotroph adenomas are usually detected as microadenomas (<10 mm), because hyperactivity of the hypothalamic–pituitary–adrenal (HPA-) axis leads to the rapid manifestation of the clinical symptoms of CD1. HPA-axis activity is regulated by limbic structures, such as the amygdala, the hippocampus, and the ACC[14]. Under the influence of the circadian rhythm and exposure to stressors, hypothalamic corticotropin-releasing hormone (CRH) secretion stimulates pituitary ACTH secretion. This, in turn, stimulates GC production by the adrenal glands. In healthy individuals, circulating cortisol inhibits CRH and ACTH secretion through a negative feedback loop, however in patients with CD this physiological control mechanism is impaired due to the autonomous secretion of ACTH by the pituitary

adenoma, resulting in unabridged hypercortisolism[15]. First-line treatment for patients with CD is transsphenoidal pituitary surgery[16]. Second-line treatment may include additional neurosurgical intervention, medical therapy, radiation therapy, or bilateral adrenalectomy. These second-line treatments often result in deficiencies in pituitary hormone production (i.e., hypopituitarism), the need for chronic replacement therapy, and adverse long-term prognoses[17]. However, usually cortisol levels normalize after removal of the adenoma, paired with concomitant somatic, cognitive, and emotional symptom reduction[18–22]. Nevertheless, a higher prevalence of psychiatric symptomatology often remains in long-term remitted CD (LTRCD)-patients in comparison to healthy controls (HCs18,[22–24]). A plausible explanation for these persistent symptoms remains unknown as of yet. Previous studies have reported a reduction in cortical thickness in patients with stress-related disorders such as generalized and social anxiety disorder[25,26] bipolar disorder[27,28], and major depressive disorder[29,30]. Two earlier studies investigated cortical thickness in CS patients and HCs: the first found no differences in cortical thickness[31] and the second reported increased cortical thickness in the lateral orbitofrontal and superior frontal cortex in children with CS in compared to HCs, however this study did not adjust for multiple comparisons[32].

Reductions in gray matter volume of the cingulate, frontal and orbitofrontal cortices, hippocampus, amygdala, inferior temporal gyrus, and striatum have been reported for stress-related disorders such as depression, anxiety and obsessive–compulsive disorder, which are also associated with cortisol dysregulation, albeit on a much smaller scale than in CD[33–37]. In CS patients, earlier studies have found loss of brain volume (for example, in the hippocampus, bicaudate, and third ventricle), which were partially reversible upon biochemical remission[21,38,39]. Analyses conducted in the same cohort of LTRCD patients as the present study have previously revealed reductions in white matter integrity throughout the brain in addition to altered resting-state connectivity between the limbic system and the subgenual ACC in comparison to HCs[24,40]. Furthermore, subcortical gray matter alterations in this patient population were examined using FSL's integrated registration and segmentation tool (FIRST). There were no differences in gray matter volume or shape for any subcortical regions, however reductions of ACC volumes were found[18]. As FSL's FIRST uses a similar segmentation approach of the subcortical regions as that of FreeSurfer, subcortical regions were not further examined in this study. The ACC is an area that has been found to remain affected upon curation of CD. Subregions of the ACC are considered critical in cognitive processing of fear and anxiety, cognitive control, emotional functioning, and reward-based decision making; damage to this

region may lead to reductions in motivation, spontaneity, and problem-solving capacity, as well as increased apathy and verbalization[18,41–43]. These findings suggest that alterations in structure and connectivity in the brain, and in particular the ACC, may explain part of the cognitive and psychiatric symptoms commonly observed both in active and remitted CD patients.

Two frequently used measures for gray matter analysis are cortical thickness, which is indicative of neuron and glia size, number, and arrangement in specific cortical regions[44–46] and cortical surface area, which is related to the number of columns in a region of interest[45–47]. Cortical thickness and surface area together constitute gray matter volume, but separately they provide more detailed information on changes in cortical structures. Therefore, cortical thickness and surface area are suggested to be of more etiological relevance than gray matter volume alone[48,49].

In the present case-control study, our primary objective was to investigate whether LTRCD patients present differences in cortical thickness and surface area in comparison with HCs. We hypothesized that reductions in cortical thickness and changes in surface area of the ACC would be associated with LTRCD. In addition, our secondary objective was to conduct an explorative whole-brain analysis in order to detect possible structural alterations in regions besides the ACC. Moreover, this study aimed to explore associations between structural alterations and measures of psychiatric symptomatology, self-reported cognitive functioning, disease duration, disease severity, and duration of remission. Here, we hypothesized that smaller cortical thickness and surface area would be associated with higher scores on scales assessing psychopathology, lower self-reported cognitive functioning scores, longer disease duration, and/or higher disease severity, and shorter duration of remission.

Methods

Subjects

All 49 LTRCD patients (aged 18–60) who were under chronic surveillance at the Leiden University Medical Center (LUMC) were invited either by letter or by telephone to participate in the study. The response rate was 96%. Of these 49 patients, 16 patients (33%) declined to participate with the (f)MRI part of the protocol. Therefore, 31 patients were screened for eligibility. Of these 31 patients, six were excluded due to one of the following exclusion criteria: neurological problems, magnetic resonance imaging (MRI) contraindications, a (history of) drug or alcohol abuse, and/or left handedness. HCs were recruited via advertisements in grocery

stores and internet, and were matched pairwise to each patient based on gender, age (between 18 and 60 years), and level of education. A further exclusion criterion regarding the HCs group was a history or presence of a psychiatric disorder. A study aimed at determining sample size estimates for cross-sectional cortical thickness studies using FreeSurfer software (the same processing stream as in the current study), found that a sample of 14 subjects per group are required to detect a thickness difference of 0.6 mm over 95% of the cortical surface using two-sided t tests (30 mm FWHM, power = 0.95, $\alpha = 1.22 \times 10^{-4}$ [50]). The final sample of the current study consisted of 25 LTRCD patients and 25 matched HCs.

The diagnosis of active CD was confirmed using inter- national guidelines and multiple positive test outcomes, such as increased urinary cortisol excretion rates, decreased overnight suppression by dexamethasone (1mg), and increased midnight salivary cortisol values. The detailed criteria have previously been published elsewhere[22]. All patients underwent transsphenoidal surgery, after which biochemical cure was confirmed using multiple test outcomes, such as normal overnight suppression of plasma cortisol levels (<50 nmol/l) by dexamethasone (1 mg), normal 24 h urinary cortisol excretion rates (<220 nmol/24 h) and normal cortisol response to CRH stimulation test or insulin tolerance test (>500nmol/l), indicating hydrocortisone independency. Patients with remaining GC dependency (n=13; 52%), received hydrocortisone replacement (on average 20 mg/day, divided over three doses), and were evaluated twice yearly. Persistent biochemical cure of CD was documented as normal levels for abovementioned diagnostic tests before participation in the study. Duration of disease was defined as the moment earliest somatic signs were presented in the patient's history and duration of remission was calculated from the date of curative transsphenoidal surgery, or in case of persistent disease, from the date of normalization of biochemical tests after postoperative radiotherapy (mean: 11.2, SD: 8.2, range 0.8–29.3 years after biochemical remission). Further detailed information on patient inclusion and characteristics have previously been published[18]. Patient characteristics and demographics are reported in Table 1, which are identical to the previously published data by Andela et al.[18]. All participants provided written informed consent, and patient and treatment characteristics were obtained from patient records. The study protocol was approved by the medical ethics committee of the LUMC, and is in accordance with the principles of the declaration of Helsinki.

Psychopathological and clinical severity assessments

Psychopathology and self-reported cognitive functioning were assessed using

various scales, for which higher sum scores indicate greater symptom severity. The Montgomery–Åsberg Depression Rating Scale (MADRS[51]), and the Inventory of Depression Symptomatology (IDS[52]) were used to assess the severity of depressive symptoms. The MADRS was assessed by the interviewer, whereas all other scales used were self-report. Anxiety was evaluated using the Beck Anxiety Inventory (BAI[53]), and phobic anxiety was measured using the total scores, as well as the agoraphobia, blood injury phobia, and social phobia subscales, of the Fear Questionnaire (FQ[54]). The Irritability Scale and the Apathy Scale were used to assess the severity of irritability and apathy, respectively[55,56]. For both questionnaires, participants were considered to be irritable or apathetic if they present a total score of 14 points or more. Failures in motor function, perception and memory were assessed using the self-report Cognitive Failure Questionnaire (CFQ[57]).

Symptom severity during active and remitted disease state were estimated using the Cushing's Syndrome Severity Index (CSI[58]). The CSI score during active disease was estimated retrospectively, whereas the score during remission was based on the last annual evaluation. In the analyses, the total CSI score was used for both active and remitted disease state. Scores on this index can range between 0 and 16, with a higher total score indicating greater symptom severity. The information required to score the CSI was obtained from the patient's clinical history and medical records. The index was scored by two independent raters that reached consensus in case of discrepancy.

MRI data acquisition

Structural magnetic resonance images were acquired using a Philips 3T system (Philips Healthcare, Best, The Netherlands; software version 3.2.1) at the LUMC. A SENSE-32 channel headcoil was used for transmission and reception of radio frequencies. A sagittal 3D gradient- echo T1-weighted sequence (echo time=4.6 ms, repetition time=9.8ms, 140 slices, scan duration 4:56min, matrix size=256 × 256, voxel size = 1.17 × 1.17 × 1.2 mm) was used to acquire anatomical images, which were examined by a neuroradiologist who was blinded for patient details. No macroscopic abnormalities were detected other than age-related white matter intensities and effects of post-transsphenoidal surgery.

Statistical analyses

Parcellation of 68 (34 left and 34 right) cortical gray matter regions as well as extraction of two whole- hemisphere measures were performed using FreeSurfer (version 5.3.0). A visual quality check and statistical outlier assessment of the 68

regions and two whole-hemisphere measures were performed by two independent individuals according to the ENIGMA imaging protocols (http:// enigma.ini.usc.edu/ protocols/imaging-protocols/).

All statistical evaluations were performed using IBM SPSS Statistics for Windows version 24 (IBM Corp. Armonk, N.Y., USA) and figures were created using the r package "ggpmisc" as an extension to the package "ggplot2". We examined differences in cortical thickness and surface area for predetermined regions of interest (ROI): the rostral ACC and the caudal ACC. Moreover, a whole-brain analysis was performed to detect possible unpredicted differences in cortical thickness and/ or surface area between LTRCD patients and HCs. Linear regression was performed with intracranial volume (ICV) as an independent variable and the unstandardized residuals were saved. Restructuring of the dataset to a wide format allowed for calculation of the difference between the residuals of patients and controls, (i.e., delta residuals), per region. The assumption of normal distribution of the delta residuals was tested using the Kolmogorov–Smirnov test, boxplots, histograms and normal and detrended normal Q–Q plots. Next, pairwise group-level comparisons between LTRCD patients and controls were per- formed using pairwise t tests (comparing each patient with its matched control) and Wilcoxon signed rank tests using the residuals of cortical thickness and surface area measures. The reported p values for the ROIs and the whole-brain analyses are two-tailed. All analyses were corrected for multiple testing using the Benjamini–Hochberg procedure[59], with the false dis- covery rate (FDR) set at 5% for 70 measures (68 cortical regions and two whole-hemisphere measures) using Cohen's d as a measure of effect size.

Finally, within the LTRCD-patient group, we investigated whether cortical thickness and surface area measures of regions that showed significant differences in the ROI and whole-brain analyses correlated with measures of psychiatric symptom severity, self-reported cognitive functioning, and clinical severity. The questionnaires used for psychopathological assessment show considerable overlap, therefore correction for multiple testing using the Benjamini–Hochberg method with an FDR set at 5% was considered too stringent. Therefore, we corrected for multiple testing using an FDR set at 20%. We report the uncorrected Pearson's correlations for normally distributed data, and the Spearman's rho for data that is not normally distributed.

Results

Participant characteristics

As previously reported in Andela et al.[18] the LTRCD- patient group did not differ from the HC group in age, gender, and education. The groups also did not differ significantly in intracranial volume (ICV). Mean disease duration was 7.9 ± (SD) 7.9 years (range 0.8–29.3), and mean duration of remission was 11.2 ± 8.2 years (range 0.8–37.0). Mean CSI score was 8.1 ± 2.0 in active CD and 2.5 ± 1.5 in LTRCD patients at the time of assessment. Compared with HCs, LTRCD patients had significantly higher scores on the MADRS and IDS (MADRS: $p < 0.001$, IDS: $p = 0.005$), the BAI ($p = 0.003$), the social phobia subscale of the FQ ($p = 0.034$), the AS ($p = 0.002$), and the CFQ ($p = 0.023$), and the total FQ score approached significance ($p = 0.051$). There were no significant differences between groups regarding scores on the IS, FQ agoraphobia, and blood injury phobia subscales (Table 1; all demographic and participant characteristics have been previously been reported in Andela et al.[18]).

Table 1. Demographics and psychometric data of LTRCD patients and matched healthy controls. Data are presented as mean ± standard deviation or number (%), with a significance level set at $P<0.05$.

	CD patients (n=25) Mean SD	**Matched controls** (n=25) Mean SD	***P* value**
Gender (male/female)	4/21	4/21	1.000[a]
Age (years)	45 8	47 7	0.471[b]
Education			0.946[a]
Low	6 (24%)	6 (24%)	
Medium	12 (48%)	11 (44%)	
High	7 (28%)	8 (32%)	
Intracranial volume	1.4510^6 0.16310^6	1.4810^6 0.14510^6	0.716[b]
MADRS	6.3 5.5	1.4 1.8	<0.0001[c]
Inventory of Depressive Symptomatology	46.8 13.0	36.3 5.8	0.005[c]
Beck Anxiety Inventory	28.4 5.7	24.0 3.1	0.003[c]
Fear Questionnaire	24.5 17.4	14.2 10.0	0.051[b]
Agoraphobia subscale	6.1 7.9	3.4 4.7	0.477[c]
Blood injury phobia subscale	6.2 8.3	3.2 4.1	0.118[c]
Social phobia subscale	12.2 8.0	7.6 4.9	0.034[b]
Irritability Scale	12.1 8.7	8.0	0.066[c]
Total score > 14	9 (36%)	6 (24%)	
Apathy Scale	13.6 6.6	7.8 3.8	0.002[c]
Total score > 14	11 (44%)	2 (8%)	
Cognitive Failures Questionnaire	38.0 16.5	27.6 9.7	0.023[b]
Disease duration (years)	7.9 7.9		
Duration of remission (years)	11.2 8.2		
Cushing's Syndrome Severity Index			
Active phase (total)	8.1 2.0		
Remission phase (total)	2.5 1.5		

MADRS = Montgomery-Åsberg Depression Rating Scale
[a] *P* values were tested with ^{2} test
[b] *P* values were tested with independent samples t-test
[c] *P* values tested with Mann-Whitney *U* test

ROI analyses

With regard to the ROI analyses, LTRCD patients showed smaller cortical thickness of the left caudal ACC (p = 0.002) and the right rostral ACC (p = 0.003) compared with HCs. Cohen's d was 0.68 and 0.65 for the left caudal and right rostral ACC respectively, indicating medium effect sizes (see Table 2 and Appendix I for a visual representation). Closer examination of the findings revealed that patients had 6% smaller left caudal ACC thickness and 5% smaller right rostral ACC thickness. ROI surface area analyses revealed no significant differences between LTRCD patients and HCs (see Table 2 and Appendix I for a visual representation).

Table 2. ROI analysis of corticol thickness and surface area measures.

Measure	Region	Mean (mm^2) (S.E)			Δ (mm^2) (S.E.)	Uncorrected *p*-value	Cohen's d
		N	Cushing's Disease	Matched controls			
Cortical thickness (mm^2)	L caudal ACC	25	2.78	2.95 (0.04)	0.18 (0.05)	0.002*	0.68
	L rostral ACC	22	2.93 (0.04)	2.89 (0.03)	-0.04 (0.04)	0.375	-0.19
	R caudal ACC	25	2.74 (0.05)	2.78 (0.06)	0.04 (0.06)	0.541	0.11
	R rostral ACC	25	2.96	3.11 (0.03)	0.15 (0.05)	0.003*	0.65
Surface area (mm^2)	L caudal ACC	25	534.8 (26.24)	546.1 (21.12)	11.3 (32.6)	0.610	0.10
	L rostral ACC	22	741.4 (25.78)	803.0 (36.06)	61.6 (42.3)	0.119	0.35
	R caudal ACC	25	665.6	652.1 (23.86)	-13.5 (30.3)	0.729	-0.07
	R rostral ACC	25	589.6	560.6	-28.9 (38.9)	0.467	-0.15

ACC = anterior cingulate cortex
* = remains significant after Benjamini-Hochberg correction (FDR=5%) for 4 comparisons.

Whole-brain analyses

In comparison to the HC group, LTRCD patients presented smaller cortical thickness of the left caudal ACC (p = 0.002), left cuneus (p = 0.004), left posterior cingulate cortex (p=0.004), left superior frontal cortex (p= 0.041), left supramarginal cortex (p = 0.044), right cuneus (p = 0.007), right pars opercularis (p = 0.037), right rostral ACC (p = 0.003), and bilateral precuneus (left: p = 0.002, right: p = 0.003). However, after correction for multiple testing using the Benjamini–Hochberg method (FDR= 5%) for 70 comparisons, only the differences in the left caudal ACC, left cuneus (Cohen's d = 0.68), left posterior cingulate cortex (Cohen's d = 0.68), right rostral ACC, left precuneus (Cohen's d = 0.70), and right precuneus (Cohen's d = 0.66), remained significant (Table 3 and Fig. 1; see Appendix II for a complete overview). Closer examination of the data revealed that patients had 6% smaller thickness of the left cuneus, 5% smaller thickness of the left posterior cingulate cortex, and 4% smaller thickness of the bilateral precuneus.

Whole-brain analyses of surface area measures revealed greater surface area of the right banks of the superior tem- poral sulcus (p = 0.011). However, this difference did not remain significant after correction for multiple testing.

Table 3. Whole-brain analysis of corticol thickness measures.

Region	N	Mean (mm^2) (S.E.) Cushing's Disease	Mean (mm^2) (S.E.) Matched controls	Δ (mm^2) (S.E.)	Uncorrected p-value	Cohen's d
L caudal ACC	25	2.78 (0.03)	2.95 (0.04)	0.18 (0.05)	0.002*	0.68
L precuneus	24	2.34 (0.03)	2.45 (0.03)	0.10 (0.03)	0.002*	0.70
R precuneus	25	2.35 (0.02)	2.45 (0.03)	0.11 (0.03)	0.003*	0.66
R rostral ACC	25	2.96 (0.04)	3.11 (0.03)	0.15 (0.05)	0.003*	0.65
L cuneus	23	1.71 (0.02)	1.82 (0.03)	0.11 (0.04)	0.004*	0.68
R cuneus	23	1.76 (0.02)	1.85 (0.03)	0.09 (0.03)	0.007	0.65
L posterior cingulate	25	2.46 (0.04)	2.60 (0.03)	0.13 (0.04)	0.004*	0.68

ACC = anterior cingulate cortex
** = remains significant after Benjamini-Hochberg correction (FDR=5%) for 70 comparisons.*

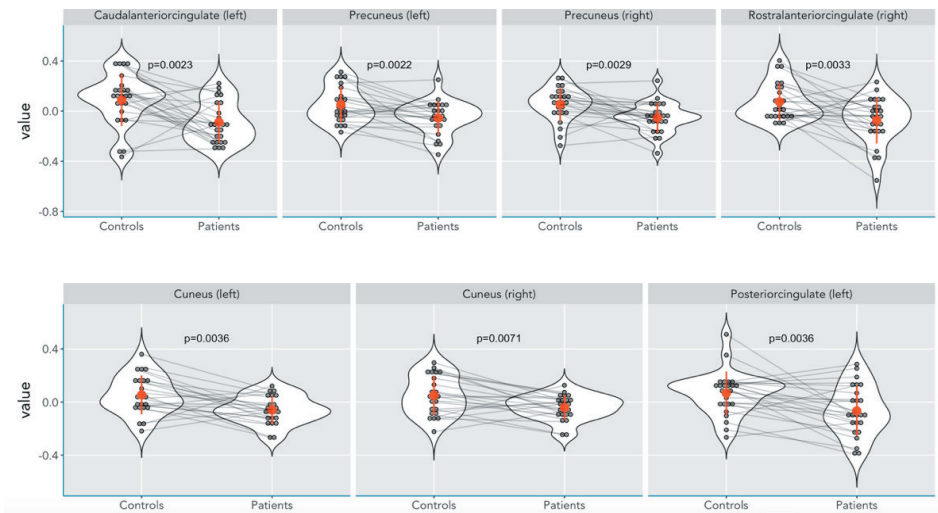

Figure 1. Violin plots of significant cortical thickness differences between LTRCD patients and HCs representing areas that remained significant after Benjamini-Hochberg correction (FDR=5%) with the exception of the right cuneus.

Correlation analyses

Correlation analyses were run for brain regions that were found to be significantly different in LTRCD patients in comparison to HCs with the psychopathology and clinical severity assessments. Within the LTRCD group, cortical thickness measures of the left caudal ACC were significantly negatively associated with disease duration ($r = -0.421$, $p = 0.036$). Considering that the total FQ score between the groups approached significance ($p = 0.051$) and the mean total FQ score between the groups differed more than 10 points, indicating a clinically relevant difference, associations between the FQ psychopathology (sub)scales and these brain regions were further investigated. Pertaining to this, cortical thickness measures of the left caudal ACC and the total FQ score were found to be negatively associated ($r = -0.512$, $p = 0.011$; see Fig. 2a and Appendix III for further details). Cortical thickness of the left cuneus was significantly negatively associated with scores on the MADRS ($r = -0.430$, $p = 0.032$) and the IDS ($r = -0.417$, $p = 0.043$; see Fig. 2b and Appendix IV). After correcting for multiple comparisons using the Benjamini–Hochberg method (FDR = 20%) for 11 tests, associations between the left caudal ACC with disease duration and the total FQ score remained statistically significant. No other significant associations between cortical thickness measures and scores on psychopathology scales, measures of disease duration, duration of remission, and clinical disease severity were found (Appendices V–VIII).

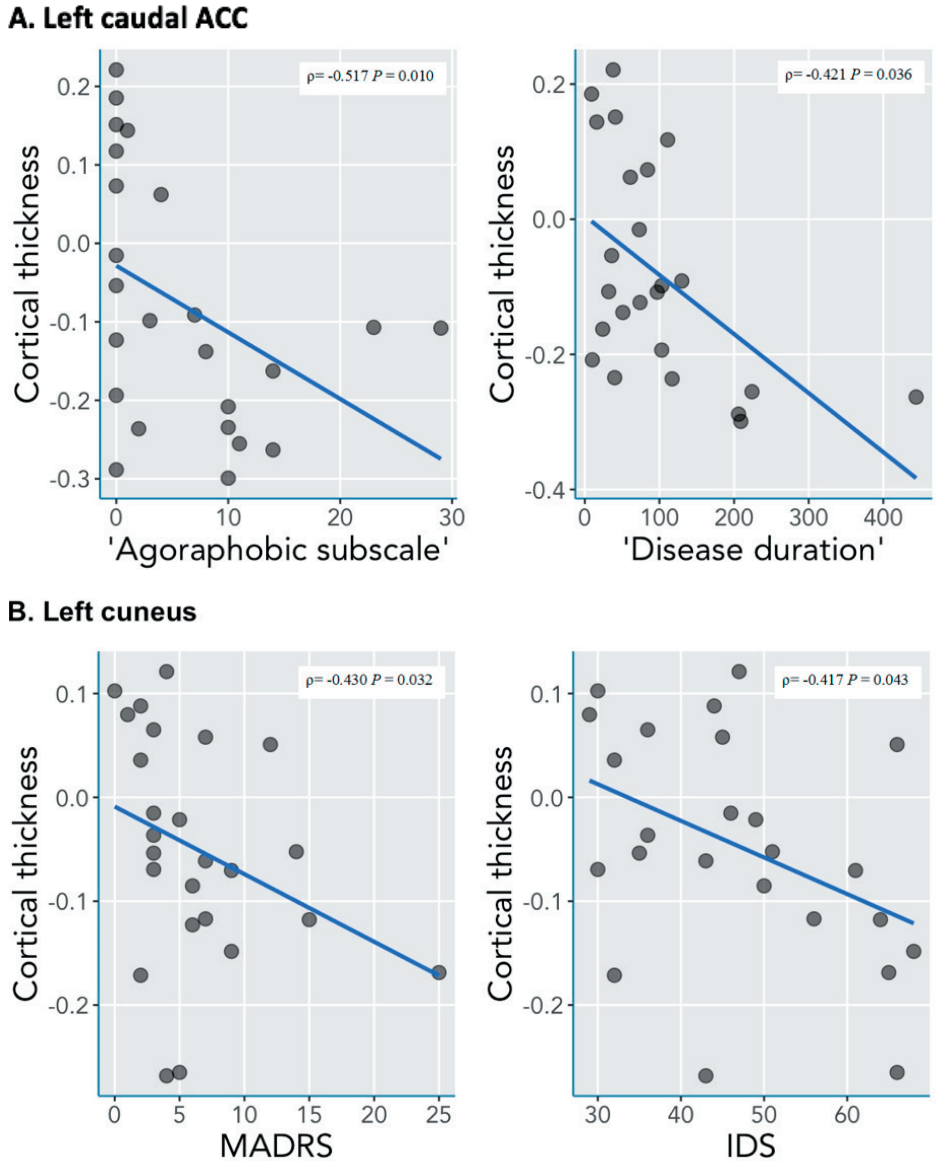

Figure 2. Significant correlations after Benjamini-Hochberg correction (FDR=20%) for 11 comparisons between cortical thickness of the left caudal ACC and disease duration in months and total FQ scores (A), and significant correlations prior to adjusting for multiple comparisons between cortical thickness of the left cuneus and the MADRS and IDS (B).

Discussion

This study aimed to investigate whether LTRCD patients and matched HCs differ in cortical thickness and/or surface area. We found smaller cortical thickness in several key regions for emotional and cognitive processing: the left caudal and right rostral ACC, the left cuneus, left posterior cingulate cortex, and bilateral precuneus in LTRCD patients compared with controls, while no significant differences in surface area between the groups were observed. Furthermore, correlation analyses within the patient group indicated that cortical thickness of the left caudal ACC was inversely associated with disease duration and total FQ score, although group differences on the total FQ score did not fully meet the significance threshold. Prior to adjusting for multiple comparisons, cortical thickness of the left cuneus was inversely associated with sum scores on the MADRS and IDS.

Our hypothesis that LTRCD patients present more thinning of the ACC in comparison with HCs was confirmed. These findings are not in line with two previous studies investigating cortical thickness in CS patients. One study did not identify any differences in cortical thickness[31,] and the second study identified increased cortical thickness in the lateral orbitofrontal- and superior frontal cortex in children with CS in compared to HCs but did not adjust for multiple comparisons[32]. As the differences found in these areas appear to be marginal, it is unlikely that these effects would have persisted after correction. A possible explanation why these earlier studies did not find results in line with ours could be due to the differences between the CS patient populations included in the studies (i.e., our study consisted solely of patients with remitted CD and the other studies also included patients with other causes of CS), or perhaps due to differences in FreeSurfer versions (e.g., the current study used a newer version than that denoted in the Crespo et al.[31] paper (v5.3.0 versus v4.3.1). Unfortunately, the Tirosh et al.[32] paper did not indicate the version of FreeSurfer they used. Using the more recent releases has been posited to provide a more accurate segmentation, although differences may also be caused due to different software builds[60]. Our findings are in accordance with previous findings of Andela et al.[13] who in the same cohort of LTRCD patients and matched HCs observed reductions in gray matter volume of parts of the bilateral ACC in LTRCD patients.

Furthermore, our results are also in line with findings from an earlier animal study, where reductions in ACC volumes were observed in rats exposed to a GC excess[12]. Limbic structures such as the ACC, hippocampus, and amygdala critically control the activity of the HPA axis[14]. These regions express high levels of glucocorticoid receptors

(GR) and mineralocorticoid receptors (MR), making them vulnerable to GC excess as seen in stress- related disorders and more severely in CD. Interestingly, GR and MR are prevalent throughout the brain and not solely in regions affected in LTRCD. The enzyme 11β-HSD2 protects MR and GR from GC excess by converting cortisol into the inactive metabolite cortisone. However, 11β-HSD2 is not expressed in limbic structures such as the ACC, allowing for MR and GR activation in this region[61,62]. It is also a possibility that structural changes are mediated by transsynaptic mechanisms.

Volume changes may reflect changes in any population of neuronal or nonneuronal glia cells in the affected areas, all of which likely are GC sensitive to some extent. Previous studies have repeatedly shown that dendrites, spines, and expression of synaptic molecules are affected by chronic stress[63–68]. A significant loss of synapses on pyramidal cells of hippocampal region CA3, as well as morphological changes in afferent mossy fibers terminating on these neurons, have been observed in animals exposed to GC excess. Moreover, remodeling of pyramidal cells in the prefrontal cortex was observed as a result of exposure to stress[69]. Such processes may cause damage to white matter tracts, which could explain previous findings of reduced white matter integrity and altered resting-state connectivity in LTRCD patients[40]. Persistent hypercortisolism may ultimately lead to loss of neurons[38]. This has been related to increased synaptic glutamate accumulation, leading to increased stimulation of N-methyl-D-aspartate receptors, and elevated post-synaptic intracellular Ca2+ levels[61,70,71]. This increases the susceptibility of postsynaptic neurons to injury and cell death, which may be an underlying cause of smaller cortical thickness as observed in the present study. It has been proposed that loss of brain volume induced by chronic hypercortisolism is likely caused by a combination of the factors described above[61,70].

Apart from the thinning of the left caudal ACC and right rostral ACC, whole-brain analyses also revealed smaller cortical thickness of the bilateral precuneus, left cuneus, and left posterior cingulate cortex in LTRCD patients. The precuneus plays a critical role in behavioral inhibition, which is implicated in cognitive and emotional functioning[72,73]. Moreover, the precuneus is involved in integration of visual and spatial information with the memory domain[74]. These are functions in which patients with LTRCD often experience persistent deficits[6–8,22]. The cuneus plays a critical role in basic visual processing, in which impairments are commonly experienced by LTRCD patients[6]. The cuneus has also been positively associated with inhibitory control in bipolar patients"[75]. The posterior cingulate cortex is a central node within the default mode network of the brain, and together with

the precuneus, has been implicated as a neural substrate for human awareness. Moreover, it has also been posed to have a prominent role in pain, episodic memory retrieval[76], and working memory performance[77]. In partial concurrence with these findings, an earlier study investigating episodic and working memory in female patients with long-term remitted CS found decreased functional brain response during episodic and working memory tests[78]. Furthermore, the precuneus, cuneus, posterior cingulate cortex, and ACC are located next to one another and show strong reciprocal connectivity, and are involved in the large-scale default mode network[40,74,79]. Given the observed cortical abnormalities of these regions in the present study, these findings may support the hypothesis that structural changes occur through transsynaptic mechanisms.

In contrast to our hypothesis, no differences were observed in cortical surface area between LTRCD patients and HCs. Several previous studies examining cortical thickness and surface area in adults with generalized- and social anxiety disorder and MDD have presented similar findings, namely reductions in cortical thickness of certain brain areas, but with no differences in cortical surface area[25,26,29]. The discrepancies between our findings in cortical thickness and surface area suggest that there are distinct (genetic and biological) pathways that affect these measures. Consistent findings in previous studies indicate that cortical thickness and surface area are genetically independent, with the result that both measures are driven by different cellular mechanisms and have different developmental trajectories[80–83]. Our discrepant findings suggest that alterations in gray matter volume that we previously observed may be explained by changes in cortical thickness alone, without changes in surface area. The null findings in our surface area analyses suggest that cortical thickness may have more etiological value than surface area. However, previous studies have shown that surface area has more influence on gray matter volume than cortical thickness[49,83,84], although this may differ for patients with hypercortisolism and may thus be condition and context dependent.

Correlation analyses revealed multiple significant negative associations between psychopathology measures and cortical thickness of the left cuneus and the left caudal ACC prior to adjusting for multiple comparisons. The psychopathology measures assessed depressive and anxiety symptoms, which are commonly observed psychopathologies in both patients with active CD and with LTRCD. Prior to adjusting for multiple comparisons, the left cuneus was found to be associated with MADRS and IDS scores. A previous study conducted within the same participant cohort as the present study compared LTRCD patients with HCs in terms

of presence and severity of psychopathology and cognitive failure. They reported significantly higher levels of depressive symptoms, anxiety, social phobia, apathy, and cognitive failure in LTRCD patients[18]. These findings also support the hypothesis that depressive symptoms and anxiety in LTRCD patients are associated with structural brain changes[22]. Furthermore, a significant negative correlation between left caudal ACC thickness and disease duration was found, offering further support that prolonged exposure to excessive amounts of cortisol may lead to more severe effects on cortical brain structures. Also, significant negative associations were found between the left caudal ACC and the total FQ score. Interestingly, an earlier study found thinning of the cingulate cortex in spider-phobic patients[42], indicating that the thinning of this brain region may be related to phobias in general. However, as the present study is cross- sectional, no causal conclusions can be drawn, and thus the possibility that structural alterations were already present before onset of CD should be considered. Nevertheless, further research into these relationships may create possibilities for developing specialized therapies for specific patient groups.

The present study provides a valuable contribution to the existing literature by demonstrating that smaller cortical thickness is at least partially responsible for smaller gray matter volumes of the ACC in patients with LTRCD. A considerable strength of our study was the matching of participants for age, gender and education, allowing for paired analysis without correction for these factors. A second strength is the homogeneity of the patient cohort in terms of treatment. It is, however, important to realize that considerable heterogeneity concerning disease duration and duration of remission still exists, which may reduce the power of the study. Nonetheless, the study had sufficient power to detect a number of structural differences even after Benjamini–Hochberg correction. This exemplifies that transient excessive exposure of cortisol excess can result in long-lasting, and possibly irreversible effects on the human brain.

A limitation of this study were the instruments used for the psychopathological assessment, in particular the CSI and CFQ. Although both have been validated repeatedly, the CSI score during active disease was estimated retrospectively and the CFQ cannot replace an elaborate neuropsychological assessment. This may have resulted in a less accurate estimation of disease severity and cognitive functioning. Next, the program FreeSurfer has difficulty with the parcellation of regions with natural anatomical variation such as the cingulate cortex, especially in the presence of a paracingulate sulcus. This affects the surrounding regions and makes accurate estimation of cor- tical thickness and surface area measures more

challenging, although brain segmentations were quality checked by means of visual inspection by two independent raters, and discrepancies between raters were reassessed by the two parties. Finally, despite the reliable quantification of factors influencing gray matter volume provided by cortical thickness and surface area, these measures do not elucidate the physiological processes involved in volumetric changes. An 1H-MRS study con- ducted in remitted CS patients revealed alterations in hippocampal Glx (Glutamate+Glutamine), NAA (N- Acetyl-aspartate), and total NAA (N-Acetyl-Aspartate+N-Acetyl-aspartyl-Glutamate[85]). Disrupted Glx balance may result in neuronal damage[86], while reduced NAA levels indicate neuronal dysfunction or loss[87,88]. Interestingly, these metabolite alterations were observed in absence of gray matter volume changes, suggesting that metabolite alterations may precede structural changes. Similarly, functional abnormalities may also be present despite a lack of structural alterations. Therefore, further research examining functional and biochemical changes is required to increase our understanding of the mechanisms underlying CD.

In conclusion, this study demonstrates that patients with LTRCD present cortical thickness rather than surface area abnormalities, building upon previous knowledge, and highlighting certain brain regions that have not been identified as different in the CD patient population to date. Differences were found in key regions for emotional and cognitive processing compared to HCs, with cortical thinning of the left caudal ACC, left cuneus, left posterior cingulate cortex, right rostral ACC, and bilateral precuneus. Moreover, within the LTRCD group, cortical thickness of the left caudal ACC was negatively associated with disease duration and total FQ score. These findings present a possible explanation for volumetric alterations observed in patients with LTRCD and suggest that longer duration of exposure to GC excess has a more severe effect on brain structures and persisting psychiatric symptomatology. It is important to note that a lateralization effect was found in almost all of the significantly different brain structures, suggesting that the left and right areas of certain cortical brain structures differ in their functionality. Disentangling the specific functionality of these brain regions may lead to further valuable insights into the effects of long-term exposure of cortisol on the brain. Future research using longitudinal study designs to examine functional and physiological changes is required to elucidate the pathways leading to persisting structural alterations in the brain of patients with CD, which may aid in improving treatment and prevention strategies for patients with CD as well as for patients with stress-related disorders[77].

Appendices

Appendix I.

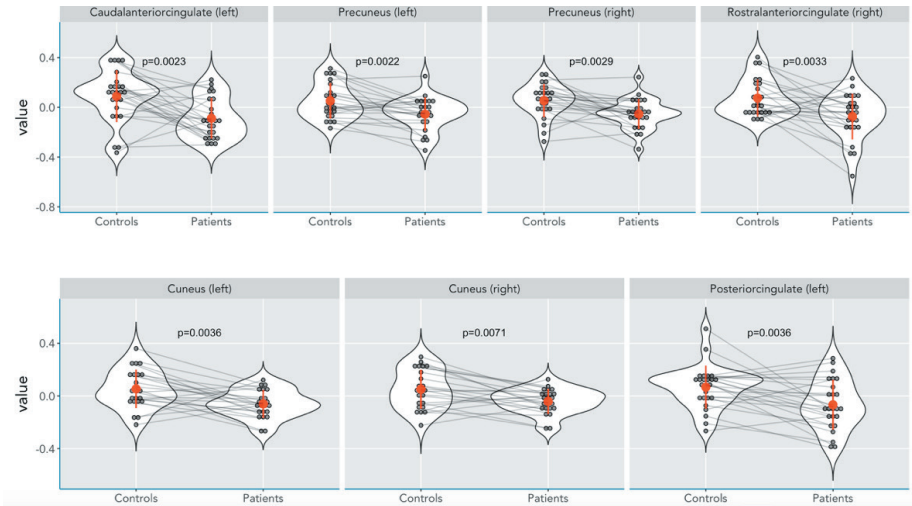

Figure. Violin plots of cortical thickness ROIs (A) and surface area ROIs (B).

Appendix I I. Complete overview of cortical thickness measures for the whole brain.

Measure	Region	N	Mean (S.E.) Cushing's Disease	Mean (S.E.) Matched controls	Δ (mm²) (S.E.)	Uncorrected p-value
Left Cortical thickness (mm²)	L banks	16	2.49 (0.05)	2.46 (0.05)	-0.03 (0.08)	0.680
	L caudalanteriorcingulate	**25**	**2.78 (0.03)**	**2.95 (0.04)**	**0.18 (0.05)**	**0.002**
	L caudalmiddlefrontal	24	2.56 (0.03)	2.63 (0.03)	0.07 (0.04)	0.116
	L cuneus	**23**	**1.71 (0.02)**	**1.82 (0.03)**	**0.11 (0.04)**	**0.004**
	L entorhinal	25	3.28 (0.06)	3.28 (0.06)	0.01 (0.08)	0.906
	L frontalpole	24	2.69 (0.05)	2.86 (0.06)	0.16 (0.09)	0.069
	L fusiform	25	2.67 (0.03)	2.64 (0.03)	-0.02 (0.04)	0.650
	L inferiorparietal	25	2.45 (0.02)	2.51 (0.03)	0.06 (0.03)	0.079
	L inferiortemporal	25	2.73 (0.02)	2.75 (0.02)	0.02 (0.03)	0.440
	L insula	24	2.95 (0.04)	3.03 (0.03)	0.09 (0.05)	0.109
	L isthmuscingulate	24	2.42 (0.04)	2.47 (0.03)	0.04 (0.05)	0.370
	L lateraloccipital	24	2.04 (0.02)	2.09 (0.03)	0.05 (0.04)	0.163
	L lateralorbitofrontal	25	2.70 (0.03)	2.71 (0.03)	0.01 (0.04)	0.726
	L lingual	24	1.85 (0.02)	1.90 (0.03)	0.05 (0.03)	0.124
	L medialorbitofrontal	23	2.47 (0.02)	2.47 (0.03)	0.004 (0.04)	0.916
	L middletemporal	18	2.86 (0.03)	2.87 (0.04)	0.008 (0.05)	0.858
	L paracentral	24	2.36 (0.05)	2.44 (0.04)	0.09 (0.07)	0.208
	L parahippocampal	25	2.77 (0.07)	2.69 (0.04)	-0.08 (0.08)	0.307
	L parsopercularis	25	2.59 (0.03)	2.63 (0.03)	0.04 (0.04)	0.382
	L parsorbitalis	25	2.74 (0.06)	2.75 (0.04)	0.01 (0.08)	0.890
	L parstriangularis	24	2.44 (0.03)	2.49 (0.03)	0.06 (0.04)	0.117
	L pericalcarine	22	1.46 (0.02)	1.49 (0.03)	0.03 (0.03)	0.301
	L postcentral	24	2.03 (0.03)	2.10 (0.03)	0.06 (0.05)	0.183
	L posteriorcingulate	**25**	**2.93 (0.04)**	**2.89 (0.03)**	**0.133 (0.04)**	**0.004**
	L precentral	23	2.51 (0.05)	2.58 (0.04)	0.07 (0.06)	0.225
	L precuneus	**24**	**2.34 (0.03)**	**2.45 (0.03)**	**0.11 (0.03)**	**0.002**
	L rostralanteriorcingulate	22	2.93 (0.04)	2.89 (0.03)	-0.04 (0.04)	0.377
	L rostralmiddlefrontal	20	2.44 (0.02)	2.44 (0.02)	0.007 (0.03)	0.842
	L superiorfrontal	24	2.76 (0.03)	2.83 (0.03)	0.07 (0.33)	0.041
	L superiorparietal	25	2.15 (0.03)	2.21 (0.02)	0.07 (0.04)	0.097
	L superiortemporal	18	2.74 (0.03)	2.77 (0.03)	0.04 (0.04)	0.411
	L supramarginal	25	2.54 (0.02)	2.62 (0.03)	0.07 (0.03)	0.044
	L temporalpole	23	3.54 (0.06)	3.69 (0.06)	0.14 (0.09)	0.117
	L transversetemporal	25	2.27 (0.06)	2.80 (0.04)	0.09 (0.07)	0.185
Right Cortical thickness (mm²)	R banks	24	2.61 (0.04)	2.57 (0.04)	-0.04 (0.06)	0.482
	R caudalanteriorcingulate	25	2.74 (0.05)	2.78 (0.06)	0.036 (0.06)	0.583
	R caudalmiddlefrontal	25	2.58 (0.03)	2.60 (0.03)	0.023 (0.04)	0.568
	R cuneus	**23**	**1.76 (0.02)**	**1.85 (0.03)**	**0.093 (0.03)**	**0.007**
	R entorhinal	25	3.53 (0.06)	3.41(0.07)	-0.115 (0.09)	0.218
	R frontalpole	25	2.78 (0.06)	2.87 (0.05)	0.083 (0.08)	0.312
	R fusiform	25	2.67 (0.03)	2.67 (0.03)	0.006 (0.04)	0.888
	R inferiorparietal	23	2.49 (0.02)	2.54 (0.02)	0.055 (0.03)	0.094
	R inferiortemporal	25	2.75 (0.03)	2.71 (0.02)	-0.036 (0.03)	0.198
	R insula	24	3.04 (0.05)	3.05 (0.03)	0.011 (0.06)	0.843
	R isthmuscingulate	24	2.48 (0.05)	2.49 (0.04)	0.014 (0.07)	0.840
	R lateraloccipital	24	2.11 (0.02)	2.17 (0.03)	0.061 (0.03)	0.082
	R lateralorbitofrontal	25	2.67 (0.03)	2.67 (0.03)	<0.001 (0.04)	0.999
	R lingual	24	1.91 (0.02)	1.94 (0.03)	0.034 (0.03)	0.341
	R medialorbitofrontal	24	2.56 (0.03)	2.60 (0.03)	0.045 (0.04)	0.281
	R middletemporal	25	2.89 (0.03)	2.91 (0.03)	0.026 (0.04)	0.517
	R paracentral	25	2.38 (0.05)	2.44 (0.04)	0.058 (0.06)	0.347
	R parahippocampal	25	2.70 (0.05)	2.72 (0.04)	0.018 (0.06)	0.770
	R parsorbitalis	25	2.68 (0.04)	2.70 (0.04)	0.014 (0.06)	0.815
	R parsopercularis	24	2.55 (0.04)	2.64 (0.03)	0.089 (0.04)	0.037
	R parstriangularis	25	2.51 (0.04)	2.51 (0.04)	0.002 (0.05)	0.967
	R pericalcarine	23	1.50 (0.02)	1.53 (0.02)	0.035 (0.03)	0.234
	R postcentral	22	2.05 (0.03)	2.08 (0.03)	0.034 (0.04)	0.386
	R posteriorcingulate	23	2.56 (0.03)	2.57 (0.04)	0.009 (0.04)	0.844
	R precentral	23	2.51 (0.04)	2.54 (0.04)	0.029 (0.04)	0.525
	R precuneus	**25**	**2.35 (0.02)**	**2.45 (0.03)**	**0.108 (0.03)**	**0.003**
	R rostralanteriorcingulate	**25**	**2.96 (0.04)**	**3.11 (0.03)**	**0.149 (0.05)**	**0.003**
	R rostralmiddlefrontal	19	2.41 (0.02)	2.45 (0.03)	0.040 (0.03)	0.263
	R superiorfrontal	25	2.78 (0.03)	2.82 (0.03)	0.047 (0.03)	0.186
	R superiorparietal	25	2.13 (0.03)	2.19 (0.03)	0.058 (0.03)	0.104
	R superiortemporal	24	2.79 (0.03)	2.83 (0.03)	0.045 (0.05)	0.347
	R supramarginal	22	2.57 (0.02)	2.58 (0.03)	0.007 (0.03)	0.839
	R temporalpole	22	3.72 (0.07)	3.78 (0.05)	0.056 (0.08)	0.503
	R transversetemporal	25	2.33 (0.06)	2.39 (0.04)	0.062 (0.08)	0.422

Appendix I I I. Correlations between cortical thickness of the left caudal anterior cingulate cortex and measures of behavioural and clinical severity within the patient group.

Behavioral and clinical severity scales	Pearson		Spearman's rho	
	Correlation coefficient	P value	Correlation coefficient	P value
MADRS			0.159	0.449
Inventory of Depressive Symptomatology	0.001	0.998		
Beck Anxiety Inventory	0.116	0.590		
Fear Questionnaire	-0.512	**0.011***		
Social phobia subscale	-0.287	0.174		
Apathy Scale	0.050	0.817		
Cognitive Failures Questionnaire	-0.021	0.924		
Disease duration			-0.421	**0.036***
Duration of remission			-0.108	0.608
Cushing's Syndrome Severity Index				
Active phase	0.008	0.971		
Remission phase	0.036	0.863		

MADRS = Montgomery-Åsberg Depression Rating Scale
* = remains significant after Benjamini-Hochberg correction (FDR=20%) for 11 comparisons.

Appendix IV. Correlations between cortical thickness of the left cuneus and measures of behavioural and clinical severity within the patient group.

Behavioral and clinical severity scales	Pearson		Spearman's rho	
	Correlation coefficient	P value	Correlation coefficient	P value
MADRS			-0.430	**0.032***
Inventory of Depressive Symptomatology	-0.417	**0.043***		
Beck Anxiety Inventory	-0.401	0.052		
Fear Questionnaire	-0.143	0.505		
Social phobia Subscale	-0.324	0.123		
Apathy Scale	-0.153	0.477		
Cognitive Failures Questionnaire	-0.262	0.215		
Disease duration			-0.260	0.209
Duration of remission			0.112	0.593
Cushing's Syndrome Severity Index				
Active phase	0.098	0.641		
Remission phase	-0.223	0.285		

MADRS = Montgomery-Åsberg Depression Rating Scale
* = remains significant after Benjamini-Hochberg correction (FDR=20%) for 11 comparisons.

Appendix V. Correlations between cortical thickness of the right rostral anterior cingulate cortex and measures of behavioral and clinical severity within the patient group.

Behavioral and clinical severity scales	Pearson		Spearman's rho	
	Correlation coefficient	P value	Correlation coefficient	P value
MADRS			0.067	0.749
Inventory of Depressive Symptomatology	-0.040	0.852		
Beck Anxiety Inventory	0.116	0.589		
Fear Questionnaire	-0.052	0.809		
Social phobia subscale	0.081	0.708		
Apathy Scale	0.291	0.168		
Cognitive Failures Questionnaire	-0.147	0.493		
Disease duration			-0.173	0.407
Duration of remission			-0.360	0.077
Cushing's Syndrome Severity Index				
Active phase	-0.059	0.781		
Remission phase	-0.313	0.128		

MADRS = Montgomery-Åsberg Depression Rating Scale
* = remains significant after Benjamini-Hochberg correction (FDR=20%) for 11 comparisons.

Appendix VI. Correlations between cortical thickness of the left posterior cingulate cortex and measures of behavioural and clinical severity within the patient group.

Behavioral and clinical severity scales	Pearson		Spearman's rho	
	Correlation coefficient	P value	Correlation coefficient	P value
MADRS			-0.103	0.626
Inventory of Depressive Symptomatology	-0.231	0.277		
Beck Anxiety Inventory	-0.229	0.282		
Fear Questionnaire	-0.207	0.333		
Social phobia subscale	-0.233	0.273		
Apathy Scale	0.012	0.956		
Cognitive Failures Questionnaire	-0.343	0.101		
Disease duration			-0.121	0.565
Duration of remission			-0.105	0.619
Cushing's Syndrome Severity Index				
Active phase	0.007	0.975		
Remission phase	-0.239	0.249		

MADRS = Montgomery-Åsberg Depression Rating Scale
* = remains significant after Benjamini-Hochberg correction (FDR=20%) for 11 comparisons.

Appendix VII. Correlations between cortical thickness of the left precuneus and measures of behavioural and clinical severity within the patient group.

Behavioral and clinical severity scales	Pearson		Spearman's rho	
	Correlation coefficient	P value	Correlation coefficient	P value
MADRS			-0.175	0.402
Inventory of Depressive Symptomatology	-0.193	0.367		
Beck Anxiety Inventory	-0.271	0.200		
Fear Questionnaire	-0.173	0.419		
Social phobia subscale	-0.381	0.066		
Apathy Scale	-0.134	0.533		
Cognitive Failures Questionnaire	-0.246	0.247		
Disease duration			-0.206	0.323
Duration of remission			-0.026	0.901
Cushing's Syndrome Severity Index				
Active phase	0.325	0.113		
Remission phase	-0.212	0.310		

MADRS = Montgomery-Åsberg Depression Rating Scale
* = remains significant after Benjamini-Hochberg correction (FDR=20%) for 11 comparisons.

Appendix VIII. Correlations between cortical thickness of the right precuneus and measures of behavioural and clinical severity within the patient group.

Behavioral and clinical severity scales	Pearson		Spearman's rho	
	Correlation coefficient	P value	Correlation coefficient	P value
MADRS			-0.167	0.426
Inventory of Depressive Symptomatology	-0.211	0.322		
Beck Anxiety Inventory	-0.278	0.188		
Fear Questionnaire	-0.115	0.592		
Social phobia subscale	-0.351	0.092		
Apathy Scale	-0.068	0.753		
Cognitive Failures Questionnaire	-0.257	0.226		
Disease duration			-0.231	0.266
Duration of remission			0.148	0.481
Cushing's Syndrome Severity Index				
Active phase	0.240	0.249		
Remission phase	-0.055	0.795		

MADRS = Montgomery-Åsberg Depression Rating Scale
* = remains significant after Benjamini-Hochberg correction (FDR=20%) for 11 comparisons.

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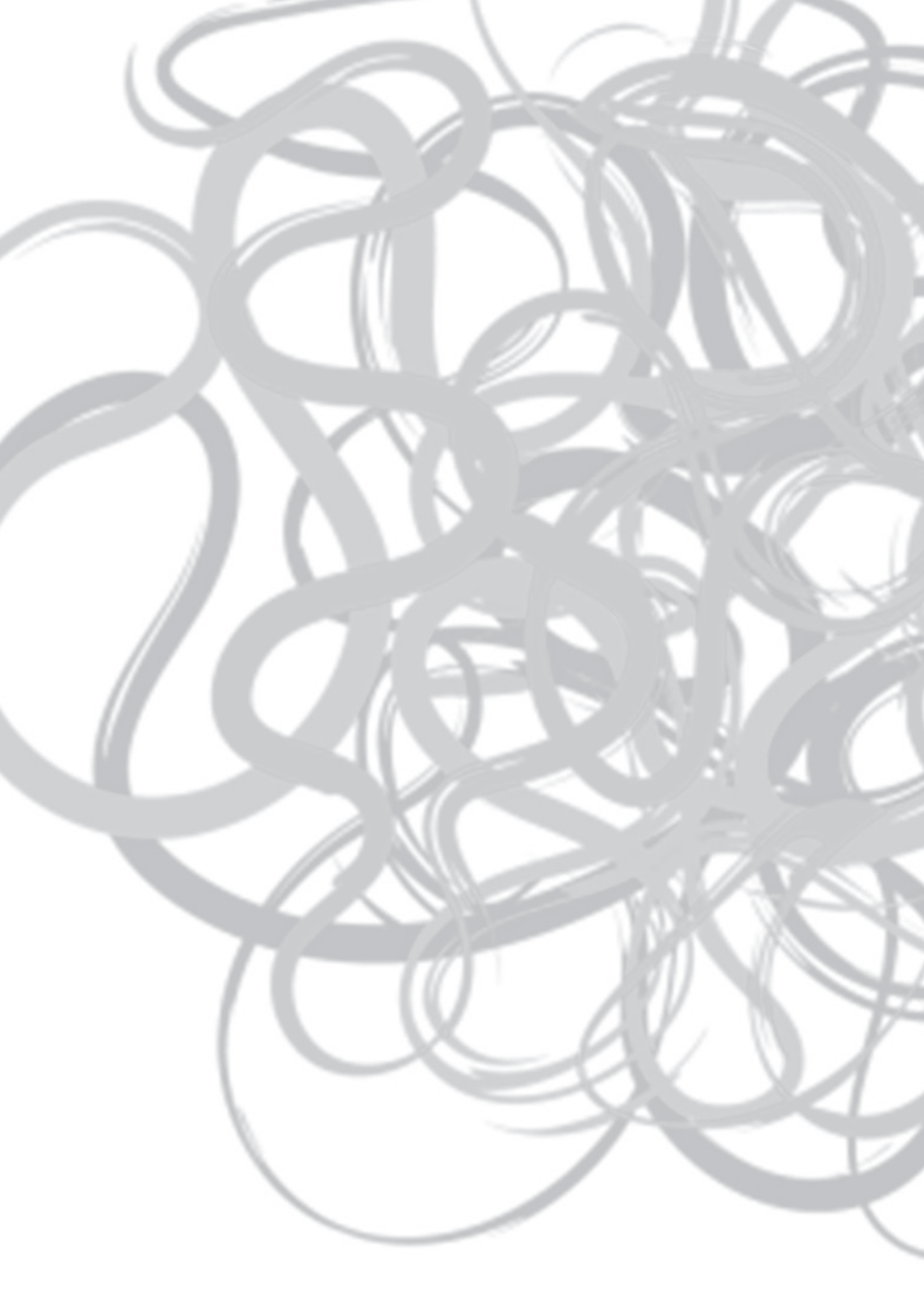

Chapter 6

Cortical thickness in Dutch police officers: an examination of factors associated with resilience

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Abstract

Previous neuroimaging studies on resilience have generally compared resilience and psychopathology after stress exposure, which does not allow for conclusions regarding correlates specific to resilience. The aim of the present study was to investigate resilience-specific correlates in cortical thickness and/or cortical surface area and their correlations with psychometric measurements, using a three-group design that included a non–trauma-exposed control group in order to disentangle effects related to resilience from those related to psychopathology. Structural magnetic resonance imaging scans were acquired from 82 Dutch police officers. Participants were categorized into resilient (n = 31; trauma exposure, no psychopathology), vulnerable (n = 32; trauma exposure, psychopathology), and control groups (n = 19; no trauma exposure, no psychopathology). Specific regions of interest (ROIs) were identified based on previous studies that found the rostral and caudal anterior cingulate cortex (ACC) to be implicated in trauma-related psychopathology. Cortical thickness and surface area of the ROIs—the rostral and caudal ACC—and of the whole brain were examined. No significant differences in cortical thickness or surface area were found between the resilient group and other groups in the ROI and whole-brain analyses. Thus, the results of the present study provide no evidence of an association between resilience to traumatic stress and measures of thickness and surface area in cortical regions of the brain in a sample of Dutch police officers.

Introduction

Due to the nature of their work, police officers and other first responders, such as firefighters, are more likely to experience traumatic events when compared to other occupational groups (Maguen et al., 2009). Although exposure to traumatic events can predispose an individual to developing psychopathology, there is no evidence that police officers suffer from more stress- related psychopathology compared to occupations that are not considered high risk; thus, one may hypothesize that police officers show resilience (Skogstad et al., 2013; van der Velden et al., 2013).
Resilience to traumatic stress can be defined as a dynamic process that enables an individual to positively adapt to and recover from a traumatic stressful event (Katz et al., 2009; Wu et al., 2013). The neural circuitry of resilience is postulated to overlap with the brain circuitry involved in emotion and stress regulation, including the limbic network (i.e., the amygdala and the hippocampus; van der Werff, van den Berg, Pannekoek, Elzinga, & van der Wee, 2013). It is thought that resilient individuals, through structural and/or functional alternations in parts of the limbic network, are more capable of upregulating their emotions and having top-down control over emotional attention, reflecting increased emotion regulation capacities (see for review, van der Werff et al., 2013). In addition, trait resilience (i.e., low neuroticism, high extraversion, and high conscientiousness) is known to be linked to the neurocircuitry involved in emotion and stress regulation (Daniels et al., 2012). Furthermore, when comparing individuals with low and high trait resilience, those with high trait resilience have been found to show higher levels of recovery and more rapid recovery of insula activity when anticipating and recovering from stress, thereby linking high trait resilience to a brain pattern that reflects efficient arousal modulation and emotional reappraisal (Waugh, Wager, Fredrickson, Noll, & Taylor, 2008). However, aside from the many studies on the neurobiology of stress-related disorders, such as posttraumatic stress disorder (PTSD), major depressive disorder (MDD), and anxiety disorders, relatively few studies have examined the neurobiology of resilience to traumatic stress. Clearly, a better understanding of the neurobiology of resilience to traumatic stress is of importance to foster improvement of treatment and prevention (Yamasue et al., 2003).

Various neuroimaging studies have identified structural abnormalities in patients with stress-related disorders. In individuals with PTSD, smaller volumes of gray matter have been found for the anterior cingulate cortex (ACC) and medial prefrontal cortex (mPFC; Kasai et al., 2008; Rauch, Shin, & Phelps, 2006; Villarreal et al., 2002; Woodward et al., 2006; Yamasue et al., 2003; J. Zhang et al., 2011). These

brain structures are part of emotion and pain processing networks (Etkin, Egner, & Kalisch, 2011; Phelps, 2004). In addition, smaller gray matter volumes for these brain structures also have been observed in MDD patients (Zhao et al., 2017).

In the context of resilience, however, findings regarding gray matter volume are inconsistent. For example, relative to PTSD patients, smaller volumes of the frontal and occipital regions have been found in resilient individuals (Fennema-Notestine, Stein, Kennedy, Archibald, & Jernigan, 2002). In contrast, a previous study on resilience to traumatic stress, which employed a three-group design that included a non–trauma-exposed control group, did not find any resilience-specific gray matter volume patterns based on the use of a whole-brain voxel-based morphometry (VBM) approach with analysis of the volume and shape of the hippocampus (van der Werff, Elzinga, Smit, & van der Wee, 2017).

Gray matter volume is determined by two independent genetic measures of cortical structures, cortical thickness and cortical surface area, which have distinct developmental trajectories (Wierenga, Langon, Oranje, & Durston, 2014; Winkler et al., 2010). Cortical thickness is thought to reflect dendritic arborization and pruning, whereas surface area may reflect folding and gyrification (Huttenlocher 1990; Rakic 2009). Therefore, changes in cortical thickness or surface area are related to different underlying biological processes (Ecker, Bookheimer, & Murphy, 2015). Thus, it is possible that, although there may not be specific patterns of gray matter volume related to resilience, one or both of the components of gray matter—cortical thickness and cortical surface area—could be implicated in resilience to traumatic stress. For example, a recent study of a sample of healthy individuals showed that a lower level of resilience was associated with a lower cortical thickness in the lateral occipital cortex, the fusiform gyrus, the inferior parietal cortex as well as the middle and inferior temporal cortex (Kahl, Wagner, de la Cruz, Kö¨hler, & Schultz, 2018). The results of several studies have suggested that cortical volume is influenced more by cortical surface area than by cortical thickness (Im et al., 2008; Winkler et al., 2010; Zhao et al., 2017). Notably, in the context of resilience, only a few studies, all of which have employed a two-group design, have reported on cortical measures, and the findings have focused predominantly on cortical thickness rather than cortical surface area and volume (Dickie, Brunet, Akerib, & Armony, 2013; Milad et al., 2005; K. Zhang et al., 2016).

To date, current models and hypotheses regarding resilience have been based mostly on studies of stress-related psychopathology that show an inconsistent pattern

(Hu et al., 2018; Katz et al., 2009; Rutter, 2012; Schmaal et al., 2017). For example, a large study investigating MDD in adults found no differences in cortical surface area between individuals with MDD and controls (Schmaal et al., 2017), whereas a previous study of individuals with a high risk for developing PTSD suggested that reduced left rostral ACC surface area might serve as a potential biomarker for PTSD risk (Hu et al., 2018).

In contrast to the inconsistent pattern for cortical surface area, cortical thickness, and in particular lower cortical thickness, has been more systematically reported in studies of stress-related psychopathology. For example, in a sample of patients with MDD, cortical thinning was found in the temporal and frontal regions (Schmaal et al., 2017). Similarly, in a sample of patients with PTSD, cortical thinning was found mainly in the frontal and temporal gyri (Geuze et al., 2008; Sussman, Pang, Jetly, Dunkley, & Taylor, 2016). Interestingly, higher cortical thickness is thought to be a potential marker of more positive treatment outcomes. For example, a thicker cingulate gyrus has been found to be a marker for potential PTSD recovery, and a thicker right caudal ACC has been associated with better symptom improvement in patients with MDD (Dickie et al., 2013; K. Zhang et al., 2016). In addition, higher cortical thickness of the ventral mPFC has been associated with extinction retention (Milad et al., 2005). Together, these results suggest that cortical thickness of the frontal regions, particularly the ACC and mPFC, may be associated with resilience to traumatic stress.

Thus far, most studies that have investigated resilience to traumatic stress have used a two-group design in which both groups consist of individuals who have been exposed to trauma—one group with psychopathology (i.e., patients) and one group with-out (i.e., trauma controls). Although such studies have been successful in detecting differences between these two groups, this design makes it impossible to distinguish whether the observed effects are related to psychopathology in the patient group or to resilience-specific factors in the trauma-exposed control group. In order to disentangle the differences between resilience and vulnerability to traumatic stress, the inclusion of a third group of individuals who have not been exposed to trauma and who are without psychopathology is needed to allow conclusions regarding resilience-specific correlates (van der Werff et al., 2013).

The aim of the present study, therefore, was to identify resilience-specific characteristics of cortical thickness and cortical surface area in resilient Dutch police officers, using a three- group design consisting of resilient (RES; trauma-exposed,

no psychopathology), vulnerable (VUL; trauma-exposed with psychopathology), and control (CON; non–trauma exposed, no psychopathology) groups. The existing literature has shown that the ACC, an important hub in emotion-regulating circuitry, is associated with psychopathology (Kasai et al., 2008; Rauch et al., 2006; Villarreal et al., 2002; Woodward et al., 2006; Yamasue et al., 2003; J. Zhang et al., 2011). In addition, an animal study that studied resilience in the context of early life stress exposure, an inoculation effect was associated with an increase in ventromedial prefrontal cortical volumes (Katz et al., 2009). Hence, we hypothesized that an increase in ACC volume would be specific to resilience. We also hypothesized that there would be a greater cortical thickness and larger cortical surface area of the ACC in the RES group relative to the other two groups. In addition, we hypothesized that the resilience-specific differences in cortical thickness and cortical surface would correlate with specific coping strategies. Furthermore, to detect possible changes in cortical thickness and cortical surface area outside of our a priori defined region of interest, we also performed an exploratory whole-brain analysis.

Methods

Participants and Procedure

Trauma-exposed executive Dutch police officers and non–trauma-exposed recruits from the police academy were recruited (van der Werff et al., 2017). A total of 149 participants were recruited using advertisements on the internal communication platform of the Dutch police. Eligible participants met the following inclusion criteria: age above 18 years, completed or attending the Dutch Police academy program, right-handed, and sufficient command of the Dutch language. Individuals were excluded upon the following exclusion criteria: (a) magnetic resonance imaging (MRI) contradictions, such as metal implants, heart arrhythmia, claustrophobia, or pregnancy; (b) history of neurological illness; (c) history of psychopathology with onset before work-related traumatic events; (d) use of psychotropic medications other than stable use of selective serotonin reuptake inhibitors or infrequent benzodiazepine use; (e) maltreated during childhood (i.e., before 18 years of age); and (f) smoking an average more than five cigarettes per day. There were 67 potential participants who did not meet the inclusion criteria and were excluded from the study. Of the remaining 82 participants, three groups could be distinguished: RES (n = 31), VUL (n = 32), CON (n = 19). The RES group consisted of police officers who experienced multiple work-related traumatic events but did not develop stress-related disorders (past or present). The VUL group consisted of police officers who had experienced multiple work-related traumatic events and

subsequently developed a stress-related psychopathology. The CON group were undergraduates at the police academy and were still in training; these participants had little to no experience in the field and were therefore naive with regard to work-related traumatic experiences. All participants provided written, informed consent and all procedures were approved by the relevant medical ethical committee (van der Werff et al., 2017). The study was designed and conducted in accordance with principles of the declaration of Helsinki.

Measures

Axis I psychiatric disorders. The Mini-International Neuropsychiatric Interview (M.I.N.I.; Sheehan et al., 1997) is an interview used to assess the presence of the most common Axis 1 psychiatric disorders according to criteria in the fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders (DSM-IV)* and 10th revision of the *International Classification of Diseases (ICD-10*; van Vliet & de Beurs, 2007). The M.I.N.I. has demonstrated good validity and reliability.

Depression. The Montgomery-Asberg Depression Rating Scale (MADRS) is a 10-item diagnostic questionnaire used to measure the severity of depressive episodes in patients with mood disorders (Fantino & Moore, 2009; Montgomery & As- berg, 1979). The symptoms are rated on a scale of 0 (*not at all*) to 6 (*definitely*). The total score classifies the patients by level depressive symptom severity, with 0–6 representing normal or absent symptoms, and 7–19 for mild, 20–34 for moderate, and 35–60 for severe symptoms. The Dutch version of this inven- tory has demonstrated good internal consistency. In the current sample, Cronbach's alpha was .88.

The Inventory of Depression Symptomatology (IDS) is a 28- item, self-report questionnaire that measures the presence and severity of depression symptoms. The symptoms are rated on a scale of 0 (*absence of pathology*) to 3 (*severe pathology*). The total score is obtained by summing the ratings of the items and ranges from 0 to 84. Cronbach's alpha values ranging from .76 to .94 have been reported ranged from .76 to .94 (Rush, Gullion, Basco, Jarrett, & Trivedi, 1996). In the current sample, Cronbach's alpha was .87.

Anxiety. The Beck Anxiety Inventory (BAI; Beck, Epstein, Brown, & Steer, 1988) was administered to assess the severity of anxiety symptoms. The BAI consists of 21 questions regarding anxiety symptoms during the past week, scores ranging from 0 (*not at all*) to 3 (*severely*). The total score ranges from 0 to 63, with a score of

0–7 classified as minimal anxiety, 8–15 as mild anxiety, 16–25 as moderate anxiety, and 30–63 as severe anxiety. The Cronbach's alpha value for the Dutch version of this inventory was found to be .82 (Bosccher, Koning, & Van, 1986). In the current sample, Cronbach's alpha was .85.

Work-related life events. The degree of exposure to work-related life events was evaluated using the Police Life Events Schedule (PLES; Carlier, Lamberts, & Gersons, 1997). The PLES is a 42-item measure of the type and number of traumatic incidents experienced by police officers and the degree to which officers felt threatened, anxious, and helpless at each of the incidents. Respondents score items on a scale of 0 (*none*) to 5 (*extreme*). The incidents are categorized as being sad/depressing or violent. The PLES has demonstrated a Cronbach's alpha value of .87 (Carlier & Gersons, 1992). In the current sample, Cronbach's alpha was .94.

Posttraumatic symptoms. The Harvard Trauma Questionnaire (HTQ) was used to evaluate the variety of trauma and the severity of the corresponding emotions. This questionnaire consists of 30 items that respondents score on a scale of 1 (*not at all*) to 4 (*extremely*). The Cronbach's alpha value has been reported as .95 in previous studies (Mollica et al., 1992). In the current sample, Cronbach's alpha was .95.

Resilience. The Connor–Davidson Resilience Scale (CD- RISC) comprises 25 items, each rated on a 5-point scale of 0 (*not at all true*) to 4 (*true nearly all of the time*), with higher scores reflecting a higher level of resilience. The scale features items related to developing strategy with a clear goal or aim, action orientation, strong self- esteem/confidence, adaptability when coping with change, social problem-solving skills, humor in the face of stress, strengthening effect of stress, taking on responsibilities for dealing with stress, secure/stable affectional bonds, and previous experiences of success and achievement, among others (Connor & Davidson, 2003). The full scale has demonstrated a Cronbach's alpha value for internal consistency of .89 (Connor & Davidson, 2003). In the current sample, Cronbach's alpha was .93.

Coping strategies. The Cognitive Emotion Regulation Questionnaire (CERQ; Garnefski, Kraaij, & Spinhoven, 2001) was used to measure different cognitive coping strategies implemented by the participants. Cognitive emotion regulation strategies were measured on a scale of 1 (*almost never*) to 5 (*al- most always*). The CERQ distinguishes between nine different cognitive emotion regulation strategies: self-blame, blaming others, acceptance, refocus on planning, positive refocusing, rumination, positive reappraisal, putting into perspective, and catastrophizing.

Individual subscale scores were obtained by summing the scores of the particular subscale, with subscale scores ranging from 4 to 20. In the current sample, the Cronbach's alpha values for the subscales ranged from .74 to .84.
Brain analysis. Structural MRIs were acquired using a Philips 3T MRI system (Philips Healthcare; Best, The Netherlands; Version 3.2.1) equipped with a SENSE32 channel head coil. Anatomical images were obtained using sagittal 3D gradient-echo T1-weighted sequence (repetition time = 9.8 ms, echo time = 4.6 ms, matrix size 256 × 256, voxel size 1.17 × 1.17 × 1.2 mm, 140 slices, scan duration: 4:56 min). For each participant, high-resolution anatomical images were examined for macroscopic abnormalities; examinations were performed by a neuroradiologist who was blinded to the clinical details of each participant. However, no such abnormalities were detected.

Data Analysis
Cortical parcellations of 68 cortical gray matter regions (34 regions in each cerebral hemisphere) were performed using FreeSurfer (Version 5.3.0). In addition, two whole-hemisphere measures were extracted using FreeSurfer. ENIGMA's quality assurance protocol was performed before analyses. The segmentations of all 68 cortical gray matter regions and the two whole-hemisphere measures were followed by a statistical outlier assessment and visually inspected by three independent researchers for segmentation errors. A participant was considered a statistical outlier if their volume was measured to be greater than 2.698 standard deviations away from the global mean. For each participant who was marked as a statistical outlier, the segmentation was reinspected in order to verify that it was properly segmented. If a participant was a statistical outlier but was properly segmented, the data were kept in the analysis. Otherwise, the participant's data were removed. No segmentation errors occurred.

All statistical analyses were executed with IBM SPSS (Version 24; IBM Corp., 2016). Assumptions of normal distribution of data and homogeneity of variances were tested using the Kolmogorov–Smirnov test and Levene's test, respectively. Cortical regions that violated these assumptions were analyzed using the nonparametric Quade's test. Analyses of covariance (ANCOVAs) were performed to examine group differences in cortical thickness and cortical surface area in the regions of interests (ROIs; i.e., the rostral and caudal ACC). Sex and intracranial volume (ICV) were included in the model as covariates to adjust for between-group differences.

To examine resilience-specific differences (i.e., a between-group difference present

in the RES vs. VUL comparison as well as in the RES vs. CON comparison), ANCOVAs were first performed to compare the RES group with the VUL group. Only the regions that were significantly different in this first comparison were further investigated using ANCOVAs to compare the RES versus CON group. Given the absence of a significant difference between the RES and the VUL groups, no further analyses were performed between the RES and CON group, as any difference found would not be specific to resilience. Similarly, an exploratory whole-brain analysis (70 measures; 68 cortical regions and two whole-hemisphere measures) was performed using ANCOVA. All comparisons were followed by a false discovery rate (FDR) correction to adjust for multiple comparisons. The FDR was set at 5% for all measures.

Correlation analyses were planned within the RES group to evaluate whether emotional cognitive coping strategies (i.e., CERQ) and levels of resilience (i.e., CD-RISC) were associated with cortical thickness parameters in areas where a resilience-specific pattern was found. For parametric data, the correlation analyses were carried out using Pearson's *r*, whereas for non- parametric data, both Kendall's tau and Spearman's rho were reported. In addition, a Bonferroni correction was applied to the correlation analyses for correction of multiple comparisons. All *p* values reported are one-tailed.

Results

Demographic and psychometric data can be found in Table 1. No significant differences were found between the RES group and the VUL or CON group regarding ICV, BAI score, or CD-RISC score. There were no age differences between the RES and VUL groups, $p = .277$, whereas a significant age difference was found between the RES and CON groups, $p < .001$. This age difference was expected, given that the participants in the CON group were undergraduates in the police academy. Furthermore, a significant difference in sex ratio was found between the RES and CON groups, $p = .033$. The RES group differed significantly in IDS score from both the VUL, $p = .013$, and CON groups, $p = .017$, with lower and higher depression scores, respectively. The CON group reported significantly lower scores, $p = .006$, on the MADRS when compared to the RES group. The HTQ scores for the RES group were significantly lower than those in the VUL group, $p = .010$, but did not differ from the CON group, $p = .159$. Moreover, PLES scores for the RES group were significantly higher than those for the CON group, $p < .001$, but did not differ from the VUL group after exclusion of a VUL-group outlier who re- ported 3,388 work-related life events, $p = .709$; this confirmed that both the RES and VUL groups were exposed

to considerably more work-related traumatic events compared to the CON group. In the context of cognitive emotion regulation, the RES group scored lower on the Blaming Others, $p = .026$, and Catastrophizing subscales, $p = .003$, compared to the VUL group. Furthermore, the RES group scored lower on the Acceptance subscale in comparison to both the CON, $p = .018$, and VUL groups, $p = .011$.

Table 1. Demographics and Psychometric Data of the Resilient, Vulnerable, and Control Groups.

Variable	Resilient Group ($n = 31$)[a]		Vulnerable Group ($n = 32$)[a]		Control Group ($n = 19$)[a]		Resilient vs. Vulnerable	Resilient vs. Control
	M	*SD*	*M*	*SD*	*M*	*SD*	*p*	*p*
Sex (female vs. male)[a]							.524[b]	.033[b]
Age (years)	40.68	11.67	43.75	11.00	25.32	4.61	.277[c]	<.001[c]
Intercranial volume	1,665,573.23	159,671.28	1,654,354.69	182,705.28	1,656,935.26	124,794.49	.858[c]	.944[c]
Assessment score								
IDS	36.39	6.82	43.94	12.65	32.58	5.32	.013[c]	.017[c]
BAI	24.00	2.73	26.31	6.56	23.94	3.0	.183[c]	.841[c]
MADRS	1.61	2.32	5.19	7.64	0.26	0.73	.168[c]	.006[c]
CD-RISC	98.23	11.92	92.25	14.44	103.89	9.57	.079[d]	.086[d]
HTQ	34.84	5.05	43.91	14.93	33.68	5.52	.010[c]	.159[c]
PLES (outlier included)	166.61	144.65	330.31	621.26	27.53	53.60	.564[c]	<.001[c]
PLES (outlier omitted)	166.61	144.65	231.68	277.73	27.53	53.60	.709[c]	<.001[c]
CERQ: Self-Blame	7.55	2.68	8.59	3.32	7.95	2.32	.211[c]	.449[c]
CERQ: Blaming others	5.74	1.79	7.16	2.58	5.42	1.71	.026[c]	.575[c]
CERQ: Acceptance	10.42	2.84	12.44	3.14	12.68	3.30	.011[c]	.018[c]
CERQ: Refocus on Planning	13.58	3.62	13.94	3.15	14.26	2.75	.678[d]	.484[d]
CERQ: Positive Refocusing	11.45	4.22	11.41	3.39	11.74	3.66	.963[d]	.809[d]
CERQ: Rumination	10.06	3.82	12.06	6.82	8.79	3.39	.183[c]	.248[c]
CERQ: Positive Reappraisal	14.55	3.41	14.16	3.81	15.37	3.44	.934[c]	.387[c]
CERQ: Putting Into Perspective	11.71	4.02	11.31	3.42	13.05	3.54	.674[d]	.236[d]
CERQ: Catastrophizing	4.87	1.50	6.34	3.01	4.74	1.19	.003[c]	.811[c]

Note. IDS = inventory of depression symptomatology; BAI = Beck Anxiety Inventory; MADRS = Montgomery-Asberg Depression Rating Scale; CD-RISC = Connor–Davidson Resilience Scale; HTQ = Harvard Trauma Questionnaire; PLES = Police Life Events Schedule; CERQ = Cognitive Emotion Regulation Questionnaire.

[a]Resilient group: $n = 10$ women, $n = 21$ men; Vulnerable group: $n = 8$ women, $n = 24$ men; Control group: $n = 12$ women, $n = 7$ men. [b]Chi-square test. [c]Mann-Whitney *U* test. [d]ndependent-samples *t* test.

Table 2 displays the mean between-group differences for cortical thickness and surface area in the ROIs (i.e., rostral and caudal ACC). These values were not significant. Because we found no significant differences between the measures for participants in the RES and VUL groups regarding the ROI analysis, we did not perform ANCOVAs to calculate the difference between the RES and CON groups.

Table 2. Cortical Thickness and Surface Area of the Rostral and Caudal Anterior Cingulate Cortex (ACC) for the Resilient and Vulnerable Groups.

Brain Hemisphere and Region	Comparison	*M* Difference	95% CI	*p*
Cortical Thickness				
Right				
Caudal ACC	RES > VUL	0.01	[−0.10, 0.11]	.901[a]
Rostral ACC	RES > VUL	0.07	[−0.05, 0.18]	.250[a]
Left				
Caudal ACC	RES > VUL	0.01	[−0.10, 0.12]	.896[a]
Rostral ACC	RES > VUL	0.06	[−0.50, 0.17]	.276[a]
Surface Area				
Right				
Caudal ACC	RES > VUL	3.46	[−92.20, 99.11]	.943[a]
Rostral ACC	RES > VUL	21.08	[−40.57, 82.74]	.497[a]
Left				
Caudal ACC	RES < VUL	−42.62	[−112.17, 26.92]	.225[a]
Rostral ACC	RES < VUL	−19.93	[−86.03, 46.18]	.549[a]

Note. *p* values are uncorrected. ACC = anterior cingulate cortex; RES = resilient group; VUL = vulnerable group.
[a]Analysis of covariance (ANCOVA).

Table 3 shows further exploratory whole-brain analysis differences at the uncorrected level between the RES and VUL groups. Significantly higher cortical thickness was found at the uncorrected $p < .05$ level in the participants in the RES group relative to those in the VUL group in the left fusiform, ^{M}difference = 0.08, 95% CI [0.03, 0.14], $p = .004$, $d = 0.8$; right pars opercularis, ^{M}difference = 0.103, 95% CI [0.03, 0.18], $p = .010$, $d = 0.7$; right lateral orbitofrontal cortex, ^{M}difference = 0.016, 95% CI [0.02, 0.16], $p = .016$, $d = 0.5$; left superior frontal cortex, ^{M}difference = 0.08, 95% CI [0.004, 0.16], $p = .040$, $d = 0.5$; and right caudal middle frontal cortex, ^{M}difference = 0.072, 95% CI [0.001, 0.14], $p = .048$, $d = 0.5$. After adjusting for multiple testing, no significant differences in cortical thickness between the participants in the RES and VUL groups remained.

Table 3. Cortical Thickness and Surface Area in an Exploratory Whole-Breain Analysis.

	Resilient vs. Vulnerable					Resilient vs. Control
Brain Hemisphere and Region	Comparison	*M* Difference	95% CI	*p*	*d*	*p*
Cortical Thickness						
Right						
Pars opercularis	RES > VUL	0.103	[0.03, 0.18]	.010[a]	0.7	.052
Lateral OFC	RES > VUL	0.090	[0.02, 0.16]	.016[a]	0.5	.068
Caudal MFC	RES > VUL	0.072	[0.001, 0.14]	.048[a]	0.5	.606
Left						
Fusiform	RES > VUL	0.080	[0.03, 0.14]	.004[a]	0.8	.081
SFC	RES > VUL	0.080	[0.004, 0.16]	.040[a]	0.5	.417
Surface Area						
Right						
Pars opercularis	RES > VUL	149.86	[23.32, 276.39]	.021[a]	0.6	.315
Left						
Pars orbitalis	RES < VUL	−35.96	[−65.86, −6.07]	.019[a]	0.5	.323

Note. *p* values are uncorrected. OFC = orbitofrontal cortex; MFC = middle frontal cortex; SFC = superior frontal cortex.
[a]Analysis of covariance (ANCOVA).

Furthermore, uncorrected significant differences between participants in the RES and VUL groups were found for the surface area of the left pars orbitalis, ^{M}difference = −35.96, 95% CI [−65.86, −6.07], p = .019, d = 0.6; and right pars triangularis, ^{M}difference = 149.86, 95% CI [23.32, 276.39], p = .021, d = 0.5. However, after adjusting for multiple testing, no significant differences in cortical thickness and surface area remained between these groups. In addition, analyses examining these regions in participants in the RES and CON groups also did not show any differences. In sum, no resilience-specific differences remained significant after FDR correction to the whole brain analysis.

Discussion

The results of the present study provide no evidence for a relation between resilience to traumatic stress and thickness and surface area measures in cortical regions of the brain in a sample consisting of resilient and vulnerable Dutch police officers, as well as a group of controls from the police academy. We hypothesized that participants in the RES group would have a higher cortical thickness compared to those in the VUL and CON groups, specifically in ACC regions. Contrary to our hypothesis, we did not find any resilience-specific differences in cortical thickness or surface area in the two ACC regions nor in other regions in the brain, either at the corrected and uncorrected statistical levels. A previous structural imaging study that used a VBM and diffusion tensor imaging approach (van der Werff et al., 2017) also did not observe abnormalities in gray matter volume, but the results of that study showed a resilience-specific white matter integrity pattern. Preliminary results of a resting- state functional MRI study in the same cohort also seems to point toward

the role of connectivity rather than gray matter structure concerning resilience.

The results of the present study appear to be at odds with prior studies on resilience. There may be a number of reasons for this. First, the discrepancy between our study and prior resilience studies could be attributed to significant differences in study design. Most studies have used two-group design instead of a three-group design that includes a non–trauma-exposed control group and, hence, these studies have not been able to identify resilience-specific findings. It is thus possible that prior findings should not be attributed to resilience specifically. Second, due to our focus on resilience in police officers, we may have selected a population of individuals who already have high levels of baseline resilience due to self-selection and screening, with the implication that we have studied especially highly resilient participants.

Although the three-group design and the sample of police officers are strengths of our study, our study also has some potential limitations. The relatively small sample size might have inflated Type II errors in the whole-brain analyses. Also, participants in the CON group were significantly younger than those in the VUL and RES groups, as they consisted of undergraduates in the police academy. Furthermore, we did not include a fourth group of unexposed controls with no police affiliation, which would have enabled us to make inferences regarding the characteristics of baseline resilience in police officers. We used a robust standard Freesurfer pipeline for the cortical segmentation, but is has been noted (Paus et al., 1996; Yucel et al., 2001) that the segmentation of regions with more natural anatomical variation can be unreliable, leading to inaccuracies in the segmentation of, for example, the paracingulate gyrus and the surrounding region. In addition, use of the predefined segmentations in FreeSurfer limited the analyses to a set of regions that may not cover all regions relevant in resilience. Finally, the operationalization of resilience used in this study does not encompass the dynamic and multidimensional nature of resilience; rather, it was based on static measures of outcome.

In conclusion, this study provides no evidence for a relation between resilience to traumatic stress and thickness and surface area measures in cortical regions of the brain in a large sample of Dutch police officers. Questions about the role of cortical surface area and thickness in the context of resilience remain and should be further investigated, preferably using longitudinal designs, and future research may also benefit from more detailed vertex-based analytic approaches to examine cortical thickness.

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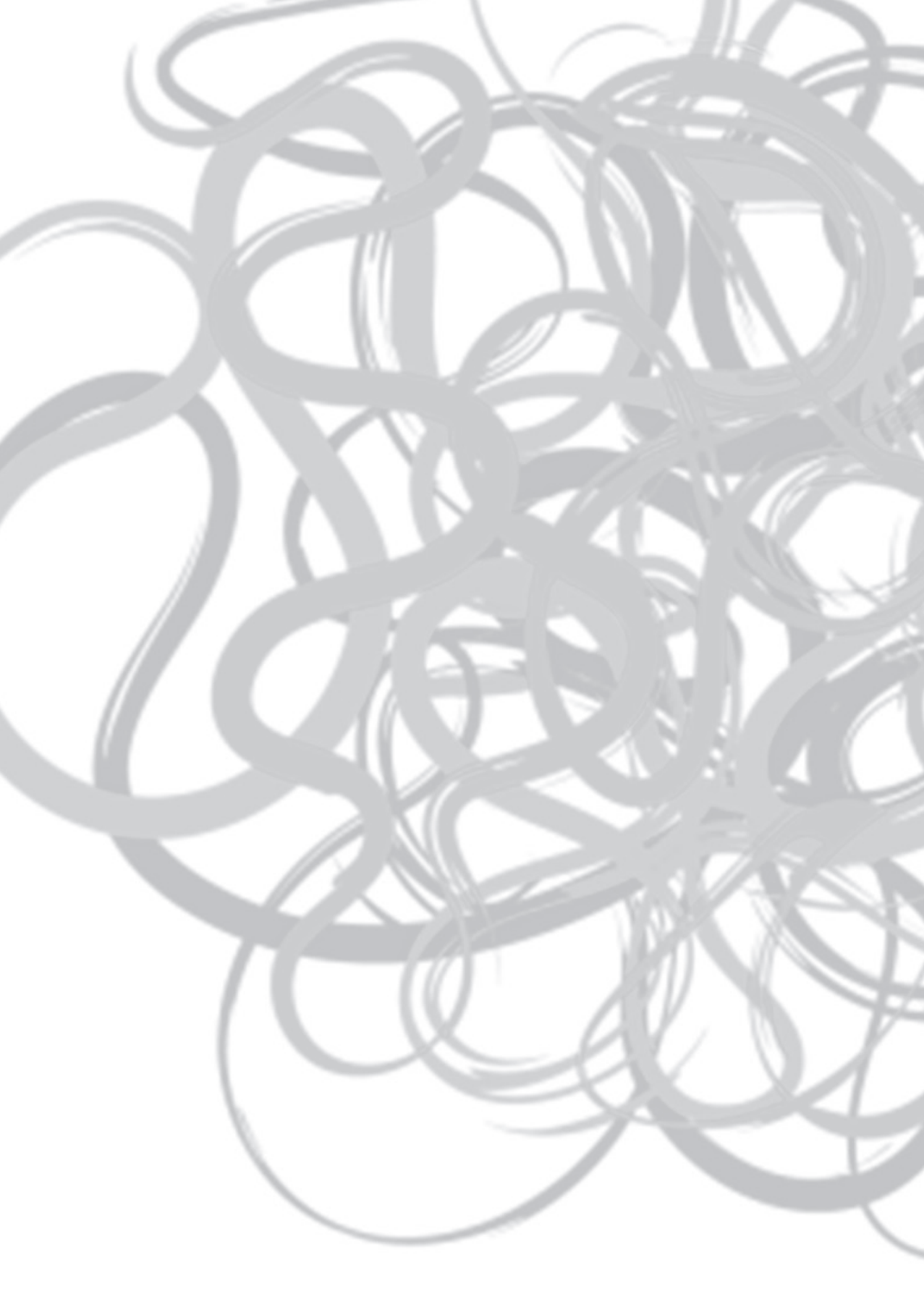

Chapter 7

Potential associations between immune signaling genes, deactivated microglia, and oligodendrocytes and cortical gray matter loss in patients with long-term remitted Cushing's disease

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Abstract

Introduction

Cushing's disease (CD) is a rare and severe endocrine disease characterized by hypercortisolemia. Previous studies have found structural brain alterations in remitted CD patients compared to healthy controls, specifically in the anterior cingulate cortex (ACC). However, potential mechanisms through which these persistent alterations may have occurred are currently unknown.

Methods

Structural 3T MRI's from 25 remitted CD patients were linked with gene expression data from neurotypical donors, derived from the Allen Human Brain Atlas. Differences in gene expression between the ACC and an unaffected control cortical region were examined, followed by a Gene Ontology (GO) enrichment analysis. A cell type enrichment analysis was conducted on the differentially expressed genes, and a disease association enrichment analysis was conducted to determine possible associations between differentially expressed genes and specific diseases. Subsequently, cortisol sensitivity of these genes in existing datasets was examined.

Results

The gene expression analysis identified 300 differentially expressed genes in the ACC compared to the cortical control region. GO analyses found underexpressed genes to represent immune function. The cell type specificity analysis indicated that underexpressed genes were enriched for deactivated microglia and oligodendrocytes. Neither significant associations with diseases, nor evidence of cortisol sensitivity with the differentially expressed genes were found.

Discussion

Underexpressed genes in the ACC, the area vulnerable to permanent changes in remitted CD patients, were often associated with immune functioning. The specific lack of deactivated microglia and oligodendrocytes implicates protective effects of these cell types against the long-term effects of cortisol overexposure.

1. Introduction

Cushing's disease (CD) is a rare and severe endocrine disease caused by a pituitary adrenocorticotropic hormone (ACTH) producing adenoma. The excessive ACTH secretion stimulates the adrenal glands to produce excessive amounts of glucocorticoids (Newell-Price et al., 2006). In healthy individuals, increases in free circulating cortisol levels inhibit ACTH secretion (i.e. the negative feedback loop). In CD patients this feedback loop is impaired, resulting in increased levels of glucocorticoids or hypercortisolism, affecting numerous organs, including the brain.

Cortisol is a pivotal mediator of the stress response (De Kloet et al., 2005). Hypercortisolism has been associated with severe physical, psychological, and cognitive impairments, resulting in a substantial deterioration in quality of life (Forget et al., 2000; Leon-Carrion et al., 2009; Michaud et al., 2009; Newell-Price et al., 2006; Nieman and Ilias, 2005; Starkman et al., 1986). Regarding the psychological and cognitive impairments, stress-related symptoms such as anxiety, depression, and mania commonly present alongside CD, as do cognitive deficits within the domains of reasoning, verbal learning, language performance, visual and spatial information processing, and memory impairments (Newell-Price et al., 2006). These symptoms all support the notion that acute as well as prolonged exposure to excessive cortisol adversely affect the central nervous system (CNS).

Current treatment strategies abrogate excessive cortisol signaling and offer substantial alleviation of several associated symptoms, but certain debilitating psychological symptoms often persist, even after long-term remission. These persistent impairments are predominantwithin the domains of cognitive function and psychopathology (Andela et al., 2013; Bas-Hoogendam et al., 2015; Dorn et al., 1995; Pereira et al., 2010; Pivonello et al., 2015; Ragnarsson et al., 2012; Sonino and Fava, 2001; Tiemensma et al., 2010). Alongside these persevering symptoms, structural changes in the brain have been found in long-term remitted CD patients in comparison to healthy controls. Specifically, magnetic resonance imaging (MRI) studies have reported widespread reductions of white matter integrity, as well as smaller anterior cingulate cortex (ACC) volumes (Andela et al., 2013; van der Werff et al., 2014). However, MRI studies alone cannot offer sufficient insight into the underlying biological processes that lead to the observed reductions in white matter integrity and atrophy in the ACC in remitted CD patients.

The ACC, as part of the limbic system, is a relevant brain area to explore further as it is involved in various cognitive and emotional functions, many of which can be persistently impaired after cure of CD. Andela et al. (2013) offered the hypothesis that intrinsic impairments and alterations in connectivity and/or biochemistry of these brain regions may have caused the structural differences observed in remitted CD patients. Such underlying biological processes could be further explored by combining information obtained from high resolution MRI scans with whole genome mRNA expression data derived from the Allen Human Brain Atlas (AHBA), a multi-modal atlas mapping gene expression across the healthy human brain (Hawrylycz et al., 2012). The comparison of regional correlations between gene expression and the MRI data may provide a better insight into which genes are likely to interact with hypercorticolism resulting in structural brain changes.

In the present study, we explore potential mechanisms through which the structure of the ACC changes when exposed to prolonged endogenous cortisol excess, by linking information derived from high resolution MRI scans with gene expression data derived from the AHBA. We examined the differential gene expression in the ACC in comparison to a control region. Subsequently, the functionality of the differentially expressed genes was characterized by means of a Gene Ontology (GO) enrichment analysis. A cell type enrichment analysis investigated whether the differentially expressed genes were enriched for certain cell type markers, followed by a disease enrichment association analysis to assess whether the differentially expressed genes were associated with any specific group of diseases. Finally, we explored the cortisol sensitivity of the differentially expressed genes.

2. Methods

2.1. Subjects and data acquisition

Data were derived from a study conducted with long-term remitted CD patients and healthy controls, aged between 18 and 60 years old. A detailed explanation of this study protocol has previously been published elsewhere (Andela et al., 2013). In brief, a total of 25 long-term remitted CD patients and 25 age-, gender-, and education matched healthy controls were included in this sample. The diagnosis of CD had been confirmed in accordance with previously described international guidelines (Tiemensma et al., 2010). All CD patients underwent transsphenoidal surgery (n = 25). Six patients received additional post-operative radiotherapy and two other patients underwent bilateral adrenalectomy. All participants were right-handed, had no contraindications for the MRI scanner, and were psychopathology-,

drug-, and alcohol abuse-free with the exception of one patient who used antidepressants. Patients who remained glucocorticoid dependent after surgery (in addition to the two patients that received postoperative radiotherapy) received hydrocortisone replacement (on average 20 mg/daily in two to three dosages), and were evaluated twice per year. The estimated disease duration was determined retrospectively using patient recall of earliest physical and/or psychological symptoms of CD. Remission duration was calculated from the date of transsphenoidal surgery, whereby remission was confirmed by multiple biochemical test outcomes (e.g. normal midnight salivary cortisol (below 5.7 nmol/L, normal overnight suppression of serum cortisol levels (<50 nmol/L) by dexamethasone (1 mg), and normal 24-h urinary free cortisol excretion rates (<220 nmol/24 h), and by means of clinical evaluation. Prior to being included is the study, persistent biochemical remission of hypercortisolism was confirmed by means of the abovementioned diagnostic tests. Informed consent was obtained from all participants and the study protocol was approved by the Leiden University Medical Center ethics review board.

Images were acquired by a Philips 3.0T Achieva MRI scanner (Philips Medical Systems, Best, The Netherlands; software version 3.2.1) using a 32-channel SENSE (sensitivity encoding) head coil. Anatomical images were obtained by means of a sagittal three-dimensional gradient-echo T1-weighted sequence (repetition time 9.8 ms, echo time 4.6 ms, matrix size 256 × 256, voxel size 1.17 × 1.17 × 1.2 mm3, 140 slices, scan duration 4:56 min), as a part of a larger scan protocol. All anatomical images were examined by a neuroradiologist blinded for participants clinical details. No further macroscopic abnormalities were found in either patients or controls, with the exception of the effects of post-transsphenoidal surgery and incidental age-related white matter hyperintensities.

2.2. Behavioral and clinical severity assessments

The Montgomery-Åsberg Depression Rating Scale (MADRS; Montgomery and Åsberg, 1979), and the Inventory of Depression Symptomatology (IDS; Rush et al., 1986) were used to assess the severity of depressive symptoms. The MADRS was assessed by an interviewer, all other scales were self-reported. Anxiety was evaluated using the Beck Anxiety Inventory (BAI; Beck et al., 1988), the social phobia, blood injury phobia, and agoraphobia subscales, the total score of the Fear Questionnaire (FQ; Marks and Mathews, 1979), and the Beck Anxiety Inventory (BAI; Beck et al., 1988). The Apathy Scale (AS) and the Irritability Scale (IS) were used to assess the severity of apathy and irritability, respectively (Starkstein et al., 2001; Chatterjee et al., 2005). Participants with total scores of more than 14 points were considered to

be apathetic or irritable. Failures in memory, perception, and motor function were assessed using the Cognitive Failure Questionnaire (CFQ; Broadbent et al., 1982), higher sum scores indicating greater symptom severity.

CD symptom severity (active and the remitted disease state) were established using the Cushing's Syndrome Severity Index (CSI; Sonino et al., 2000). The CSI score during active disease was assessed retrospectively. The remission score was based on the last annual evaluation. Total CSI scores were used for both active and remitted disease states. Scores on this index can range between 0 and 16, with higher total scores indicating greater symptom severity. The information necessary to score the CSI was obtained from the patient's medical records and clinical history. The index was scored by two independent raters who reached consensus in the case of discrepancy.

2.3. Allen Human Brain Atlas (AHBA)

The Allen Human Brain Atlas (AHBA) is a genome-wide transcriptional atlas of the pathology-free human brain, providing gene expression data derived from six healthy human brains between the ages of 24–57 (Hawrylycz et al., 2012, 2015). More than 500 regions were sampled from each hemisphere, and 19,992 genes were extracted using multiple probes. Microarray data were downloaded from the AHBA database and probes were mapped to genes as previously described (Keo et al., 2017). Z-scores for normalized gene expression levels from the AHBA were calculated separately for each of the six individual brains. A major strength of the AHBA is that the gene expression data is mapped to the Montreal Neurological Institute (MNI) space (Collins et al., 1998), a standardized phantom brain that can be used to compare neuroimaging data across different brains.

2.4. Mapping AHBA to MRI data

As described in more detail by Andela et al. (2013), structural data were analyzed using FSL-VBM (a voxel-based morphometry style analysis; FMRIB's software library; Smith et al., 2004). In this study, smaller gray matter volumes were found in a large part of the bilateral ACC in remitted CD patients in comparison with controls (617 voxels; $p < 0.05$, 2 mm isotropic). We selected this region as our region of interest (ROI). Throughout the manuscript the ROI will be referred to as the ACC. A control region was selected by identifying the cortical area that showed the least differences between healthy controls (HCs) and patients with remitted CD (Andela et al., 2013). This region was identified using t-statistic thresholds of 0.7 to 0.0001 and 0.0001 to 0.7 respectively, leading to a control region consisting of segments

of the dorsolateral prefrontal cortex (see Fig. 1). As these regions are anatomically distinct, there are likely to be functional differences between these regions. However, as this control region is anatomically adjacent to the ACC, anatomically driven transcriptional differences are consequently minimized (e.g. Huntenburg et al., 2018). The ABHA samples were then mapped to the ACC and control region based on their associated MNI coordinates.

2.5. Differential gene expression in the ACC

Differences in gene expression between regions were examined by comparing expression level of genes between the ACC to the control region by means of two-tailed independent t-tests. Genes were considered to be differentially expressed at a Benjamini-Hochberg (HB; Benjamini and Hochberg, 1995)-adjusted p-value of <0.05 and effect size of log2(fold-change) >1.

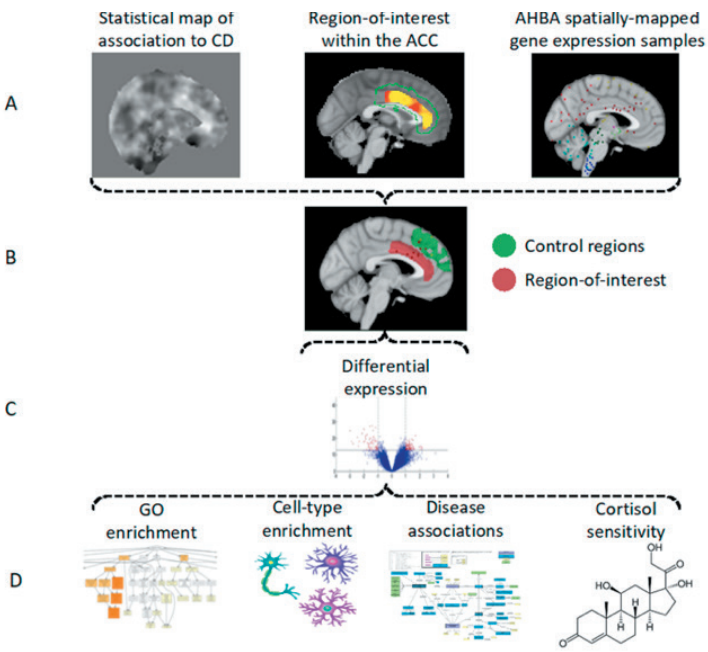

Figure 1. Study overview. (A) The Anterior Cingulate Cortex (ACC; region of interest) from our earlier study on grey matter volumes in patients with remitted Cushing's Disease (Andela et al., 2013) and the gene expression data derived from the AHBA (B) The identification of a control region (close in proximity to the ACC with a similar neuronal characteristic and showing the least differences to the ACC using a stringent threshold and the ROI mapped onto the AHBA gene expression data (C) Differential gene expression analysis (D) Enrichment or depletion for Gene ontology, cell type markers, disease associations, and cortisol sensitivity of differentially expressed genes.

2.6. Gene ontology (GO) enrichment analysis

To characterize the functionality of the differentially expressed genes, a Gene Ontology (GO) enrichment analysis was performed using GOrilla (Gene Ontology Enrichment Analysis and Visualization Tool), an online tool for identifying and visualizing enriched GO terms (Eden et al., 2009). Enrichment analysis was carried out for differentially over- and underexpressed genes. Gene symbols were used as identifiers, and a background list of the top 20% of genes with the highest expression level in the cortex was used to correct for non-selective ontology terms. GO terms with BH-corrected p-value <0.05 were considered significant. The following ontologies were used: 'GO: Biological Process' and 'GO: Molecular Function'.

2.7. Cell type enrichment analysis

In order to determine whether the differentially expressed genes were enriched for certain cell type markers, we conducted a cell type enrichment analysis. As our AHBA samples were located in the cortex region, a set of brain-region specific markers was used, focusing on 28 cell types (Mancarci et al., 2017). Markers were downloaded from the NeuroExpresso database (http://neuroexpresso.org) using markers from all brain regions. Entrez IDs of the mouse cell-type specific markers were converted to human homologs (homologene R-package version 1.4) and filtered for genes present in the AHBA dataset. Two markers with different mouse gene IDs (14972, H2-K1, microglial, and 15006, H2-Q1 serotonergic), were converted to the same human gene ID (3105, HLA-A), and therefore removed before analysis.

2.8. Enrichment analysis of disease-associated genes

A disease association enrichment analysis was conducted using disease gene sets from DisGeNET (http://www.disgenet.org/; Pi˜nero et al., 2016), in order to assess whether the differentially expressed genes are associated with any specific group of diseases. A table of 628,685 gene-disease associations covered 24,166 diseases that were tested for. Genes that were associated to waste-hip ratio and height were used in the analysis as control (i.e. non-disease) conditions (Lin et al., 2017; Heid et al., 2010).

2.9. Assessment of cortisol sensitivity of the ACC

We assessed whether the differentially expressed genes in our dataset were known to be regulated by glucocorticoids (GCs) in the brain, or in cell cultures derived from the central nervous system. We compared our list of differentially expressed genes with a list of genes that have been published by Juszczak and Stankiewicz (2018), who identified 113 genes that were consistently regulated by GCs across

studies. We also investigated GR and MR binding loci in the rat hippocampus under increased levels of corticosterone, the predominant glucocorticoid in this species (van Weert et al., 2017). Using these gene sets we identified GR-specific, MR-specific, as well as GR-MR overlapping DNA binding loci, which we used as potential target genes. Genes were converted from rat to human orthologues by use of the Toppfun Suite (Chen et al., 2009). In order to predict glucocorticoid sensitivity of the current differentially expressed genes, we assessed whether these target genes were enriched in the differentially expressed genes.

2.10. Enrichment statistics for GO, cell type, disease-associated genes and receptor binding

Enrichment statistics were determined based on Fisher's Exact Tests. Odds ratios (ORs) were calculated as a measurement of effect size with regard to the enrichments, with an OR > 1 and 0 < OR < 1 indicating enrichment and depletion, respectively. The BH method was used for all p-values to correct for multiple testing. A BH corrected p-value of <0.05 was considered to be significant.

2.11. Literature search on the differentially expressed genes

For all differentially expressed genes, a literature search was subsequently performed to determine previously found associations with normal and pathological processes or states. Furthermore, with regard to Cushing's Disease, specific known associations with the HPA-axis and/ or glucocorticoid responsiveness were investigated. This was done by means of a PubMed search using the following search term strategy: ("gene" [all fields]) AND ("HPA-axis" [all fields] OR "cortisol" [all fields] OR "glucocorticoid" [all fields] OR "GR" [all fields]). Interactions between genes were determined by means of searching gene pairs in Google Scholar.

3. Results

3.1. Patient characteristics

As previously published in Andela et al. (2013), mean disease duration was 7.9 years (ranging from 0.8 to 29.3 years), and mean duration of remission was 11.2 years (ranging from 0.8 to 37.0 years). The mean Cushing's Syndrome Severity Index score was 8.1 during the active period of CD, and 2.5 in the remitted CD-patients at the time of assessment (see Table 1 for further details).

Table 1. Demographics and psychometric data of remitted CD patients. Data are presented as mean ± standard deviation or number (%).

	CD patients (n = 25) Mean ± SD
Gender (female)	21(84%)
Age (years)	45 ± 8
Education	
Low	6 (24%)
Medium	12 (48%)
High	7 (28%)
Intracranial volume $\cdot 10^6$ (cm^3)	1.450 ± 0.163
MADRS	6.3 ± 5.5
Inventory of depressive Symptomatology	46.8 ± 13.0
Beck Anxiety Inventory	28.4 ± 5.7
Fear questionnaire	24.5 ± 17.4
Agoraphobia subscale	6.1 ± 7.9
Blood injury phobia subscale	6.2 ± 8.3
Social phobia subscale	12.2 ± 8.0
Irritability scale	12.1 ± 8.7
Total score >14	9 (36%)
Apathy scale	13.6 ± 6.6
Total score >14	11 (44%)
Cognitive failures questionnaire	38.0 ± 16.5
Disease duration (years)	7.9 ± 7.9
Duration of remission (years)	11.2 ± 8.2
Cushing's syndrome severity index	
Active phase (total)	8.1 ± 2.0
Remission phase (total)	2.5 ± 1.5

MADRS = Montgomery-Åsberg Depression Rating Scale.

3.2. Differentially expressed genes in the ACC in comparison to the control region

Given the small size of the regions analyzed, we aggregated all samples from the six donors in the AHBA. This led to a total of 31 samples in the ACC and 29 samples in the control region (see Table 2 for further details). Using a differential expression analysis, we identified 300 differentially expressed genes (BH-adjusted $p < 0.05$ and log2(fold- difference) >1 (see Fig. 1). Of these 300 differentially expressed

genes, 58 genes were overexpressed and 242 were underexpressed in the ACC in comparison to the control region (see Supplementary Tables S1 and S2) (Fig. 2). The top three most significantly overexpressed genes in the ACC in comparison to the control region were KIAA0748, also known as Thymocyte-expressed, positive selection-associated 1 (TESPA1; FDR = $4.80 \cdot 10^{-5}$, log2(FC) = 2.76), ONECUT2, One Cut Homeobox 2, (FDR = $1.94 \cdot 10^{-6}$, log2(FC) = 2.45), and CALML3, Calmodulin 3, (FDR = $3.44 \cdot 10^{-7}$, og2(FC) = 2.13).

Endothelial cell-specific molecule 1 (ESM1), Ubiquitin Specific Peptidase 54 (USP54), and Putative translationally-controlled tumor protein-like protein TPT1P8 (TPT1P8), also known as FKSG2, were the most underexpressed genes in the ACC in comparison to the control region (FDR = $6.43 \cdot 10^{-4}$, log2(FC) = 3.13; FDR = $5.28 \cdot 10^{-4}$, log2(FC) = -2.14; and FDR = $5.16 \cdot 10^{-3}$, log2(FC) = -2.13, respectively).

Table 2. Overview of samples from the six AHBA donors in the AAC and the control region.

	Donor 9861	Donor 10021	Donor 12876	Donor 14380	Donor 15496	Donor 15697	All donors
ACC	1	12	3	3	5	7	31
Control region	13	2	4	3	3	4	29

3.3. Functionality and cell type specificity of differentially expressed genes in the ACC

The GO term enrichment analysis found none of the enriched GO terms from the list of overexpressed genes to remain statistically significant after BH-adjustment for multiple testing. Genes with lower expression in the ACC compared to the control region were enriched for GO terms that were, amongst others, involved in synapse pruning, immune system processes, antigen processing, leukocyte-, macrophage- and (micro)glial activation. 34 GO-terms remained significant after correction for multiple testing at a BH adjusted p-value of < 0.05, although several of these terms were partially overlapping (see Supplementary Table S3 for further details). Based on the GO terms, the most frequently occurring genes involved in the processes (assigned to between 9 and 22 of the 34 significant GO-terms), were Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), Integrin Subunit Alpha M (ITGAM), CD36, Interleukin 33 (IL33), Complement Component 3 (C3), human leukocyte antigen (HLA)-DRB1/-DQB1/-DMB/-DRB3/-DRB4 (all belonging to the HLA class II), and arachidonate 5-lipoxygenase (ALOX5; see Table 3 for further details and cell-types; Mancarci et al., 2017).

We then assessed whether a set of differentially expressed genes were particularly expressed in a cellular subtype and found that underexpressed genes were significantly enriched for deactivated microglia cells (7 markers; BH-corrected p-value = 0.001), and oligodendrocyte cells (12 markers, BH-corrected p-value = 0.036; see Supplementary Table S4 for a complete overview).

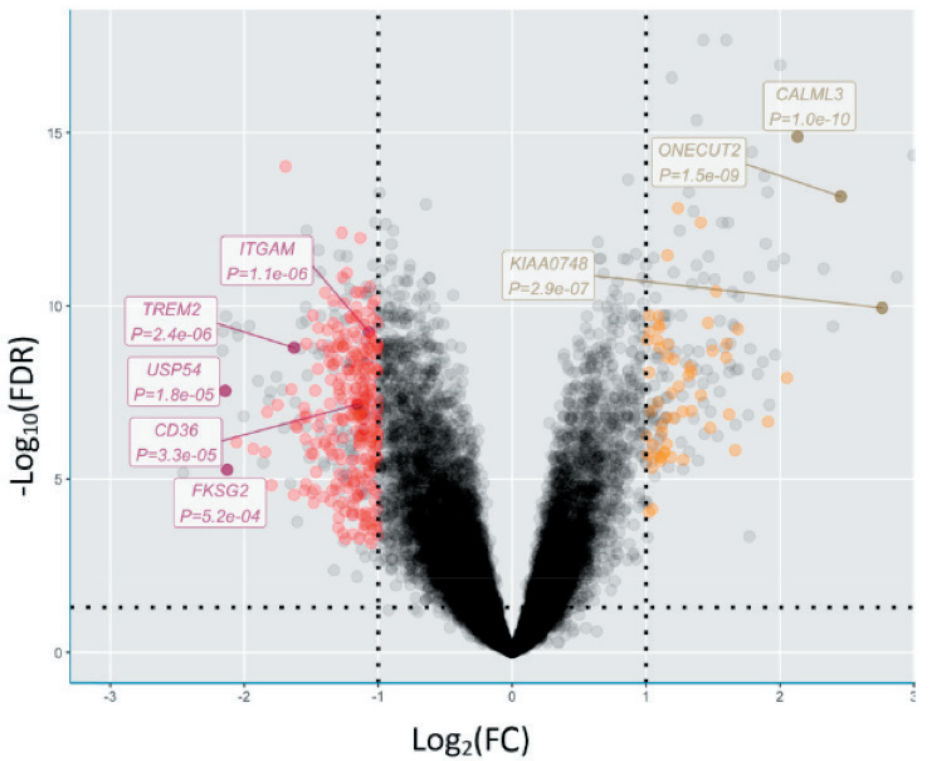

Figure 2. Genes significantly expressed after BH-correction at higher and lower levels (orange and red dots respectively) in the ACC relative to control regions are depicted on a volcano plot. Non-statistically significant differentially expressed genes in the cortical regions are depicted in black. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3. Overview frequency and cell type of often occurring genes in the 34 GO terms that remained significant after BH correction.

Gene	Number of times occurring	Cell type
ITGAM	23/34 (67.6%)	Microglia
TREM2	22/34 (64.7%)	Microglia
CD36	19/34 (55.9%)	Endothelial
IL33	16/34 (47.1%)	Oligodendrocytes and Astrocytes
C3	14/34 (41.2%)	Endothelial
HLA class II	12/34 (35.3%)	Microglia
ALOX5	9/34 (26.5%)	Microglia and Oligodendrocytes

3.4. Differentially expressed genes in the ACC are not associated with any diseases

Using disease gene sets from DisGeNet (Pi˜nero et al., 2016) containing 24,166 diseases, we tested the over- and underexpressed genes in the ACC for enrichment in psychiatric-brain diseases (e.g. MDD), -non-psychiatric brain diseases (e.g. Huntington's Disease), and non-disease traits (e.g. height and waist-hip-ratio). After BH-adjustment, no associations with any of these diseases remained significant.

3.5. Cortisol sensitivity of the ACC

Finally, we assessed whether the differentially expressed genes in our dataset were known to be regulated by glucocorticoids (GCs) in the brain or in cell cultures derived from the central nervous system. We compared our list of differentially expressed genes with a list of 113 genes that were found to be consistently regulated by GCs across studies (Juszczak and Stankiewicz, 2018). We found a non-significant overlap of one gene, namely LYVE1. We further compared our differentially expressed genes with genes that have been identified to be regulated by GCs and mineralocorticoids (MCs) in rats (van Weert et al., 2017), we found a non-significant overlap of the following genes: ASPA, CALML3, XCR1, and USP54, however not LYVE1.

4. Discussion

The current study aimed at exploring potential mechanisms underlying the persistent structural alterations of the ACC in patients with long-term remitted CD by linking information derived from high resolution MRI scans with gene expression data derived from the AHBA. By combining structural MRI data with gene expression data, we identified 300 differentially expressed genes in the ACC in comparison to the control region, several of which were immune signaling genes. In line with this

finding, the GO term enrichment analyses indicated that underexpressed genes were enriched for functionality involving, amongst others, immune functioning. Furthermore, cell type specificity analyses indicated that low-expressed genes were enriched for deactivated microglia and oligodendrocytes. No associations were found between the differentially expressed genes and specific diseases. Finally, our findings indicated no enrichment of glucocorticoid target genes in the ACC in comparison to the control region.

A potential limitation of our data study may be the relatively small ROI and sample sizes. This may make it difficult to detect certain effects due to a possible lack of power. Also, we are assessing gene expression levels in the healthy brain and not in those of remitted CD patients. This means that the differential expression may indicate vulnerability as present prior to CD, but tells us nothing about what the actual effects of cortisol exposure have been. Possible ways in which we could extend our findings to include the actual effects of cortisol are by extrapolating our current findings to existing mouse models (e.g. Drelon et al., 2016; Leccia et al., 2016), or by examining these areas in remitted CD patient brains post-mortem. However, material from patients with remitted CD will be extremely scarce. Furthermore, we used mouse data for our cell type enrichment analysis. There may therefore be a limited overlap with human data, thu replicating these findings in a robust human dataset is advisable. Finally, it is important to bear in mind that data derived from the CD patient population are cross-sectional, and we are therefore unable to determine whether these patients presented alterations in the ACC prior to the development of the disease.

The differential gene expression analysis indicated that KIAA0748, ONECUT2, and CALML3 were the most highly overexpressed genes in the ACC in comparison to the control region, and ESM1, USP54, and TPT1P8 were the most underexpressed. KIAA0748, ONECUT2, ESM1, USP54 have been found to be associated with various (auto-)immune diseases (e.g. Yao et al., 2015, 2018; Shen et al., 2019; Seo et al., 2020; Fraile et al., 2016; Jin et al., 2020; Xu et al., 2019; Li et al., 2019); and CALML3 has recently been found to affect the onset of Alzheimer's Disease (AD; Chen et al., 2019). The GO analysis conducted using the over- and underexpressed genes found that several of the most under- expressed genes, i.e. TREM2, ITGAM, CD36, IL33, HLA-DRB1/-DQB1/-DMB/-DRB3/-DRB4 (all belonging to the HLA class II), and ALOX5, were enriched for GO terms that were, amongst others, involved in immune-signaling. Interestingly, these genes have also been found to be associated with numerous (auto-)immune diseases, and strikingly all have been suggested to

be linked with AD. Specifically, variants in TREM2 have been strongly implicated in the pathogenesis of AD (Jonsson et al., 2013; Guerreiro et al., 2013). Moreover, a recent study investigating TREM2 knockout in human microglia cells in a xenograft mouse model using aggravated pathology found that TREM2 knockout microglia show lower viability, which is in line with a protective effect of this gene (McQuade et al., 2020). ITGAM has been proposed to be a candidate susceptibility locus for AD (Shulman et al., 2014), and CD36 gene polymorphisms have been associated with AD (Serý et al., 2017, 2020). Furthermore, IL33 has been identified as a candidate gene for AD (Chapuis et al., 2009), and several of the HLA class II genes, as well as ALOX5, have been found to be associated with AD (Lehmann et al., 2001; Wang et al., 2017; Serý et al., 2017).

The enrichment analysis for cortical cell-type markers found an underrepresentation of deactivated microglia in the ACC in comparison to the control region. Microglia form the first line of defense in the case of injury, disease, or invading pathogens in the CNS (Nimmerjahn et al., 2005), and actively partake in maintenance and plasticity of the adult CNS by secreting neurotrophic factors such as BDNF and cytokines (Parkhurst et al., 2013). They also refine the neuronal circuit by pruning axonal terminals and synapses (Parkhurst et al., 2013; Salter and Beggs, 2014). Microglia are generally known to polarize in two directions from a resting (M0) state: classical (M1) activation, known as the mediator of pro-inflammatory responses and alternative (M2) activation, which is responsible for resolution and repair (Zheng and Wong, 2019). The M2 microglial phenotypes are divided into M2a, M2b, and M2c (Franco and Fernandez-Suarez, 2015; Mantovani et al., 2004; Martinez et al., 2008). M2c microglia, also known as acquired deactivated microglia, have been found to be deactivated by adjacent cells through processes that are guided by local and systemic homeostatic signals, but are still poorly understood (Saijo and Glass, 2011; Starossom et al., 2012). M2c microglia have been found to participate in neuroprotection and to release certain anti-inflammatory cytokines (Zhang et al., 2018), as well as be involved in matrix deposition and tissue remodeling after inflammation has been downregulated (Mantovani et al., 2004). An underrepresentation of deactivated microglia in the ACC in the remitted CD patient population may be a possible explanation for the persevering alterations in the ACC, as well as the lasting impairments within a number of cognitive domains, as there are apparently few M2c microglia present to repair the possible 'damage' to this area.

Additionally, an underrepresentation of oligodendrocytes was found in the ACC

in comparison to the control region. Oligodendrocytes are largely responsible for the remyelination process (Alonso, 2000; Miyata et al., 2011), and damage to oligodendrocytes has been found to lead to a reduction or cessation in action potential velocity, leading to mental or physical disability (Karadottir and Attwell, 2007). Earlier animal studies have found an association between prolonged exposure to elevated corticosteroid levels and the inhibited proliferation of oligodendrocyte precursors throughout the white matter of the brain (Alonso, 2000; Miyata et al., 2011; Willette et al., 2012). A study investigating white matter integrity in the same patient population as the current study found reductions of fractional anisotropy (FA; a measurement used to assess white matter microstructure) values in nearly all of the white matter tracts throughout the brain (van der Werff et al., 2014). Although the study was cross-sectional and thus no causal conclusions can be drawn, all patients in this study had been exposed to hypercorticolism, leading to the authors suggestion that prolonged exposure to increased levels of cortisol causes reduced white matter integrity, either directly or indirectly. The lower FA values found can be linked to earlier findings showing that corticosterone treatment in mice can lead to an increased distance between nerve fibers in fiber tracts (Miyata et al., 2011). This is likely due to a consequence of direct glucocorticoid receptor activation in oligodendrocytes that lead to an increased branching of these cells and possibly to lower levels of myelination. In summary, the relative underrepresentation of oligodendrocytes present in the ACC may also offer a possible explanation for the persistent alterations in this area, as well as for the lasting cognitive impairments within the long-term remitted CD patient population. Oligodendrocytes are clearly affected during CD, and their underrepresentation in areas with long-term changes may indicate an insufficiently large buffer against the deleterious effects of excessive cortisol exposure.

With regard to the cortisol sensitivity of the ACC, our findings were opposite to what we had hypothesized. Earlier studies have found that MR and/or GR expression in the ACC is high (e.g. Hawrylycz et al., 2012), although this has not been found to be predictive of structural changes following chronic overexposure to glucocorticoids (Andela et al., 2013). Our findings indicated that the ACC does not seem to be enriched for glucocorticoid responsive genes, neither at the mRNA level, nor for DNA-binding loci for the GR and the MR.

In sum, these findings provide further insight into the possible molecular mechanisms underlying the vulnerability for persistent alterations in the ACC of patients with long-term remitted CD. Future studies should explore how oligodendrocyte and

deactivated microglia cell numbers confer vulnerability, and also characterize genes that are differentially expressed as a (long-term) consequence of the cortisol overexposure. Moreover, it would also be of interest to explore the epigenetic regulation of certain genes that were identified, as well as possible gene-environment interactions. It is important to note that although we have not studied the ACCs of remitted CD patients, the cortisol excess does meet a 'naïve' brain, of which some areas have been found to be vulnerable, whereas others have not. Our findings present differences in basal gene expression in vulnerable areas that define the initial situation in which vulnerability is inherent and well documented, and importantly, also tell us which genes are less likely to be part of this initial vulnerability as no differences were found in MR or GR expression, in HSD1, and in chaperones. Future further (experimental) studies in the ACCs of remitted patients using post-mortem tissue or in Cushing's mouse models (e.g. Amaya et al., 2021) are necessary to validate these findings. These findings may ultimately aid in developing novel treatments for stress-related disorders of the brain, and for CD patients in particular. In conclusion, we found that certain underexpressed genes in the ACC, a region afflicted in remitted CD, were often associated with immunology functioning, as well as a lack of deactivated microglia and oligodendrocytes in the ACC, implicating immune functioning in general and suggesting a protective role of these cell types in relation to the long-term effects of excess cortisol exposure in the brain.

Supplementary tables

Supplementary Table 1. Gene symbols of the 58 significantly overexpressed genes in the ACC compared to the control region

#	Gene	BH-adjusted p-value	Log2(FC)	#	Gene	BH-adjusted p-value	Log2(FC)
1	*KIAA0748*	4.8E-05	2.76	**31**	*VWC2L*	0.00020131	1.16
2	*ONECUT2*	1.9E-06	2.45	**32**	*ABHD12B*	0.0024415	1.15
3	*CALML3*	3.4E-07	2.13	**33**	*HIST1H1D*	0.00114689	1.15
4	*AC079341.1*	3.6E-4	2.05	**34**	*OSR1*	0.00313916	1.14
5	*CTXN3*	1.3E-3	1.91	**35**	*LOC100130811*	0.003082	1.14
6	*AC010087.3*	8.8E-05	1.68	**36**	*GSTA3*	0.00176408	1.12
7	*EPN3*	2.9E-3	1.67	**37**	*CREB3L3*	0.00144554	1.12
8	*ANKRD56*	1.0E-3	1.62	**38**	*CD70*	0.00360575	1.11
9	*C13orf39*	0.0001348	1.6	**39**	*GPX3*	0.00402601	1.11
10	*AC017096.1*	0.00020131	1.6	**40**	*AC116165.2*	0.00354063	1.11
11	*FOXP2*	2.9793E-05	1.52	**41**	*DKK2*	8.5189E-05	1.1
12	*AC109486.1*	0.00016555	1.5	**42**	*COL22A1*	0.00015231	1.1
13	*C6orf105*	0.00153647	1.48	**43**	*PRKG2*	7.8751E-05	1.1
14	*C7orf62*	7.4172E-05	1.46	**44**	*RP1-152L7.5*	0.00006123	1.09
15	*GEFT*	0.00060117	1.41	**45**	*P2RX6*	0.00073769	1.09
16	*C12orf64*	4.106E-06	1.41	**46**	*AC132186.2*	0.00012163	1.08
17	*AGPAT9*	0.00030327	1.34	**47**	*AC099797.1*	0.00355346	1.05
18	*TGFBI*	0.0009426	1.34	**48**	*LOC389831*	0.00251141	1.05
19	*C13orf16*	0.0002648	1.32	**49**	*LAIR2*	0.01596857	1.04
20	*KLF4*	0.00034234	1.31	**50**	*SGK493*	0.00487298	1.04
21	*KRT31*	0.00385741	1.28	**51**	*SHD*	0.0009461	1.03
22	*MUC20*	0.00096254	1.27	**52**	*SOCS7*	9.4916E-05	1.03
23	*TWIST2*	0.0004627	1.24	**53**	*SMYD1*	5.9949E-05	1.02
24	*CCL27*	2.7072E-06	1.24	**54**	*CRTAC1*	0.00031035	1.02
25	*C4orf22*	0.00021141	1.21	**55**	*GAL*	0.01758361	1.02
26	*C21orf110*	0.00115177	1.2	**56**	*LOC732327*	0.00340362	1.01
27	*IL7R*	0.0005663	1.19	**57**	*LOC646548*	0.0001214	1.01
28	*UBQLNL*	0.00359144	1.19	**58**	*CBLN1*	0.00196426	1
29	*SPTSSB*	0.00013992	1.16				
30	*ADRA2A*	1.0566E-05	1.16				

Supplementary Table 2. Gene symbols of significantly underexpressed genes in the ACC compared to the control region

#	Gene	BH-adjusted p-value	Log2(FC)	#	Gene	BH-adjusted p-value	Log2(FC)	#	Gene	BH-adjusted p-value	Log2(FC)
1	*ESM1*	0.000643	-3.131971	**33**	*CISH*	0.00147	-1.44661	**65**	*T*	0.000109	-1.297565
2	*USP54*	0.000528	-2.141561	**34**	*IGFN1*	0.000086	-1.446063	**66**	*OPALIN*	0.014623	-1.29625
3	*FKSG2*	0.005164	-2.127462	**35**	*PPM1F*	0.001065	-1.433598	**67**	*C18orf56*	0.020569	-1.293979
4	*XIRP1*	0.002321	-2.058309	**36**	*C3*	0.000141	-1.419813	**68**	*RP11-379K17.4*	0.003293	-1.292907
5	*AC217771.1*	0.002809	-1.932542	**37**	*HIST1H3J*	0.01126	-1.407132	**69**	*MAS1L*	0.00059	-1.292138
6	*NACAP1*	0.003092	-1.843889	**38**	*HCAR3*	0.002477	-1.398194	**70**	*CYTH4*	0.000135	-1.289241
7	*C7orf52*	0.000974	-1.828502	**39**	*CYBB*	0.000031	-1.395657	**71**	*LAPTM5*	0.000508	-1.289139
8	*LRRC37A16P*	0.008016	-1.796806	**40**	*APOL4*	0.00228	-1.395187	**72**	*IL27*	0.005852	-1.285478
9	*RP1-74M1.1*	0.000794	-1.751832	**41**	*C1QB*	0.000091	-1.377739	**73**	*PIK3R5*	0.000364	-1.278479
10	*ARHGAP6*	0.000001	-1.691281	**42**	*TNFAIP8L2*	0.000166	-1.366946	**74**	*DGKH*	0.003823	-1.275499
11	*RP13-102H20.1*	0.000506	-1.647887	**43**	*LINC00302*	0.000228	-1.364728	**75**	*GHRLOS*	0.001176	-1.271973
12	*TFAP2D*	0.010628	-1.628211	**44**	*PIK3AP1*	0.002772	-1.363659	**76**	*FCGR1B*	0.000006	-1.271732
13	*TREM2*	0.000152	-1.62805	**45**	*FCGR3A*	0.000764	-1.35984	**77**	*HLA-DRB1*	0.000661	-1.271721
14	*ADAMDEC1*	0.0014	-1.617845	**46**	*MGC34800*	0.012626	-1.353962	**78**	*FOLR2*	0.00002	-1.268012
15	*LRRC2*	0.001518	-1.603071	**47**	*ARVCF*	0.001631	-1.352179	**79**	*AC087645.1*	0.032335	-1.265673
16	*KCTD4*	0.00086	-1.552604	**48**	*LOC392145*	0.000383	-1.351312	**80**	*TTLL10*	0.013341	-1.265597
17	*LOC392352*	0.009379	-1.55075	**49**	*RIPK3*	0.009267	-1.342552	**81**	*LYZL1*	0.000148	-1.264477
18	*LOC100133583*	0.00111	-1.550365	**50**	*LOC493754*	0.003335	-1.341574	**82**	*SLC6A19*	0.001229	-1.26386
19	*EEF1A1P32*	0.00212	-1.543428	**51**	*CD300A*	0.00025	-1.339029	**83**	*HLA-DRB3*	0.002063	-1.261032
20	*TYMS*	0.007758	-1.538527	**52**	*PABPC3*	0.00215	-1.338831	**84**	*ZACN*	0.000145	-1.258726
21	*SLC5A12*	0.000135	-1.535445	**53**	*CCR1*	0.000149	-1.337939	**85**	*RHBDF2*	0.000042	-1.253818
22	*LOC100129104*	0.001131	-1.532802	**54**	*KCNQ1*	0.000075	-1.335623	**86**	*TTBK1*	0.01707	-1.24624
23	*UBE2NL*	0.003216	-1.500601	**55**	*VSIG4*	0.000039	-1.334618	**87**	*HELB*	0.03786	-1.24412
24	*PRSS36*	0.010023	-1.491626	**56**	*OLFML3*	0.00024	-1.332063	**88**	*C1QA*	0.000102	-1.242582
25	*ITGB2*	0.00006	-1.485939	**57**	*C10orf113*	0.009201	-1.317093	**89**	*UGT3A1*	0.000493	-1.238639
26	*ZNF962P*	0.003284	-1.483219	**58**	*CGB1*	0.007774	-1.314111	**90**	*CX3CR1*	0.000138	-1.238131
27	*HLA-DQB1*	0.013347	-1.481177	**59**	*SYK*	0.00015	-1.307994	**91**	*DOCK8*	0.000018	-1.234026
28	*C6orf124*	0.009127	-1.472043	**60**	*DHRS9*	0.00022	-1.304074	**92**	*CAPZA2*	0.007372	-1.231083
29	*HLA-DMB*	0.000524	-1.469848	**61**	*DUSIL*	0.001192	-1.301545	**93**	*LOC442293*	0.008759	-1.226626
30	*ASB18*	0.002411	-1.46391	**62**	*ALOX5*	0.000084	-1.300801	**94**	*GHSR*	0.000281	-1.226355
31	*DCAF8L2*	0.005493	-1.462881	**63**	*ICMT*	0.027369	-1.298883	**95**	*INHBC*	0.000286	-1.215329
32	*BHLHE22*	0.00028	-1.449861	**64**	*LINC00475*	0.000438	-1.298307	**96**	*POLR2A*	0.000063	-1.210874

Supplementary Table 2. Gene symbols of significantly underexpressed genes in the ACC compared to the control region (continued)

#	Gene	BH-adjusted p-value	Log2(FC)	#	Gene	BH-adjusted p-value	Log2(FC)	#	Gene	BH-adjusted p-value	Log2(FC)
97	*PAGE4*	0.000758	-1.209436	**129**	*CD36*	0.000782	-1.158675	**161**	*GKN1*	0.001195	-1.105922
98	*AC013553.1*	0.00106	-1.209154	**130**	*SIGLECP3*	0.000194	-1.155155	**162**	*DDX11L2*	0.000279	-1.105832
99	*PGDS*	0.000339	-1.203295	**131**	*CXXC11*	0.000708	-1.152977	**163**	*JAM3*	0.025207	-1.103934
100	*LOC149832*	0.000196	-1.199155	**132**	*TCP1P3*	0.010404	-1.152398	**164**	*KRTAP2-3*	0.000077	-1.103781
101	*ARPC3P3*	0.017512	-1.195586	**133**	*HLA-DRB4*	0.001093	-1.151974	**165**	*RNASE6*	0.00066	-1.103514
102	*GGNBP1*	0.003932	-1.193093	**134**	*ITGAX*	0.000249	-1.15014	**166**	*C13orf30*	0.007079	-1.102135
103	*CYTL1*	0.000065	-1.192892	**135**	*SLC18A1*	0.010783	-1.14557	**167**	*UG0898H09*	0.000332	-1.10062
104	*HRASLS2*	0.005571	-1.191768	**136**	*FCRLA*	0.002589	-1.143966	**168**	*KIF20A*	0.000172	-1.100388
105	*LOC652494*	0.001066	-1.191537	**137**	*KRTAP11-1*	0.002157	-1.14022	**169**	*CCL22*	0.000233	-1.099942
106	*AC084851.1*	0.01887	-1.191151	**138**	*CHIA*	0.000363	-1.139375	**170**	*AQP3*	0.000943	-1.099861
107	*ADAMTS14*	0.032904	-1.188686	**139**	*GLYATL1P4*	0.002337	-1.138993	**171**	*BTBD16*	0.024564	-1.092944
108	*GOLGA6C*	0.014243	-1.1872	**140**	*SSTR4*	0.01834	-1.138461	**172**	*HNRNPCL1*	0.020022	-1.092215
109	*BMF*	0.000097	-1.184658	**141**	*AL359392.2*	0.037019	-1.137754	**173**	*GPR142*	0.017669	-1.091013
110	*FSHB*	0.007905	-1.182569	**142**	*C5ORF27*	0.001052	-1.13442	**174**	*FLYWCH2*	0.003734	-1.090978
111	*PPBP*	0.001137	-1.181566	**143**	*FBP1*	0.000006	-1.133798	**175**	*ZDHHC19*	0.001603	-1.085208
112	*CYP2B7P1*	0.000172	-1.181442	**144**	*SUCNR1*	0.001047	-1.13215	**176**	*MRGPRD*	0.000129	-1.084214
113	*C4ORF10*	0.007289	-1.179522	**145**	*RBM22*	0.001528	-1.129837	**177**	*C1DP3*	0.000877	-1.083969
114	*CYB5R2*	0.011806	-1.174476	**146**	*PRSS58*	0.000031	-1.127723	**178**	*DCST2*	0.000584	-1.083159
115	*TBX19*	0.000289	-1.171713	**147**	*NR2F2*	0.003781	-1.126786	**179**	*MDFI*	0.000211	-1.082027
116	*KIAA1772*	0.000644	-1.170781	**148**	*OR2B2*	0.001187	-1.123974	**180**	*APBB1IP*	0.000054	-1.079027
117	*SPRR2C*	0.000877	-1.169085	**149**	*HOXC4*	0.006499	-1.123726	**181**	*OR51S1*	0.005369	-1.070911
118	*MS4A7*	0.000484	-1.168639	**150**	*C20orf195*	0.001963	-1.12343	**182**	*SPANXN2*	0.004563	-1.070139
119	*SLC37A2*	0.000183	-1.167494	**151**	*RPS10P7*	0.001896	-1.123268	**183**	*ITGAM*	0.000097	-1.068902
120	*G6PC*	0.001687	-1.167205	**152**	*RBP7*	0.009865	-1.120622	**184**	*PROKR1*	0.000271	-1.067033
121	*DEFB123*	0.005833	-1.165392	**153**	*IGSF6*	0.00041	-1.119765	**185**	*ASPA*	0.037009	-1.065427
122	*LINC00602*	0.000498	-1.164531	**154**	*EME2*	0.019056	-1.117081	**186**	*FSTL3*	0.000155	-1.063874
123	*EBI3*	0.00006	-1.164307	**155**	*SUSD3*	0.000155	-1.114983	**187**	*FCER1G*	0.000026	-1.062954
124	*C20orf54*	0.000031	-1.164201	**156**	*OR10H2*	0.00048	-1.113771	**188**	*LOC643421*	0.0057	-1.062897
125	*HMGCS2*	0.000047	-1.162735	**157**	*APOD*	0.00086	-1.11244	**189**	*NR5A2*	0.002631	-1.059087
126	*C1orf226*	0.000111	-1.160511	**158**	*PATE3*	0.000078	-1.110198	**190**	*ACCN3*	0.002279	-1.059046
127	*AIF1*	0.000061	-1.160064	**159**	*TBC1D3*	0.000648	-1.108674	**191**	*AGXT2L1*	0.000443	-1.058594
128	*LYVE1*	0.000264	-1.158721	**160**	*CSNK1A1L*	0.029286	-1.108302	**192**	*DIRC1*	0.00052	-1.057317

Supplementary Table 3. Overview of GO terms for down-regulated genes that remained significant after BH correction

GO term	Description	P-value	FDR q-value	Enrichment (N, B, n, b)
GO:0098883	synapse pruning	2.73E-08	2.56E-04	18.00 (3402,6,189,6)
GO:0002478	antigen processing and presentation of exogenous peptide antigen	2.20E-07	1.03E-03	7.50 (3402,24,189,10)
GO:0019884	antigen processing and presentation of exogenous antigen	3.49E-07	1.09E-03	7.20 (3402,25,189,10)
GO:0042116	macrophage activation	3.61E-07	8.49E-04	9.60 (3402,15,189,8)
GO:0048002	antigen processing and presentation of peptide antigen	5.39E-07	1.01E-03	6.92 (3402,26,189,10)
GO:0019882	antigen processing and presentation	1.76E-06	2.75E-03	6.21 (3402,29,189,10)
GO:0002269	leukocyte activation involved in inflammatory response	1.99E-06	2.67E-03	12.00 (3402,9,189,6)
GO:0001774	microglial cell activation	1.99E-06	2.34E-03	12.00 (3402,9,189,6)
GO:0002504	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	3.60E-06	3.77E-03	9.00 (3402,14,189,7)
GO:0002495	antigen processing and presentation of peptide antigen via MHC class II	3.60E-06	3.39E-03	9.00 (3402,14,189,7)
GO:0002504	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	3.60E-06	3.77E-03	9.00 (3402,14,189,7)
GO:0002495	antigen processing and presentation of peptide antigen via MHC class II	3.60E-06	3.39E-03	9.00 (3402,14,189,7)
GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	3.60E-06	3.08E-03	9.00 (3402,14,189,7)
GO:0061900	glial cell activation	4.74E-06	3.71E-03	10.80 (3402,10,189,6)
GO:0002376	immune system process	5.95E-06	4.30E-03	1.83 (3402,491,189,50)
GO:0002685	regulation of leukocyte migration	7.27E-06	4.89E-03	4.18 (3402,56,189,13)
GO:0150062	complement-mediated synapse pruning	9.24E-06	5.80E-03	18.00 (3402,4,189,4)
GO:0019221	cytokine-mediated signaling pathway	1.76E-05	1.03E-02	2.51 (3402,172,189,24)
GO:0006955	immune response	2.04E-05	1.13E-02	2.07 (3402,295,189,34)
GO:0002274	myeloid leukocyte activation	3.17E-05	1.66E-02	2.89 (3402,112,189,18)
GO:0002252	immune effector process	4.18E-05	2.07E-02	2.39 (3402,181,189,24)
GO:0002281	macrophage activation involved in immune response	4.42E-05	2.08E-02	14.40 (3402,5,189,4)
GO:0002281	macrophage activation involved in immune response	4.42E-05	2.08E-02	14.40 (3402,5,189,4)
GO:0006911	phagocytosis, engulfment	5.61E-05	2.51E-02	7.71 (3402,14,189,6)
GO:0002682	regulation of immune system process	5.73E-05	2.45E-02	1.88 (3402,363,189,38)
GO:0002275	myeloid cell activation involved in immune response	6.43E-05	2.63E-02	2.97 (3402,97,189,16)
GO:0002366	leukocyte activation involved in immune response	6.51E-05	2.55E-02	2.75 (3402,118,189,18)
GO:0002263	cell activation involved in immune response	6.51E-05	2.45E-02	2.75 (3402,118,189,18)
GO:0045321	leukocyte activation	6.58E-05	2.38E-02	2.32 (3402,186,189,24)
GO:0001775	cell activation	7.11E-05	2.48E-02	2.22 (3402,211,189,26)
GO:0099024	plasma membrane invagination	8.93E-05	3.00E-02	7.20 (3402,15,189,6)
GO:0010324	membrane invagination	8.93E-05	2.90E-02	7.20 (3402,15,189,6)
GO:0097242	amyloid-beta clearance	1.01E-04	3.16E-02	9.00 (3402,10,189,5)
GO:0071404	cellular response to low-density lipoprotein particle stimulus	1.27E-04	3.85E-02	12.00 (3402,6,189,4)
GO:1902563	regulation of neutrophil activation	1.27E-04	3.73E-02	12.00 (3402,6,189,4)
GO:0150064	vertebrate eye-specific patterning	1.69E-04	4.81E-02	18.00 (3402,3,189,3)
GO:2000416	regulation of eosinophil migration	1.69E-04	4.67E-02	18.00 (3402,3,189,3)

Supplementary Table 4. Overview of the cell-type enrichment analysissignificant after BH correction

Cell-type	Markers	P-value	Benjamini-Hochberg p-value	Odds Ratio
Deactivated microglia	95	3.630e-05	0.001	7.07
Oligodendrocyte	1	0.003	0.036	12.38
Microglia	30	0.06	0.53	2.87
Astrocyte	1	1	1	0.42
Basket	9	1	1	27.15
Bergmann	7	1	1	1.33
Brainstem Cholin	1	1	1	27.15
Cerebellar granule cells	42	1	1	4.28
Dentate Granule	5	1	1	5.43
Dopaminergic	3	1	1	27.15
Ependymal	6	1	1	0.96
Forebrain Cholinergic	1	1	1	7.40
GabaPV	4	1	1	11.63
GabaReln	5	1	1	6.26
GabaRelnCalb	1	1	1	27.15
GabaSSTReln	4	1	1	9.05
GabaVIPReln	10	1	1	7.40
Gluta	119	1	1	27.15
Gogli	85	1	1	9.05
Hypocretinergic	103	1	1	3.88
Activated microglia	8	0.27	1	1.97
Noradrenergic	23	1	1	4.79
Purkinje	27	0.28	1	3.15
Pyramidal	1	1	1	27.15
Serotonergic	6	1	1	6.26
Spinal Cord Cholinergic	6	1	1	6.26
Spiny	10	1	1	3.88
Thalamus Cholinergic	16	1	1	2.47

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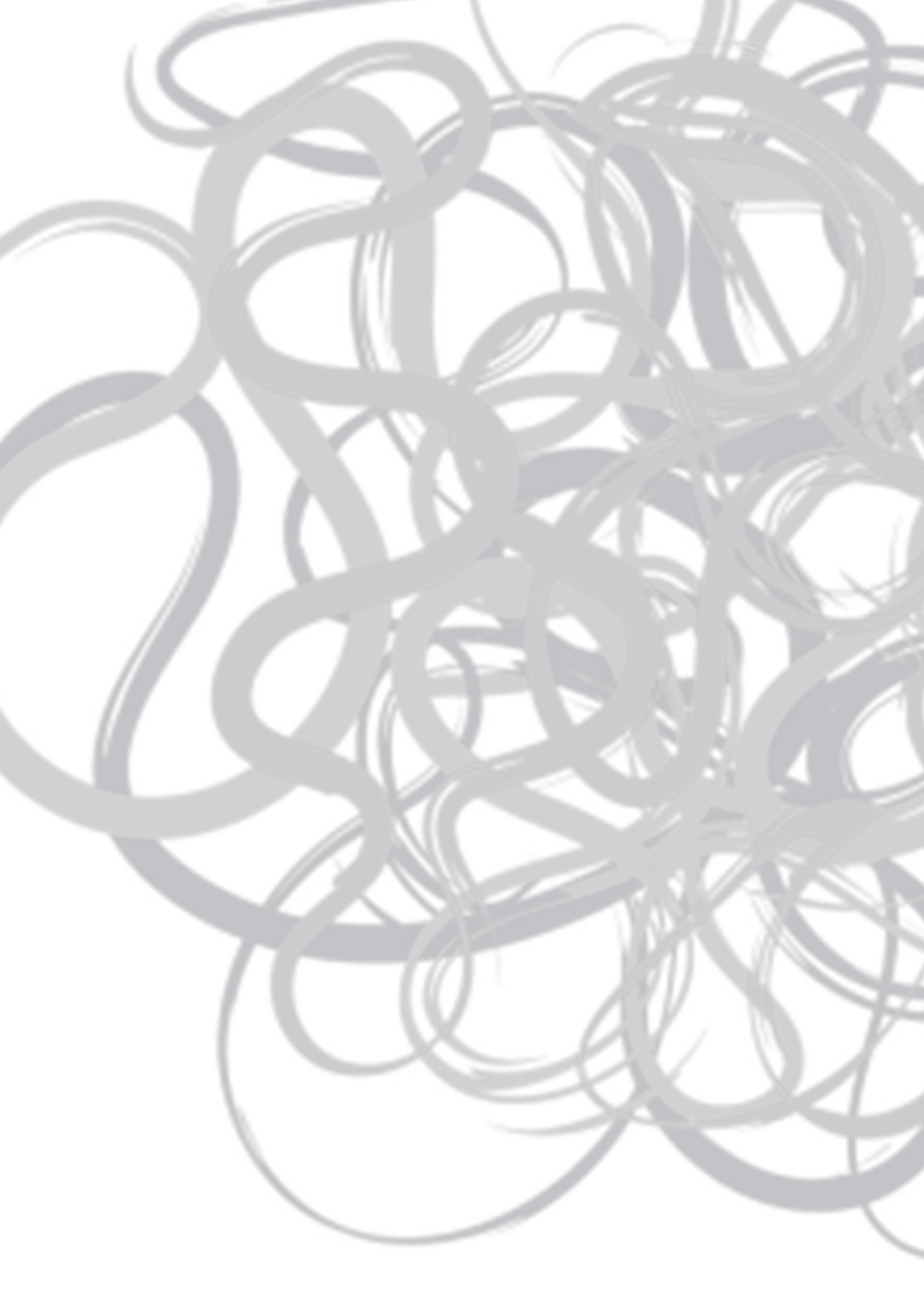

Chapter 8

Long-term effects of Cushing's disease on visuospatial planning and executive functioning

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Abstract

Background

Patients with remitted Cushing's Disease (CD) often present persisting impairments in executive and cognitive functioning domains. Little research has been conducted regarding the functional neural correlates of an important executive functioning skill, namely the ability to plan, in these patients. We used functional magnetic resonance imaging (fMRI) to examine visuospatial planning related brain activity in patients with remitted CD and matched controls.

Methods

fMRI scans were made using a 3-Telsa scanner while remitted CD patients (n=21) and age-, gender-, and education matched healthy controls (HCs; n=21) completed a parametric Tower of London (ToL) task. Psychological and cognitive functioning were assessed using validated questionnaires. Clinical severity was assessed retrospectively using the Cushing's syndrome Severity Index (CSI).

Results

CD Patients were on average 45.1 (SD=7.1) years old, 81% female, and in remission for mean 10.68 (SD=7.69) years. No differences were found in number of correct trials, response times per ToL trial, or in the region of interest analyses. Exploratory whole-brain analyses found that CD patients showed more activation in several brain regions associated with higher cognitive processes on 2-, 3-, and 5-step trials compared to HCs. Over-recruitment of the right parietal operculum cortex in the patients was significantly negatively associated with the prior active disease state on the CSI (r=-0.519, p=0.02).

Conclusions

The increased brain activation during the ToL in remitted CD patients versus controls signals over-recruitment of certain brain areas involved in higher cognitive processes. CD may thus result in long-lasting, subtle scarring effects during demanding executive functioning tasks, despite remission.

Introduction

Cushing's disease (CD) is characterized by hypercortisolism caused by a pituitary adenoma secreting excessive amounts of adrenocorticotropic hormone (ACTH; Nieman & Ilias, 2005). A variety of psychiatric symptoms can be induced by hypercortisolism, whereby the most common is major depressive disorder. However, mania, anxiety, and cognitive dysfunction also often co-occur (Sonino & Fava, 2001). Although CD can be effectively treated (usually by means of transsphenoidal surgery), increased mortality (van Haalen et al., 2015), residual psychopathological and physical morbidity (Resmini, 2014; Tiemensma et al., 2010; Ragnarsson et al., 2012), and reduction in quality of life (Van Aken et al., 2005) often remain. Furthermore, several important skills within the cognitive functioning domain have also often been found to remain impaired (Ragnarsson et al., 2012, Hook et al., 2007; Tiemensma et al., 2012).

It is likely that these residual symptoms are associated with the detrimental effects of long-term exposure to hypercortisolism on brain function and structures (Andela et al., 2013). Several neuroimaging studies have observed changes in both brain structure and function in patients with current CD (e.g. Starkman, Gebraski, Berent, & Schteingart, 1992; Andela et al., 2013; Maheu et al., 2008). With regard to the structural changes of the brain, certain abnormalities appear to persist after successful treatment of CD. The often found decreased hippocampal volume in patients with current CD seems to normalize after remission of the disease (e.g. Starkman et al., 1999; Tiemensma et al., 2010; Van der Werff et al., 2014). In contrast to this, altered gray matter volumes of certain brain regions, such as the anterior cingulate cortex (ACC), tend to persist after remission (Andela et al., 2013; Bauduin et al., 2020).

Regarding functional brain alterations in patients with remitted CD specifically (i.e. not in patients with remitted Cushing's syndrome), functional magnetic resonance imaging (fMRI) studies have revealed abnormalities in brain activity in this patient population in comparison to healthy controls (HCs). An fMRI study using an emotional faces paradigm found remitted CD patients had less activation in the medial prefrontal cortex in comparison to HCs (Bas-Hoogendam et al. 2015). Resting-state functional MRI (rs-fMRI) studies with remitted CD patients have found increased resting-state functional connectivity (RSFC) between the limbic network and the ACC, the default mode network in the left lateral occipital cortex (Van der Werff et al., 2015), and elevated RSFC in the medial temporal lobe, the hippocampus, and the

prefrontal cortex networks (Stomby et al., 2019). In contrast to this, the functioning of the executive control network in these studies was found to be similar in remitted CD patients and HCs. This could be explained by the fact that a rs-fMRI does not include specific goal-oriented tasks that requires high cognitive effort. Thus, it may the case that differences in functional activity within this network only manifest in the remitted CD population when the cognitive demands are higher, as is the case in patients with other stress-related psychopathologies, such as depression and post-traumatic stress disorder (PTSD, Wang et al., 2008; Aizenstein et al., 2009; Daniels et al., 2010).

Cognitive functioning has been examined by means of standard neuropsychological testing in current CD patients (Ragnarsson et al., 2012; Tiemensma et al., 2010), as well as in remitted CD patients after a follow-up period of up to 18 months (Hook et al., 2007). These studies found that cognitive and executive functioning (i.e., psychomotor functioning, visuoconceptual tracking, processing speed, auditory attention, auditory working memory, verbal fluency, reading speed, and brief attention) are (and perhaps remain) impaired in active and remitted CD patients. Cognitive planning encompasses the neurological processes that are involved with the strategy formulation, coordination, evaluation, and selection of a thought sequence, and the necessary actions that are needed in order to achieve that goal (Morris et al., 1997). Reductions of these cognitive abilities in patients with remitted CD may lead to lasting effects on planning abilities, affecting one's daily functionality, psychological state, and quality of life. Although these aforementioned studies have found a number of impairments in the cognitive functioning domain, it is currently unclear as to whether impairments are also detectable in the brain activity patterns of this patient population.

In this study, we examined whether patients with remitted CD display altered performance and brain activity patterns in comparison to healthy controls (HCs) with regard to cognitive planning and executive functioning using the Tower of London (ToL) task (Shallice, 1982). Based on previous research on cognitive functioning in CD patients, we hypothesized that remitted CD patients will complete less trials correctly, complete less trials in total, and take more time to complete a ToL trial in comparison to healthy controls. Furthermore, taking the differences in brain activation found in earlier studies with CD patients into account (i.e. Andela et al., 2013; van der Werff et al., 2015), we hypothesized increased activation in the ACC, an area involved in several complex cognitive functions and critically active when engaging in a cognitively demanding task (Fincham & Anderson, 2006), in

comparison to matched HCs. In addition, we performed an exploratory whole-brain analysis to examine whether other task-related differences in activation can be identified. Furthermore, potential associations between brain activity, psychological, cognitive, and clinical measures were explored.

Methods

Subjects

Participants were all remitted CD patients (aged 18-60 years) who were being monitored at the Leiden University Medical Center (LUMC). Of the 49 invited participants, 96% responded to the invitation, and based on primary in- and exclusion criteria, 31 patients were ultimately screened for further study eligibility (details with regard to this study protocol have previously been published elsewhere; Andela et al., 2013). HCs were recruited via advertisements in grocery stores and internet. HCs were matched to each patient based on gender, age, and level of education. A HC specific exclusion criterion was a history of or current psychiatric disorder. Further exclusion criteria for both the remitted CD and HC groups were neurological problems, MRI contraindications, (history of) drug or alcohol abuse, and/or left-handedness. Six remitted CD patients were excluded due to one of these exclusion criteria. Finally, one remitted CD and their matched HC were excluded because behavioral data was not recorded, leaving the final sample to consist of 24 remitted CD patients and 24 matched HCs.

Biochemical, radiological, and clinical observations conform current international guidelines were used to diagnose CD. Detailed information with regard to these criteria have previously been published elsewhere (Tiemensma et al., 2010). All CD patients received transsphenoidal surgery following the diagnosis of active CD. After surgery, CD remission was confirmed by means of clinical evaluation and multiple biochemical test outcomes (for example, normal 24-hr urinary cortisol excretion rates (<220 nmol/24-hr), normal midnight saliva cortisol (below 5.7 nmol/L), and normal overnight suppression of plasma cortisol levels (<50 nmol/l) by dexamethasone (1 mg)). Patients with remaining glucocorticoid dependency were substituted with hydrocortisone (on average 20 mg/day, divided over three doses), and evaluated twice yearly. Prior to study participation, persistent biochemical cure of CD was confirmed in concurrence with the abovementioned diagnostic tests. Disease duration was identified as the moment earliest somatic signs were presented in a patient's history. Duration of remission was calculated from either the date of curative transsphenoidal surgery or from the date of normalization

of biochemical tests in the case of initial persistent disease persistence following surgery. Written informed consent was obtained from all participants and the study protocol was approved by the medical ethical committee of the LUMC. The protocol was written in accordance with the principles of the Helsinki declaration. Patient and treatment characteristics were obtained from patient medical records.

Behavioral and clinical severity assessment

Psychopathology and cognitive functioning were assessed using the following scales: the 10 item Montgomery-Åsberg Depression Rating Scale (MADRS; Montgomery & Åsberg, 1979), and the 28 item Inventory of Depression Symptomatology (IDS; Rush et al., 1986) to assess the severity of depressive symptoms. An interviewer assessed the MADRS, all other scales used were self-report. Anxiety was evaluated using the blood injury phobia, social phobia, blood injury subscales, and total score of the 15 item Fear Questionnaire (FQ; Marks & Mathews, 1979), and the 21 item Beck Anxiety Inventory (BAI; Beck et al., 1988). The 14 item Irritability Scale (IS) and the 14 item Apathy Scale (AS) were used to assess the severity of irritability and apathy, respectively (Starkstein et al., 2001; Chatterjee et al., 2005). Participants with total scores of more than 14 points were considered to be irritable or apathetic. Failures in memory, motor function, and perception were assessed using the 25 item Cognitive Failure Questionnaire (CFQ; Broadbent et al., 1982). Higher sum scores indicate greater symptom severity.

CD symptom severity during the active and the remitted disease state were established using the 8 item Cushing's syndrome Severity Index (CSI; Sonino et al., 2000). The CSI score during active disease was estimated retrospectively. The remission score was based on the last annual evaluation. Total CSI scores were used for both active and remitted disease states and scores on this index can range between 0 and 16 (higher total score indicates greater symptom severity). The necessary information in order to score the CSI was obtained from the patient's clinical history and medical records. The index was scored by two independent raters that reached consensus in case of discrepancy. Finally, prior to and after the fMRI ToL task, anxiety levels were monitored by means of a Visual Analogue Scale (VAS; Huskisson, 1974) ranging from 0 to 100, where a higher score indicates a higher level of anxiety.

Task paradigm

An event-related parametric version of the ToL was used. A detailed description of this task has been previously published (van den Heuvel et al., 2003). In brief,

participants were presented with either a baseline or test trial. In the baseline trials, participants were requested to count the number of yellow and blue beads presented on the screen. In the test trials, participants were requested to count the minimum number of steps from the 'start' condition to the 'goal' condition. The test trials ranged from 1 to 5 steps (see Figure 1 for examples). The task was pseudorandomized and self-paced, with a maximum response duration of 60 seconds for each trial. The trial was presented by means of E-Prime software (Psychological Software Tools, Pittsburg, PA, USA). Responses and response times were logged by means of button boxes. No feedback was given with regard to the answers. Image Acquisition

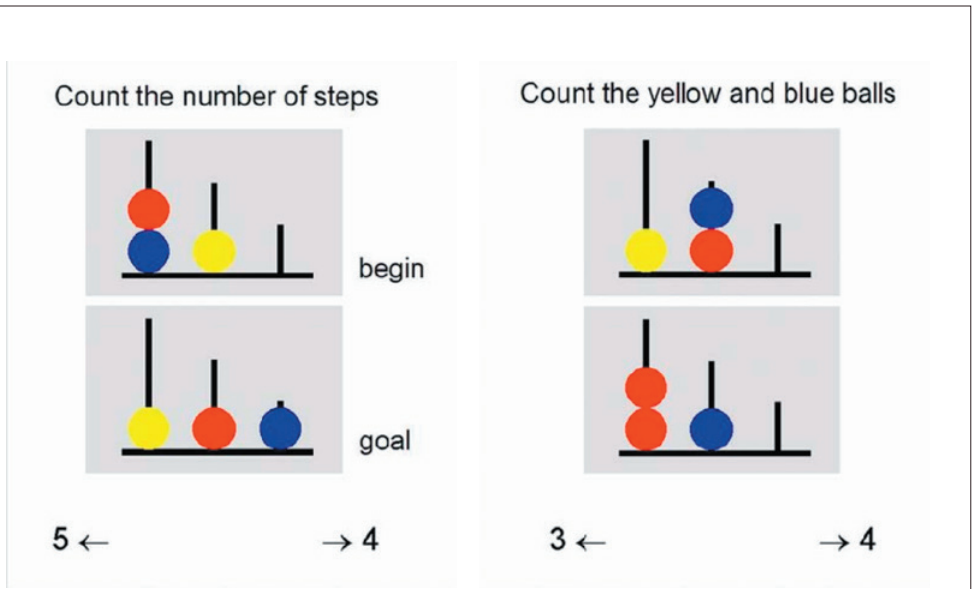


Figure 1. Example of a 5-step planning trial in the Tower of London (left figure), and a baseline trial with no planning involved (i.e. participants were asked to count the number of yellow and blue balls presented (right figure)).

Image Acquisition

The ToL paradigm was part of a larger fMRI protocol, which included a resting-state scan and an emotional faces paradigm. In each session, the ToL was administered as the first fMRI paradigm in each session. The task duration was 17 minutes and 36 seconds. Imaging data were acquired in the LUMC using a Philips 3T system (Philips Healthcare, Best, The Netherlands; software version 3.2.1). A SENSE-32 channel headcoil was used for transmission and reception of radio frequencies. For each

subject, anatomical imaging was acquired by means of a transvers 3D gradient-echo T1-weighted sequence (repetition time (TR) = 9.8, echo time (TE) = 4.6 ms, flip angle = 8º, Field of view (FOV) matrix size = 256 x 256, voxel size = 1.17x1.1.7x1.2 mm, 140 slides), which were examined by a neuroradiologist blinded for patient details. Other than age-related white matter intensities and effects of post-transsphenoidal surgery, no further macroscopic abnormalities were detected. ToL fMRI echo-planar images (EPI) were acquired using a T2*-weighted gradient-echoplanar imaging sequene (EPI) (TR = 2200 ms, TE = 30 ms, flip angle = 80º, 38 transverse slices, no slice gap, FOV = 220 × 220 mm, voxel size = 2.75 × 2.75, 3 mm slice thickness), which was then registered to the MNI T1-template brain.

Data analysis

Task performance and clinical characteristics

Psychometric and task performance data were analyzed using IBM SPSS Statistics for Windows version 24 (IBM Corp. Armonk, N.Y., USA). If the data did not meet the assumptions required for parametric analyses, the appropriate nonparametric tests were performed (i.e. Mann-Whitney U test). VAS scores and performance were analyzed using paired samples t-tests. Proportion correct scores and mean response times per trial were entered as dependent factors in the analyses.

Image processing

Preprocessing and analyzing of the ToL data was conducted using FSL v.5.0.8. Preprocessing included artefact removal with FSL FIX (Salimi-Khorshidi et al., 2014), motion correction (realignment), grand mean scaling, and spatial smoothing with 6mm Gaussian kernel. ICA-AROMA (Pruim et al., 2015) was used for motion artefact removal, and high-pass filtering was used. FSL FEAT was used to create first-level statistical parametric maps.

The fMRI ToL paradigm was modelled in an event-related manner with regressors (i.e. explanatory variables) made by convolving each event-related stimulus function (baseline, 1-5 step trials), with a canonical hemodynamic response function, and then modulated using reaction times. Low-frequency noise was stripped by applying a high-pass filter (set at a cut-off of 128 seconds) to the time series at every voxel.

Main effects of task and between-group comparisons

Analysis were conducted in line with the ToL analysis approach reported in van Tol et al. (2011). Contrast images for task load, which ranged from trial type 1-5 with weighting [-1.5, -1.0, -0.5, 1, 2] based on level of cognitive demand respectively, were

calculated per subject on a voxel-by-voxel basis and then entered into a second-level analyses for between group comparisons (remitted CD and HC). Thus, in all cases, activation of the regions specified were modulated by the complexity of the task.

Thresholds for the main effects of the task and between group comparisons were corrected using a cluster z-threshold of 2.3 with $p < 0.05$. Between group comparisons were conducted using the ACC as a region of interest (ROI). This region was defined using the Harvard-Oxford Cortical Structural Label Atlas implemented in FSLeyes (version 5.0.10). This was followed by an exploratory whole-brain analysis per trial step. Both the ROI- and the exploratory analyses were carried out using FSL FEAT fMRI analysis and analyzed as matched pairs in order to identify differences in planning activity between the remitted CD population and the HCs (Beckmann, Jenkison, & Smith, 2003; Woolricht et al., 2004). Significant clusters per trial step were tested for correlations between measures of psychiatric symptom severity, cognitive functioning and clinical severity and corrected for multiple testing. As the questionnaires used for the behavioral assessment show considerable overlap, correction for multiple testing using the Benjamini-Hochberg (Benjamini and Hochberg, 1995) method with an FDR set at 5% was considered too stringent. For this reason, we corrected for multiple testing using an FDR set at 20%. We report the uncorrected Pearson's correlations for normally distributed data, and the Spearman's rho for data that is not normally distributed.

Results

Sample characteristics

Three subjects (1 remitted CD patient and 2 HCs) and their respective matched pairs (thus n = 6), were excluded from the analyses because they did not meet the prespecified overall performance percentage of more than 75% correct responses. This was done in order to increase the likelihood of capturing task-based planning activity and to reduce possible non-task related bias, resulting in a total of 21 pairs of participants. Remitted CD patients and the HCs were well-matched as they did not differ significantly in gender, age, education, and intercranial volume (ICV). Mean MADRS, IDS, BAI, and AS scores differed significantly between remitted CD and HC groups (all $p < 0.02$), whereas mean scores on the total FQ score and its subscales, and the IS did not (see Table 1 for further details). Mean disease duration in the remitted CD was 7.6 years and duration of remission, 10.7 years. Mean scores on the CSI were 7.95 (SE = 0.428) during the active phase and 2.33 (SE = 0.340) upon remission of CD.

Table 1. Demographic and clinical characteristics remitted CD (RCD) patients and matched HCs. Data are presented as mean ± standard deviation or number (%), with a significance level set at $p<0.05$.

	RCD patients (n=21)	HCs (n=21)	*p*-value
Gender (female, (%))	17(81%)	17(81%)	1.00[a]
Age (years ± SD)	45.9 ± 7.1	44.6 ± 7.7	0.57[b]
Education (years ± SD)			1.00[a]
Low	5 (23.8%)	5 (23.8%)	
Medium	10 (47.6%)	10 (47.6%)	
High	6 (28.6%)	6 (28.6%)	
ICV mm^3 (mean ± SD)	$1.51{\cdot}10^6 \pm 1.41{\cdot}10^5$	$1.52{\cdot}10^6 \pm 1.69{\cdot}10^5$	0.76[b]
MADRS (mean ± SD)	5.43 ± 3.91	1.38 ± 1.80	<0.001[c]
Inventory of Depressive Symptomatology (mean ± SD)	45.55 ± 12.60	36.10 ± 6.07	0.02[c]
Beck Anxiety Inventory (mean ± SD)	28.15 ± 6.10	24.05 ± 3.34	0.02[c]
Fear Questionnaire (mean ± SD)	22.85 ± 17.10	14.52 ± 9.94	0.07[b]
Agoraphobia subscale	5.30 ± 6.69	2.67 ± 3.26	0.52[c]
Blood injury phobia subscale	6.45 ± 9.04	3.76 ± 4.28	0.73[c]
Social phobia subscale	11.10 ± 7.33	8.10 ± 4.89	0.13[b]
Irritability Scale	11.90 ± 8.99	8.52 ± 6.45	0.23[c]
Apathy Scale	13.6 ± 6.6	7.8 ± 3.8	0.002[c]
Cognitive Failures Questionnaire	35.60 ± 14.17	29.0 ± 9.46	0.09[b]
Disease duration (years)	7.55 ± 8.39		
Duration of remission (years)	10.68 ± 7.69		
Cushing's Syndrome Severity Index (CSI)	7.95 ± 1.96		
Active phase (total)			
Remission phase (total)	2.33 ± 1.56		

ICV = Intercranial volume; MADRS = Montgomery-Åsberg Depression Rating Scale

[a] *p*-values were tested with X^2 test

[b] *p*-values were tested with independent samples t-test

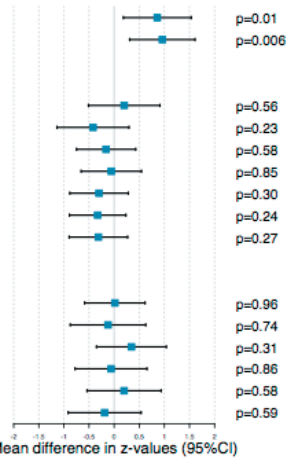
[c] *p*-values tested with Mann-Whitney *U* test

Behavioral results

Mean VAS scores, mean accuracy scores, and mean response types per trial type are reported in Table 2. Remitted CD patients reported significantly higher levels of anxiety in comparison to healthy controls (p = 0.02) both before and after the task (p = 0.006). Overall, mean accuracy decreased with increasing task load. This did not differ significantly between the groups on any of the step trials. Also, performance speed increased as task load increased in both groups, although no differences in response times on any of the trial steps were found. An overview of the mean number of trials per trial type and group can be found in Appendix 1. Although the remitted CD group completed less trials per step in comparison to the HC group, this did not differ significantly between groups on any of the trial steps.

Table 2. Overview of proportion correct answers and response times (in seconds) per group (remitted CD (RCD) and Healthy Controls (HC).

Variable	RCD (n = 21)	HC (n = 21)	Mean difference	p-value
VAS prior to ToL (total score)	37.86 (29.63; 46.09)	19.81 (11.68; 27.94)	18.05 (3.87; 32.22)	p=0.01
VAS after ToL (total score)	34.05 (27.03; 41.07)	16.14 (9.04; 23.24)	17.90 (5.78; 30.03)	p=0.006
Proportion correct:				
- Baseline	0.98 (0.97; 0.99)	0.97 (0.96; 0.98)	0.00 (-0.01; 0.02)	p=0.56
- 1 step	0.93 (0.91; 0.95)	0.96 (0.94; 0.98)	-0.04 (-0.10; 0.03)	p=0.23
- 2 steps	0.91 (0.89; 0.93)	0.92 (0.90; 0.94)	-0.02 (-0.08; 0.05)	p=0.58
- 3 steps	0.91 (0.87; 0.95)	0.91 (0.87; 0.95)	-0.01 (-0.08; 0.06)	p=0.85
- 4 steps	0.79 (0.75; 0.83)	0.83 (0.79; 0.87)	-0.05 (-0.14; 0.05)	p=0.30
- 5 steps	0.73 (0.67; 0.79)	0.80 (0.74; 0.86)	-0.07 (-0.19; 0.05)	p=0.24
- Total	0.92 (0.91; 0.93)	0.93 (0.92; 0.94)	-0.17 (-0.49; 0.15)	p=0.27
Response time(s):				
- Baseline	3.26 (2.95; 3.57)	3.24 (2.81; 3.67)	0.12 (-0.52; 0.54)	p=0.96
- 1 step	4.86 (2.84; 6.88)	5.00 (2.47; 7.53)	-0.14 (-1.01; 0.73)	p=0.74
- 2 steps	6.73 (5.67; 7.79)	6.01 (5.30; 6.72)	0.72 (-0.74; 2.19)	p=0.31
- 3 steps	8.44 (7.24; 9.64)	8.62 (7.33; 9.91)	-0.18 (-2.24; 1.88)	p=0.86
- 4 steps	13.15 (10.72; 15.58)	12.15 (10.29; 14.01)	1.00 (-2.72; 4.72)	p=0.58
- 5 steps	15.32 (13.73; 16.91)	16.23 (13.76; 18.70)	-0.91 (-4.41; 2.59)	p=0.59



* *p*-values were tested using paired samples t-tests
**VAS: Visual Analogue Scale measuring anxiety prior to the ToL task and after the ToL task

fMRI results

Main task effects

No participants were excluded from the analyses due to movement or scanning artifacts. The task effects across all participants identified two significant activity clusters: (i) in the superior frontal gyrus and the frontal pole, and (ii) in the cingulate gyrus (posterior division) and the precuneus cortex ($p < 0.05$ for both clusters after cluster correction; see Table 3 and Figure 2). No main effects of increasing task load were found in both the remitted CD group and the HC group. This is likely due to a lack of power due to less trials in the more difficult steps (Appendix I). No significant activity clusters were found in the ROI. Significant activity clusters were found in the parietal operculum cortex on 2 step trials ($z = 3.75$, $p = 0.005$ after cluster correction), and in the supramarginal gyrus on 3 step trials in the remitted CD patient group ($z = 3.27$, $p = 0.02$ after cluster correction) in comparison to HCs. Group comparisons on the other trial steps did not reveal further significant differences (see Table 3). However, at a lower threshold (1.9), significant activity clusters were found in the remitted CD group on 4 and 5 step trials (see Appendix II).

Table 3. Mean task activation and planned paired comparisons of activity related to increasing task load at threshold 2.3.

Mean task activation/ paired comparison	Area	Side	Cluster size	Peak Voxel (MNI) x (mm)	y (mm)	z (mm)	*p*
Mean task activation	Superior Frontal Gyrus/ Frontal Pole	L	801	-6	56	26	0.004**
	Cingulate gyrus, posterior division/ Precuneus Cortex	R	554	2	-40	44	0.03**
ROI							
ACC RCD*>HC	None						
ACC RCD<CD	None						
1 Step							
RCD>HC	None						
RCD<HC	None						
2 steps							
RCD>HC	Parietal Operculum Cortex	R	664	60	-36	26	0.005**
RCD<HC	None						
3 steps							
RCD>HC	Supramarginal Gyrus	R	527	58	-42	20	0.02**
RCD<HC	None						
4 steps							
RCD>HC	None						
RCD<HC	None						
5 steps							
RCD>HC	Occipital Fusiform Gyrus/ Lingual Gyrus	R	599	20	-74	-18	0.01**
RCD>HC	Supramarginal Gyrus	R	478	58	-38	28	0.04**
RCD<HC	None						

*RCD = remitted Cushing's Disease patients

*Thresholded using Cluster correction $z = 2.3$; $p < 0.05$.

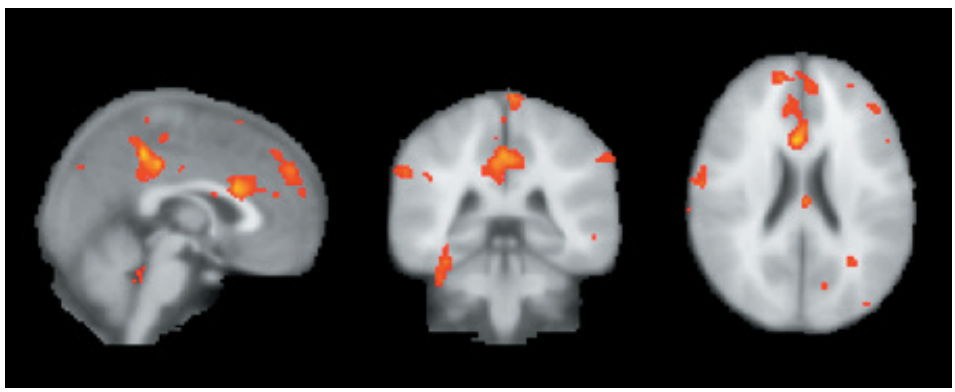


Figure 2. Mean activity during task performance across the subjects displayed at cluster z-threshold of 2.3 with $p<0.05$ [-39, 36, 30].

Correlation analyses

After adjusting for multiple comparisons using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995) using an FDR set at 20%, activation in of the right parietal operculum cortex in remitted CD patients was found to be significantly negatively associated with the prior active disease state on the CSI ($r = -0.519$, $p = 0.02$). No other significant associations between significantly activated clusters and scores on behavioral scales, measures of disease duration, duration of remission, and clinical disease severity were found.

Discussion

In this study, we investigated whether patients with remitted CD displayed altered performance and brain activity patterns in comparison to HCs using the ToL, a parametric visuospatial planning task. No differences in performance were found between the groups, neither in the number of trials completed correctly, nor in the number of trials completed in total, nor in the amount of time needed to complete the trials. Although mean task activation was identified in two brain clusters, our ROI analysis of the ACC did not yield any significant difference in activation between the groups. However, an exploratory whole-brain analysis identified areas of increased brain activity in the remitted CD group on 2-, 3-, and 5-step trials. Finally, a negative association between activation of the right parietal operculum cortex in remitted CD group with the prior active disease state as measured on the CSI was found. These findings indicate that CD may result in subtle scarring effects despite long-term remission in certain brain areas when completing demanding executive functioning tasks.

As mentioned earlier, previous research conducted with remitted CD patients found impairments in multiple domains of neurocognitive functioning (i.e. Hook et al., 2007; Ragnarsson et al., 2012; Tiemensma et al., 2010; Zarino et al., 2019). We therefore hypothesized that remitted CD patients would also present impairments on the ToL task by completing less trials correctly, completing less trials in total, and taking more time to complete a ToL trial in comparison to HCs. Surprisingly, remitted CD patients showed no cognitive and executive functioning deficits in comparison to HCs as measured on the ToL. As several studies have identified visuospatial impairments in active CD patients (Siegel et al., 2020), albeit using other measurement instruments, our findings suggest that certain visuospatial impairments may improve upon remission of CD. However, further insight into whether remission of CD also remits all or most visuospatial impairments should be

confirmed in longitudinal studies comparing performance on the ToL in the active disease state with the long-term remission state.

With regard to fMRI brain activation patterns on the ToL, an earlier study with HCs aimed at validating the ToL paradigm for fMRI found mean task activation in a number of brain areas (i.e., in the dorsolateral prefrontal cortex, the cingulate cortex, the cuneus, the supramarginal and angular gyrus in the parietal lobe, and the frontal opercular area of the insula, Lazeron et al., 2000). We were unable to replicate these results in current sample of HCs as it was too small, however we did investigate mean task activation over the whole sample. Although we did not expect to find precisely the same activation in all of the brain areas found in the aforementioned study due to the differences in our study populations and MRI scanners, we did expect to find a certain amount of overlap. We identified mean group activation in two separate brain clusters: (i) the superior frontal gyrus and the frontal pole, and (ii) the posterior division of the cingulate gyrus and the precuneus cortex. Our first cluster (i.e., the superior frontal gyrus (part of the dorsolateral prefrontal cortex), and the frontal pole), partially overlapped with one of the activated areas found in the Lazeron et al. (2000) paper (i.e. the dorsolateral prefrontal cortex). This area has been found to be involved in the management of uncertainty, where increasing uncertainty leads to increased activation (Volz, Schubotz, & von Cramon, 2005), and the frontal pole has been implicated in cognition, perception, and working memory (Bludal et al., 2014). With regard to the second cluster identified (i.e. the cingulate gyrus (an area in the cingulate cortex) and the precuneus cortex), this largely overlaps with an area found in the Lazeron et al. (2000) study (i.e. the cingulate cortex and the precuneus). These overlapping findings increase the validity of our current findings, and provide further evidence regarding the specific brain areas that are recruited during visuospatial planning tasks.

Considering the differences in brain structure and activation found in earlier studies with this same population of remitted CD patients (i.e. Andela et al., 2013; van der Werff et al., 2015), we hypothesized to find increased activation in the ACC, an area involved in several complex cognitive functions and critically active when engaging in a cognitively demanding task (Fincham & Anderson, 2006), in comparison to matched HCs. However, we did not find any differences in activation between the groups. This indicates that although the ACC has previously been implicated in displaying altered resting-state brain activity, altered gray matter volumes, and altered white matter integrity in this same patient group (van der Werff et al., 2015; Andela et al., 2013; Van der Werff et al., 2014), they are not overrecruited during

the ToL task. It could, however, be the case that both patients and HCs overrecruit the ACC in this type of visuospatial planning and executive functioning task, as it is an area involved in several complex cognitive functions (Stevens et al., 2011).
As mentioned earlier, certain regions were not identified in our mean group activation that were identified in the Lazeron et al. (2000) paper. Interestingly, several of these areas were found to be more activated in the remitted CD group on a number of the trial steps. Increased right parietal operculum cortex recruitment was found as a function of increased planning load on 2-step trials in the remitted CD group. This is an area involved in mathematical thought, visuospatial cognition, and imagery of movement, among other functions (Witelson, Kigar, & Harvey, 1999). Also, increased right supramarginal gyrus recruitment was found as a function of increased planning load on 3- and 5-step trials. This brain area has been found to be involved in complex cognitive functions, such as calculation and visuospatial awareness (De Schotten et al., 2005). These findings indicate that remitted CD patients need to overrecruit these brain regions to attain a similar performance level as the HCs. Moreover, increased recruitment in the occipital fusiform implicated in higher processing for visual information such as the processing of color information, word recognition, and working memory capacity, amongst others (Ramachandran, 2011; McCandliss, Cohen, & Dehaene, 2003; Brunyé, Moran, Holmes, Mahoney, & Taylor, 2017), and lingual gyri was found on the 5-step trials. Both regions (i.e. the occipital fusiform and lingual gyri) have demonstrated to play an important role in color perception (i.e. Sakai et al., 1995; Sereno et al., 1995). In sum, this indicates that remitted CD patients primarily characterize themselves in increased recruitment of the abovementioned brain regions on certain trial steps, and not in executive and cognitive functioning as measured in the number of trials answered correctly or the time needed to answer each trial.

Although no differences in altered brain activity were found on the 4-step trials, we believe this was likely due to lack of power (i.e. too few trials to be able to identify a possible effect). We therefore ran further exploratory whole-brain analyses at a lower threshold (i.e. 1.9) for all trial steps (see Appendix II). Although we cannot interpret these results as we interpret the results set at the more stringent and accepted threshold, we did find increased activation in the precuneus of the remitted CD group, an area that was also found to be overrecruited in the mean task activation. Moreover, activation in this area was also observed in 5-step trials at this lower threshold. Previous studies have shown activation in the precuneus during action generation tasks (Allendorfer et al., 2012), as well as in visuospatial and -motor imagery (Cavanna & Trimble, 2006; Kawashima, Roland, & O'Sullivan,

1995). It has also been suggested to be involved in the direct visual route from vision to action, functioning in extracting visual-motor and spatial relationship features (Wang et al., 2019).

A negative association was found between the right parietal operculum cortex in remitted CD patients and the CSI score of the prior active disease state, although this result should be interpreted with caution as the FDR rate used was adjusted due to overlap in the behavioral assessment measures. However, this association indicates that the more severe the active disease state (as was measured using the CSI), the less activation in the right parietal operculum cortex, a region that has been found to be involved in mathematical thought, specifically in the knowledge of numbers and their relations (Blackmore & Firth, 2005). This seems to imply that this brain region may be less proficient in increasing activation in remitted CD patients who have experienced a more severe active phase of CD. There were no further significant associations found between activated brain clusters and scores on behavioral scales, measures of disease duration, and duration of remission.

The hypothesis has been posited that studying patients with remitted CD could offer further insight into the effects of prolonged cortisol exposure on, amongst others, the brain, as these findings may in turn be (partially) generalizable to other remitted stress-related disorders (such as depression and/or anxiety), as well as to conditions treated with synthetic glucocorticoids. A previous study investigating the neural correlates of the ToL task in out-patients with (remitted) depression and anxiety found that only patients with a current moderate or severe depression had increased dorsolateral prefrontal cortex activation as a function of increasing task load, whereas patients with current mild or remitted depression, with a current diagnosis of anxiety disorder(s) (such as generalized anxiety disorder and/or panic disorder and/or social anxiety disorder) did not, in comparison to HCs (van Tol et al., 2011). Thus, it seems that the prolonged excess exposure of endogenous cortisol on the brain in the magnitude as is the case with CD, leads to seemingly permanent alterations in brain activation of certain brain regions after long-term disease remission in contrast to, for example, patients with remitted depression.

Due to the cross-sectional nature of this study, causal conclusions cannot be drawn as we cannot be certain whether the found differences in psychopathology or brain activity were present prior to the onset of CD. Also, we cannot know for certain whether other factors that occurred over the course of the seven-year remission period influenced the observed changes in the neural networks. A further possible

limitation of this study is the use of the CSI to evaluate disease severity during the active phase. Although this instrument has been validated repeatedly, it does make use of retrospective assessments, which may lead to less accurate estimations. Study strengths were the homogeneity of the patient population (i.e. all of the patients included in the study were treated by means of transphenoidal surgery), and the selection of the age-, gender-, and education matched HCs. Nevertheless, heterogeneity was present in the remitted CD patient group regarding duration of the disease and duration of remission, and this therefore may have decreased the precision of the effect estimates of the study.

In conclusion, we found no evidence for pervasive cognitive impairments for the domain of visuospatial planning and executive functioning as measured on the ToL task in remitted CD patients. We did find differences in brain activation in the remitted CD patient group on 2-, 3- and 5-step trials, namely an over-recruitment of a number of brain regions predominately involved with higher cognitive functioning. This increased activation implies that remitted CD patients require increased effort of certain brain regions to successfully complete this visuospatial planning task, although they do not need more time to do so accurately, indicating a subtle scarring due to CD. In the future, longitudinal studies are necessary to provide further insight with regard to the onset and course of alterations in cognition and brain activity patterns in the CD patient population during the active disease state and the transition into remission.

Appendix I. Overview of number of trials per step per group (remitted CD (RCD) and Healthy Controls (HCs) on the Tower of London task.

	RCD (n=21; no. of trials, (%))	HCs (n = 21; no. of trials, (%))	Rounded mean no. of trials per participant remitted CD group (%)	Rounded mean no. of trials per participant HC group (%)
Baseline	1376 (40.6)	1410 (40.5)	66 (40.7%)	67 (40.4%)
1 step	647 (19.1)	669 (19.2)	31 (19.1%)	32 (19.3%)
2 steps	535 (15.8)	539 (15.5)	25 (15.5%)	26 (15.7%)
3 steps	347 (10.2)	358 (10.3)	17 (10.5%)	17 (10.2%)
4 steps	292 (8.6)	303 (8.7)	14 (8.6%)	14 (8.4%)
5 steps	195 (5.7)	200 (5.7)	9 (5.6%)	10 (6.0%)
Total	3392 (100.0)	3479 (100.0)	162 (100%)	166 (100%)

Appendix I I. Effects of paired testing per trial step at threshold 1.9 for remitted CD (RCD) and Healthy Controls (HCs).

Paired comparison	Area	Side	Cluster size	Peak Voxel (MNI) x (mm)	y (mm)	z (mm)	*p**
1 step							
RCD>HC	Cingulate Gyrus, posterior division	R/L	1361	6	-48	20	0.005
RCD<HC	None						
2 steps							
RCD>HC	Supramarginal gyrus, posterior division/ parietal operculum cortex	R	2242	60	-36	26	6.53e-05
RCD<HC	None						
3 steps							
RCD>HC	Supramarginal Gyrus (posterior division)/	R	1105	58	-42	20	0.02
RCD>HC	Intracalcarine Cortex	L	1008	-16	-70	4	0.03
RCD<HC	None						
4 steps							
RCD>HC	Precuneous Cortex	R	965	0	-44	56	0.03
RCD<HC	None						
5 steps							
RCD>HC	Lingual Gyrus/Occipital Fusiform Gyrus	R	5398	-2	-36	-10	1.01e-09
RCD>HC	Supramarginal Gyrus, posterior division	R	1692	58	-38	28	<0.001
RCD>HC	Precentral Gyrus	L	1289	-58	4	36	0.007
RCD>HC	Precuneous Cortex	R	1265	2	-44	66	0.008
RCD<HC	None						

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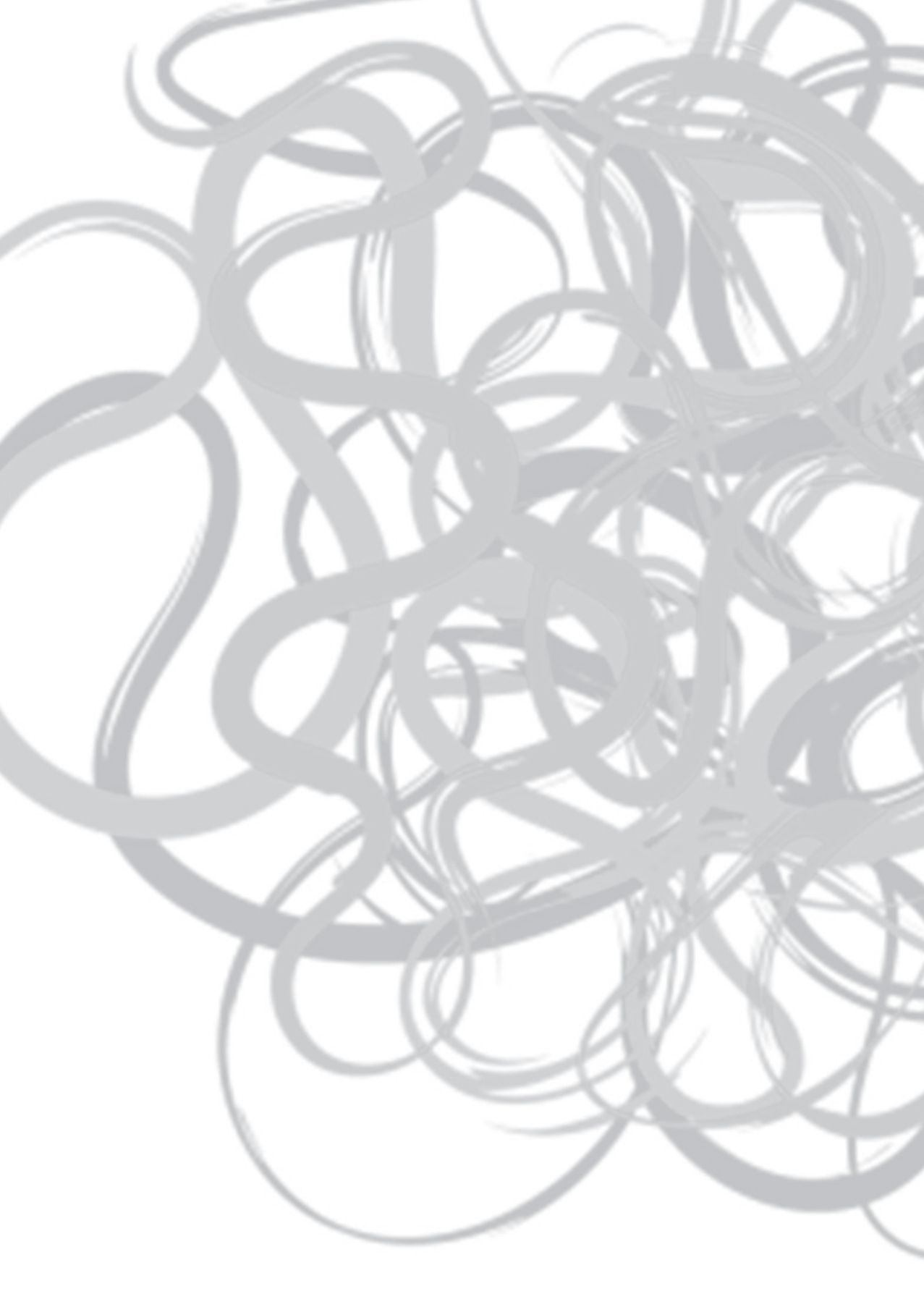

Chapter 9

General discussion

The aim of this thesis was to further unravel the role of stress systems in the pathophysiology of stress-related psychiatric disorders by exploring elements of regulation and dysregulation of the two major stress systems (i.e. the ANS and the HPA-axis), and their relation with psychological and psychiatric symptoms.

Two main hypotheses were posited at the beginning of this thesis. The first was that sAA, unlike salivary cortisol, can differentiate between certain stress-related disorders. The second was that exposure to high levels of endogenous cortisol over a long period of time, as is the case in Cushing's disease, will result in persisting abnormalities of certain brain regions and circuits. In the following sections the results from our studies are discussed and integrated alongside contemporary study findings where possible.

Section I: General implications with regard to sAA

In **Chapter 2** we explored elements of regulation and dysregulation of the two major stress systems (i.e. the ANS and the HPA-axis), and their relation with psychological and psychiatric symptoms by investigating diurnal salivary alpha amylase (sAA) and salivary cortisol levels in patients with mood-, anxiety-, and symptom somatic (MAS)-disorders and healthy controls (HCs). Although an ample amount of research has been conducted with regard to diurnal salivary cortisol (sC), few naturalistic studies have investigated diurnal sAA levels in patients with MAS-disorders to date. Our hypotheses were that MDD patients would show the same diurnal sAA pattern as HCs, however, we expected the morning sAA levels to be higher in the MDD patient group compared to both the HC and other MAS-disorders groups. Furthermore, we expected to find an elevation of sAA in the AUCg in both the patient groups in comparison to HCs. Seven saliva samples were collected over the course of 24 hours in a naturalistic setting (i.e. at awakening on day 1, 30 minutes after awakening, 45 minutes after awakening, 1 hour after awakening, at 10:00 p.m., 11:00 p.m., and at awakening at day 2). As expected and in line with previous research, sC was able to differentiate between stress-related psychiatric disorders on the one hand, and HCs on the other hand, but not between the stress-related disorders. Our main results with regard to sAA showed that sAA levels at awakening in the MDD group were higher than those in the other MAS-disorder patient group and in the HC group on both day 1 and day 2, a novel finding and in line with one of our hypotheses. In contrast to our second hypothesis, we did not find an elevated AUCg in the MDD and other MAS-disorders patient groups compared to the HCs. Furthermore, we found that the mean sAA levels at awakening to be positively associated with the brief symptom inventory (BSI) depression subscale scores, indicating that sAA levels are higher in those with more severe depression. Whereas two previous studies with depressed patients and HCs have found evidence that sAA is able to differentiate between these two groups at awakening and in the morning, respectively[1,2], our findings provide the first scientific evidence for the differentiating quality of naturalistic sAA levels at awakening in patients with MDD in comparison to both HCs as well as patients with other MAS-disorders.

In light of the findings in Chapter 2, we further investigated the possible role of sympathetic nervous system (SNS) activity in stress-related disorders in **Chapter 3**. In line with the goals of the DSM-5 and National Institute of Mental Health Research Domain Criteria (RDoC), which aims at identifying new ways of classifying psychiatric disorders based on dimensions of neurobiological measures and observable behaviour, a transdiagnostic dimensional approach was applied. We examined the

relationship between sAA and social withdrawal (SW), which has been posited to be a more stable endophenotype that is more closely connected to biological pathways than psychiatric disorders are[3,4]. Previously, evidence has been found supporting the likelihood of a temporal relationship starting with SW, leading to subsequent depression. Furthermore, increased SW has been found to be a mediating variable in the relationship between salivary cortisol and depression. We were interested in exploring possible associations between SNS activity and SW, and hypothesized that higher sAA levels would be positively associated with higher levels of SW. We included sAA en sC samples from patients with MAS-disorders HCs, and ordered them along the dimension of three SW subscales (i.e. the BSI social withdrawal subscale[5], the SF-36 social withdrawal subscale[6], and the DAPP-SF social avoidance subscale[7]). We did not find any associations between sAA and the SW subscales, however several associations were found between the BSI social withdrawal subscale and sC.
Furthermore, increased SW has been found to be a mediating variable in the relationship between sC and depression. We were therefore interested to explore whether SW was a mediating variable in the relationship between sAA and depression, however although we found further evidence for the aforementioned relationship between sC and depression, we did not find any evidence for this relationship with regard to sAA. Therefore, our findings do not support our hypothesis that SNS activation is involved in SW, though it may also be the case that the measurement instruments that were used in this study were not sensitive enough to measure the complex SW construct. On the other hand, we were able to replicate earlier associations between salivary cortisol and SW, although the effect sizes were small. In the future, better validated measurement instruments of SW (such as the World Health Organization Disability Assessment Schedule 2.0 social withdrawal scale[8]), should be employed to further explore these possible associations. Moreover, future studies should also attempt to differentiate between state and trait characteristics of SW[9].

Taken together, our studies in **Chapters 2 and 3** are innovative in several ways. First, they further assessed diurnal sAA in more detail than previous research conducted to date. Second, we found that sAA levels, specifically those at awakening, have the potential of differentiating between MDD patients and patients with other MAS-disorders, a novel finding. Third, we found that it is unlikely for SNS activation to be involved in SW. However, further research is necessary to determine if, and if so, to what extent, our current findings can be replicated. As the studies conducted in this thesis were of a cross-sectional nature, no conclusions can be made regarding

the temporal directions and causal pathways. Furthermore, these studies made use of a number of saliva samples over the course of the day, whereas collecting multiple samples over the course of the day for a longer period of time will likely yield more significant insights into its role in the disease course. This would aid in new insights as to whether sAA could be seen as a risk marker (i.e. neurological or biological traits indicating a predisposition towards developing a disease, but not part of the causal chain), or risk factor (implicating elevated sAA levels as a causal factor in the development of a stress-related disorder), the latter of which is unlikely. Importantly, our studies were again able to highlight the advantages of measuring ANS and HPA-axis activation in saliva. Specifically, saliva sampling is a relatively inexpensive, non-invasive (thus painless) sampling method that can be performed in one's natural habitat and under normal conditions, which makes it possible to collect multiple saliva samples in a stress-free environment. In sum, our findings add to the increasing body of evidence indicating that the (inter-)relations between ANS activation and stress-related disorders warrants further exploration.

Section II: General implications with regard to Cushing's disease

The second aim of this thesis was to further explore the possibly persistent brain abnormalities in patients with long-term remitted Cushing's disease likely due to the long-term exposure to cortisol. This is important for several reasons. The first is the clinical importance to this patient population itself. As mentioned previously, patients that are in remission of Cushing's disease often experience persistent deficits within certain cognitive and psychiatric domains[10-12], even after several years of biochemical curation (i.e. cortisol levels that lie within the normal range). This phenomenon clearly warrants further investigation. Second, study findings are of importance for other patients suffering from autoimmune diseases who are prescribed immunosuppressive drugs (i.e., glucocorticoids). Excessive use of glucocorticoids can induce Cushing's syndrome, a syndrome that is similar to Cushing's disease, but where the cause is of an exogenous origin, instead of endogenous (i.e. an adenoma on the pituitary gland or an adrenal gland tumor). Remitted Cushing's syndrome patients have also reported to experience similar and persistent deficits in the same domains as those reported by patients with remitted Cushing's disease. Further research investigating the possible long-term side-effects of dosages regarding augmented glucocorticoid therapy on the brain and its functioning could yield additional important insights. Finally, earlier research has posited that Cushing's disease might be a suitable naturalistic model to explore the (possibly lasting) effects of endogenous cortisol overexposure on the brain[86]. Patients with other psychiatric stress-related disorders also experience elevations in

cortisol levels over longer periods of time, although to a lesser extent. By comparing brain abnormalities found in patients with remitted Cushing's disease with brain abnormalities found in patients with other (remitted) stress-related psychiatric disorders we can further explore the validity of this proposed model.

In **Chapter 4**, we built further upon structural and functional abnormalities found to be associated with Cushing's syndrome as reported in the elaborate review by Andela and colleagues[13]. Specifically, we evaluated the newest findings with regard to the gray- and white- matter structural abnormalities that have been found in patients with active Cushing's disease and Cushing's syndrome, and also the extent of reversibility of these abnormalities. With regard to the gray matter structural abnormalities, we found volume reductions of the hippocampus and a prefrontal region involving the medial frontal gyrus (MFG) and the anterior cingulate cortex (ACC). Regarding reversibility of these abnormalities, hippocampal volume was found to be partially reversible, whereas the alterations in the MFG and ACC seem to be more persistent. (Regions of) the ACC have been found to be critically involved in cognitive control, cognitive processing of anxiety and fear, emotional functioning, and reward-based decision making[e.g.14]. Therefore, it seems likely that damage to this region may lead to reductions in motivation, spontaneity, problem-solving capacity, and increased apathy, domains that have often also been found to be impaired in patients with stress-related disorders. This may explain part of the cognitive and psychiatric symptoms commonly observed both in active and remitted CD patients.

In this review, two further important aspects were emphasized. The first is that the alterations in the ACC seem to be a persistent effect of exposure to hypercorticolism, indicating that this may be part of the pathophysiological pathway that keeps the persisting reported symptomology intact. The second is that there is a lack of well-designed studies that use advanced neuroimaging methods and analysis techniques, as well as a lack of studies that investigate the possible underlying microbiological processes of Cushing's disease. We highlighted that MRI studies alone can offer only part of the insights necessary, but that by converging MRI data with other data modalities our knowledge regarding these processes could be extended. Specifically, we posited that by combining data from the Allen Human Brain Atlas, a multi-modal atlas mapping gene expression across the healthy human brain[15], further insights into these mechanisms could be attained. For example, possible genes that interact with hypercorticolism, and thus may influence the structural changes that have been identified in the brain, may be uncovered.

In light of the abovementioned lack of well-designed studies using advanced

neuroimaging methods and analysis techniques, we further investigated the ACC by examining the cortical thickness and cortical surface area of remitted Cushing's disease patients and their matched HCs in **Chapter 5**. Specifically, we investigated cortical thickness and cortical surface area separately in long-term remitted CD patients, and age-, gender-, and education matched HCs. In line with the Andela et al.[13] results, we identified the ACC as region of interest (ROI), followed by an exploratory whole-brain analysis. In line with findings from a previous meta-analysis and a systematic review with MDD patients[16,17], we hypothesized that long-term remitted CD patients would have thinner ACC cortices in comparison to HCs. Our three most important findings were (i) that remitted Cushing's disease patients had reduced cortical thickness of numerous cortical areas, amongst which the left caudal ACC, (ii) that no differences were found in cortical surface area, and (iii) that the cortical thickness of the left caudal ACC was inversely associated with and disease duration. Taken together, these findings again highlight the importance of further exploring the ACC, and also offer evidence that the length of exposure to cortisol leads to cortical thinning of this brain area. Importantly, these results highlight the added value of examining cortical thickness and cortical surface area separately, instead of as a whole. Finally, it is also of importance to branch beyond the use of single data modalities such as MRI to study this disease, as insights into, for example, the possible underlying microbiological processes that lead to these seemingly persistent alterations in this, and perhaps other stress-related disorder patient populations cannot be identified using MRI data alone.

In **Chapter 6**, we explored the ACC from a resilience perspective in an attempt to identify neural correlates of resilience against traumatic experiences. As police officers are often first-responders, they are more likely to experience traumatic events in comparison to other occupational groups[18]. However, no evidence has been reported that police officers experience higher rates of psychopathology in comparison to occupations that are deemed as less high-risk. Police officers thus seem particularly resilient.

In order to explore the biological underpinnings of this phenomenon, we included a sample of Dutch police officers and police academy recruits, and categorized them by means of a three-group design (i.e. trauma and psychopathology, trauma and no psychopathology, and recruits with neither trauma nor psychopathology). Previous studies have found smaller hippocampal size to be associated with stress-related disorders[e.g.19-21], active Cushing's disease, and Cushing's syndrome[22,23] on the one hand, and increased hippocampal size to be related to resilience[24,25] on the other

hand. It has therefore been posited that increased hippocampal size may be a biomarker of resilience. However, a voxel-based morphometry analysis in this same population did not find any differences in the hippocampus between the three groups included in this study (i.e. vulnerable', 'resilient', and 'controls')[26]. In addition to the studies conducted in patients with stress-related disorders that have found the ACC to be involved in higher cognitive processes and emotion regulation, studies have also found associations between the ACC and psychopathology[27,28]. Based on these findings and those in a more recent systematic review, we hypothesized that the ACC may be associated with resilience in this patient population in a similar way, as this area has been found to be thicker in many resilient populations[29]. We investigated cortical thickness and surface area separately in this patient population using the ACC as region of interest (ROI), followed by a whole-brain analysis. In contrast to our hypotheses, we did not find any differences in cortical thickness or cortical surface area between the resilient group and the other two groups in both the ROI or in the whole brain analyses. It was a surprising finding that the vulnerable group did not show any brain alterations in comparison to the resilient group specifically, as we would at least expect the ACC in the vulnerable patient population to be altered, in line with the previous findings in the remitted Cushing's disease population. However, perhaps the cortisol exposure and disease duration in the vulnerable patient population were less extensive than in the remitted Cushing's disease patient group, as even the vulnerable group included in this study were previously preselected on certain resilience-specific criteria in order to be admitted to the police academy. These findings suggest that either there are no resilience specific correlates regarding cortical thickness and cortical surface area, or that the sample included in this study showed insufficient variability on reliance to test this hypothesis.

To briefly recap, previous research has identified several domains which seem to remain impaired in patients with (long-term) remitted Cushing's disease. Amongst others, persisting deficits have been found in emotional and executive functioning[12,30-33], psychopathological morbidity[30-32], as well as a reduced quality of life[34]. An important skill necessary to function in daily life is the cognitive skill of planning. In **Chapter 7**, we therefore explored whether cognitive planning and executive functioning differs between the same remitted Cushing's disease patient population and their matched HCs as those in Chapter 6. Earlier studies examining the cognitive and executive functioning of patients with remitted CD using standard neuropsychological tests have found seemingly lasting impairments within both of these domains. We further explored these deficits using the fMRI Tower of London

(ToL) task, an often-used task to measure possible neurobiological alterations in brain activity patterns regarding cognitive planning and executive functioning[22]. We hypothesized that cognitive planning in the remitted Cushing's disease patient group would be impaired, and that this patient population would display increased activation in the ACC compared to their age-, gender-, and education matched HCs. We asked the participants to complete the ToL task in a 3T-MRI scanner. No differences were found in cognitive planning or in ACC activation compared to the matched HCs. However, the exploratory whole-brain analysis identified the over-recruitment of several brain regions associated with higher cognitive processes on most trial steps in comparison to HCs. This highlights the importance of conducting an exploratory whole-brain analysis alongside the apriori defined hypothesis driven ROI analysis. In sum, although we found differences in brain activation, we did not find any evidence for pervasive cognitive impairments in remitted Cushing's patients regarding visuospatial planning and executive functioning as measured on the ToL task, demonstrating brain flexibility upon recovery of Cushing's disease to a certain extent. Our findings suggest that the over-recruitment of a number of brain regions is necessary for remitted Cushing's patients to successfully complete the ToL task at the same level that matched HCs do. Taken together, although several studies using different measurement instruments than the ToL have identified impairments within the domain of visuospatial planning in similar patient populations [(e.g.35)], our findings suggest that certain visuospatial impairments may improve in remitted Cushing's disease patients.

Finally, as mentioned previously in Chapter 4, MRI studies alone cannot offer enough insight into the underlying biological processes that may lead to the observed alterations regarding atrophy and white matter integrity in the ACC of remitted Cushing's disease patients. In **Chapter 8** we therefore combined our high-resolution MRI scans with Allen Human Brain Atlas (AHBA) whole genome mRNA expression data[15] to further explore the possible microbiological processes underlying CD. As this was an exploratory analysis, we did not specify any hypotheses a priori. First, our differential gene expression analysis found that the majority of the differentially expressed genes identified were immune signaling genes, and that of these genes, the underexpressed genes were often enriched for functionalities largely involving immune-signaling. Interestingly, the top ten most underexpressed genes found in our study have all been previously associated with Alzheimer's disease in a variety of different ways[37-45]. Our most important finding was the underrepresentation of deactivated microglia and oligodendrocytes in the ACC, which may explain the persevering alterations found in the ACCs of remitted Cushing's disease patients,

as well as the (possibly related) cognitive impairments. Specifically, deactivated microglia have been found to release a number of anti-inflammatory cytokines, participate in neuroprotection[46], matrix deposition, and tissue remodeling[47], whereas oligodendrocytes are largely responsible for the remyelination process[48-49], and damage to oligodendrocytes has been found to lead to mental or physical disability[50]. In support of the found underrepresentation of oligodendrocytes, previous research has found prolonged exposure to corticosteroids to be associated with the inhibition of oligodendrocyte precursor proliferation throughout the white brain matter[85], and widespread reductions of white matter integrity throughout the brain of remitted Cushing's disease patients[86]. Although we did not study the ACCs of remitted Cushing's disease patients directly in this study, our findings denote differences in basal gene expression, and indicate which genes are less likely to be part of this initial vulnerability.

As mentioned previously, earlier research has postulated that Cushing's disease is a suitable naturalistic model to explore the (possibly lasting) effects of endogenous cortisol overexposure on the brain. Although subtler, patients with stress-related disorders also experience elevations in cortisol levels over longer periods of time. Additionally, there is an overlap in the psychiatric symptomatology that both (remitted) Cushing's disease patients and patients with other psychiatric stress-related disorders present. In order to further explore this model, we assessed whether we could identify any overlap in brain abnormalities between those found in our remitted Cushing's disease patient population and those found in patients with other stress-related psychiatric disorders.

To date, ample previous research has highlighted associations between structural brain abnormalities in patients with other stress-related disorders[e.g.51-61], and to a lesser extent in remitted patients with other stress-related disorders[e.g.55-57,61]. Longitudinal studies in these patient populations are scarce[e.g.61]. Although overlap was found between certain brain areas (e.g. areas that have been implicated in executive and emotional functioning) in the current stress-disorder patient population[e.g.51-60], the alterations identified in the remitted Cushing's patients were much more widespread. Less overlap was found between the remitted stress-disorder patients61 and the remitted Cushing's disease patients. As the levels of endogenous cortisol are much higher in patients with Cushing's disease compared to patients with other stress-related disorders, it seems that this excessive exposure leads to more persistent and widespread brain alterations upon remission than is the case with other stress-related psychiatric disorders. However, it is important to

denote that the patient populations included in the aforementioned studies were patients that generally did not report high symptom severity levels.
With regard to fMRI studies, we identified a previous study that investigated the neural correlates of the ToL task with the same paradigm as used in our study in out-patients with depression and anxiety[36]. They found that patients with a current moderate or severe depression had increased dorsolateral prefrontal cortex activation as a function of increasing task load, whereas patients with current mild or remitted depression, current anxiety disorder(s) (such as generalized anxiety disorder and/or panic disorder and/or social anxiety disorder) did not, in comparison to HCs. Thus, in line with the structural study findings, it seems that prolonged exposure of endogenous cortisol on the brain in the vast amounts as is the case with Cushing's disease leads to more widespread alterations that seem to persist after disease remission, as seen in the over-recruitment of certain brain regions in the remitted Cushing's patient population on the ToL task.

In sum, by studying brain characteristics in remitted Cushing's disease patients in comparison to HCs, we further explored possible pathophysiological pathways through which the long-term exposure to excessive cortisol leads to possibly lasting alterations in brain structures and brain activation patterns.

Our research offers support for the idea that long-term overexposure to high levels of cortisol, as is the case in Cushing's disease, has lasting effects on the brain. It remains unclear however, what level and duration of cortisol exposure may lead to these seemingly permanent detrimental effects as seen in remitted Cushing's disease patients.

The following section will highlight possible future directions in order to further our research.

Directions for future research
The results presented in this thesis offer support for the hypotheses (i) that sAA is a biomarker that can differentiate between certain stress-related disorders, and (ii) that exposure to high levels of endogenous cortisol over a long period of time, as is the case in Cushing's disease, will result in persisting brain abnormalities of certain brain regions. However, with regard to both hypotheses, further research is necessary.

With regard to the first hypothesis, future research should (i) replicate and refine our current findings, and (ii) use larger data collections and more advanced

sampling methods. MDD is an extremely complex and heterogeneous disorder. Stress-related disorders are caused by many pathological mechanisms that interact with each other in complex manners. Currently, we have an imperfect psychiatric classification system, and no invasive diagnostic methods. For example, it has been reported that in the DSM-IV and DSM 5 there are 227 possible ways to reach a diagnosis of MDD. However, symptom criteria are usually met in 170 different ways (i.e. one-quarter of the possible criteria combinations generally do not occur), and nine combinations have been found to account for more than 40% of the diagnoses[62]. If the qualitative differences for six of the compounds are considered (for example, differentiating hypersomnia from insomnia), this can even lead to 10,377 unique symptom profiles[63], of which 1030 unique profiles were identified in depressed patients that partook in the STAR*D study[64]. In light of this, it is important that future studies further disentangle whether the sAA levels at awakening are associated with specific symptoms or symptom profiles of MDD. This knowledge is necessary in determining whether our findings have any predictive, diagnostic, or treatment value for individual patients. Future analyses could begin, for example, by determining whether sAA elevations at awakening are associated with certain depression symptom profiles or individual symptoms in large datasets. Also, studies should also compare remitted from current MDD patients and use prospective designs to help determine whether sAA elevations are a state or rather a trait characteristic. Studies should also include participants at risk for developing a depressive disorder in order to determine whether the elevated sAA levels at awakening are present prior to disease onset or represent a 'scar'-effect, if these elevated levels should persist in remitted MDD patients.

Furthermore, future research should implement more advanced data collection and sampling methods. Since we began collecting our saliva samples in 2007, there have been several advancements in the methods used to collect these samples, further increasing sample validity and quality. For example, wearable and flexible sensors are non-invasive, safe, easy and pain-free, allowing for high quality salvia analysis[65]. These sensors offer valuable detailed insights due to their potential to provide continuous, real-time physiological information. If this is not feasible, it is preferable to use the passive drooling method, as this method has been found to minimalize potential sources of error[66], which have been associated with cotton salivettes in previous studies. Furthermore, saliva flow rate can influence salivary measures and should therefore also be considered[67], as well as mouth position during saliva collection[68]. Besides cortisol and sAA, there are many other components of saliva that may be of value to psychiatry research. Studies focusing on immunoglobulin A

(sIgA), lysozyme, melatonin, chromogranin A (CgA), and fibroblast growth factor 2 (FGF-2) have all been found to promising markers of stress, anxiety, or depression to a certain extent[69], and could also provide valuable insights.

In future studies data collection methods could also be elaborated on. Over the course of the last decade, research based on "big data" has increased exponentially. Indeed, big data can be instrumental in uncovering robust patterns in psychological and psychiatric states[70] using, for example, machine learning approaches. However, size alone does not necessarily lead to better data[71], as there are several issues that can influence the quality of data collected (e.g. issues during data collection, issues while processing data, biases, as mentioned previously)[72]. Also, fully personalized treatments or precision medicine cannot be achieved with this type of data.

The findings presented in this thesis were group level findings, and are therefore not directly implementable for individual patients in clinical settings. "Small data" or high-intensive data collection within an individual, coined as small as it concerns data from a single unit (for example, one person, one hospital, one clinic etc.), complements the big data approach, and offers an elegant solution to investigate on an individual level. By collecting vast amounts of detailed time series data, small data aims at matching the right intervention at the right time to a specific unit by taking all unit specific characteristics into account. Small data refers to the rigorous use of specific N-of-1 data in order to achieve detailed individual-level descriptions, predictions, and, eventually control for that one unit[73]. N-of-1 data can have very large datasets in terms of length of time series data (e.g. years), and data types (e.g. genomics, microbiomics, and metabolomics). In single patients, sequential saliva samples are more feasible that sequential blood withdrawals. Also, small data collection targets helping individuals, not transportable knowledge first, and thus has can lead to more rapid clinical consequences for an individual, and is therefore exceptionally valuable.

Finally, studying the genetic underpinnings of sAA in patients with MDD can be an alternative way to learn more about the genetics of this complex psychiatric disorder. Ultimately, this could lead to a better understanding of MDD and possibly increase its applicability as a biomarker, as it would enable us to take account of the genetic determinant of sAA in epidemiological studies. As twin studies have estimated the heritability of MDD at 30 to 40% and sAA levels have been found to be influenced by ancestry, heritability and genetics continue to be a particular field of interest[74-76]. Genome wide association studies (GWAS) can lead to a better

understanding of the genetic architecture of complex traits and can consequently aid in detecting associations between common DNA variants and human disease and disorders[77]. For example, a meta-analysis of GWAS on MDD resulted in the finding of 44 independent and significant genetic loci, supported by multiple single-nucleotide polymorphisms (SNPs)[78]. Furthermore, a recent genome-wide meta-analysis was able to identify 102 independent genetic variants associated with depression, including genes and gene pathways related to synaptic structure and neurotransmission[79]. However, this has not yet resulted in the finding of genetic variants that determine differential sAA levels. Performing a GWAS on sAA and combing this with the already available genome wide association data on depression, could unravel the genetic landscape of sAA and its connection with MDD. Such knowledge could aid studies with Mendelian Randomization designs, which may help to uncover or refute the potential effects of sAA in different diseases[80].

With regard to further Cushing's disease research, the largest current deficit is the lack of longitudinal studies, and the small and heterogenous samples included in the studies to date. Due to the cross-sectional nature of the current study designs, we remain unable to draw conclusions with regard to causality of the seeming pervasive symptomatology seen in patients with remitted Cushing's disease. Longitudinal studies (to the extent that this is possible; i.e. from the point of diagnosis onwards) using a wide array of modalities, would offer valuable insights. Besides sequential routine outcome monitoring (ROM) and salivary biomarker assessments, (f)MRI could be used to evaluate brain volume and structures involved in cognitive function, integrity of white matter, structural connectivity, as well as functional connectivity over time. This would also offer further insights regarding the neurobiological processes supporting the correction of certain brain regions upon remission of hypercorticolism. Future studies could also benefit from larger data sets. This is, of course, difficult as the estimated prevalence of endogenous Cushing's syndrome has been estimated to be 1 in 26.000, of which Cushing's disease has been found to represent more than two-thirds of all cases[81]. A possible solution for this could be to establish a rare disease working group in which data is pooled, like the working groups initiated by the Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) consortium. As individual Cushing's disease trajectories show large amounts of variation, more robust datasets may aid in understanding certain frequently occurring phenomena in this patient population (e.g. why certain patients require cortisol supplementation after surgery or why certain patients redevelop a pituitary tumor).

Patients with Cushing's disease have also been found to have a globally diminished cerebral metabolism in comparison to controls[82]. Studying glucose utilization by means of positron emission tomography (PET) using the radiotracer F18-fluorodeoxyglucose (which enables the assessment of cerebral glucose metabolism) may offer insights into the psychiatric and cognitive problems often observed in this patient population. Currently, a study is investigating the possible prevention of neuropsychiatric adverse perioperative effects often caused by dexamethasone (a potent glucocorticoid activator that suppresses cortisol production, depleting its mineralocorticoid (MR) binding) in patients with brain tumors by reinstating MR activity by cotreating with hydrocortisone[83]. Based on previous study conducted in childhood leukemia patients the results of this cortisol refill approach seemed to be beneficial in reducing the occurrence of serious neuropsychological adverse effects[84]. The findings from this study will likely be hypothesis-generating, highlighting new paths towards a more advanced treatment of Cushing's disease.

Furthermore, as previously mentioned, the point when the effects of overexposure to endogenous cortisol become seemingly lasting, remains unclear. Research conducted to date in patients with other stress-related disorders have often included patients low to moderate symptom severity, possibly painting a skewed picture. Perhaps patients with more extreme forms of stress-related psychiatric symptomatology and higher cortisol levels over a longer period of time experience similar brain alterations as the (remitted) Cushing's disease population. It would therefore be of interest to transdiagnostically explore whether a certain 'tipping point', or point where brain alterations become seemingly pervasive, can be identified in patient populations suffering from more severe stress-related symptomatology. In support of this notion, one study with (remitted) MDD patients with more persistent forms of MDD (e.g. longer disease duration, repeated relapses or multiple episodes) to be associated with a greater impact on regional brain volumes[61]. A second study found higher severity of depression symptoms in MDD patients to be associated with thinner rostral anterior cingulate cortices[62]. In order to explore this, brain structure and brain function should be investigated in (remitted) MAS-disorder patients that experience(d) moderate to severe stress-related symptoms, longer duration of symptoms, multiple relapses or multiple episodes. Important in this is that the same functional tasks that have been investigated in the (remitted) Cushing's disease population are used in order to aid generalizability of the findings. In this, it may also be of interest to further explore the role of the autonomic nervous system (ANS) in these patient populations, using sAA levels as a marker for ANS activation as sAA can easily be derived from the same saliva sample as that in which salivary cortisol is localized.

Finally, in order to validate the findings from Chapter 8, further (experimental) studies using post-mortem tissue of remitted Cushing's disease or Cushing's mouse models are necessary. These findings may eventually aid in developing novel treatments for certain stress-related disorders of the brain, and specifically for Cushing's disease patients.

General conclusion

At the beginning of this thesis, two research aims were formulated. The first aim was to disentangle the role of stress systems and their possible involvement in the etiology and pathophysiology of certain stress-related psychiatric disorders (i.e. mood-, anxiety- and somatic symptom (MAS)-disorders). This was done by exploring elements of regulation and dysregulation of the two major stress systems (i.e. the ANS and the HPA-axis). In order to measure this, we used salivary alpha amylase and salivary cortisol as markers for sympathetic nervous system (SNS) - and HPA-axis -activation, respectively. Our hypothesis was that sAA, unlike salivary cortisol, would be able to differentiate between certain MAS-disorders. Our research supports this hypothesis as we found that sAA levels at awakening were able to differentiate between MDD and other MAS-disorders.

The second aim of this thesis was to investigate the possibly lasting detrimental effects of long-term exposure to excessive levels of endogenous cortisol on the brain, as is the case with Cushing's disease patients. Our results indicate that certain brain areas are affected by hypercortisolemia and do seem to recover upon disease remission, however, certain cortical areas involved in higher cognitive functioning seem to remain permanently altered.

In sum, our findings yield further insights into the etiology, pathophysiology, and neurobiology of stress-related psychiatric disorders, with which we aim to ultimately aid in the identification or refinement of early detection tools, more advanced treatments, and more successful prevention strategies.

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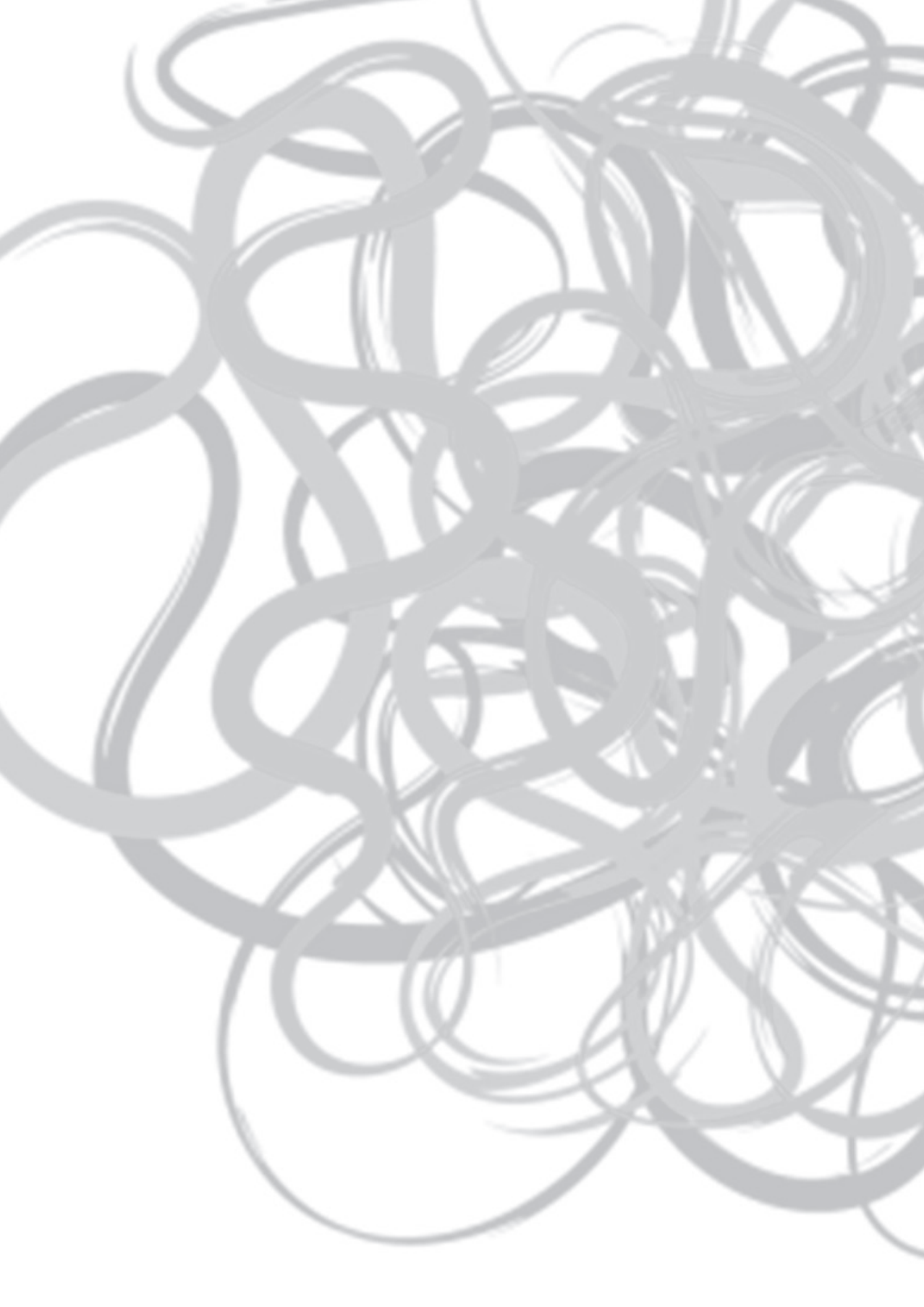

Appendix

Nederlandse samenvatting
Curriculum vitae
List of publications
Dankwoord

Nederlandse samenvatting

Stresssystemen zijn essentieel om je aan te kunnen passen aan een veranderende omgeving. Onze stressrespons stelt ons in staat om zo snel en efficiënt mogelijk te reageren op een stressor en om ons lichaam zo snel mogelijk weer terug te brengen naar een evenwichtige toestand. Maar er is ook een keerzijde aan activatie van het stresssysteem. Eerder onderzoek heeft uitgewezen dat overmatige of langdurige blootstelling aan stress het risico op een breed scala aan onder andere psychiatrische symptomen verhoogt. Er is echter nog meer onderzoek nodig naar hoe blootstelling aan stress leidt tot psychiatrische symptomen en of deze effecten blijvend zijn of zich ook kunnen herstellen.

Het doel van dit proefschrift is om de rol van stresssystemen in de pathofysiologie van stress-gerelateerde psychiatrische stoornissen verder te ontrafelen. Dit is gedaan door elementen van regulatie en ontregeling van de twee belangrijkste stresssystemen, namelijk het autonome zenuwstelstel (ANS) en de hypothalamus-hypofyse-bijnier (HPA)-as), en de relatie tussen deze stresssystemen en psychiatrische symptomen te onderzoeken.

Aan het begin van dit proefschrift zijn twee hoofdhypothesen gesteld. Ten eerste dat het enzym alfa-amylase (een marker van ANS-activatie), in tegenstelling tot het stresshormoon cortisol (een marker van HPA-as activatie), onderscheid kan maken tussen depressie en andere stress-gerelateerde psychiatrische stoornissen. Ten tweede dat blootstelling aan hoge niveaus van endogene cortisol gedurende een lange periode, zoals bij de ziekte van Cushing, zal leiden tot aanhoudende afwijkingen van bepaalde hersengebieden relevant voor psychiatrische symptomen. Daarnaast is bekeken of de ziekte van Cushing daarmee een model zou kunnen zijn voor de effecten van langdurige blootstelling aan hoge niveaus cortisol bij stressgerelateerde stoornissen. In de volgende paragrafen worden de resultaten van deze studies besproken.

Deel 1: Algemene implicaties met betrekking tot speeksel alfa amylase

In **hoofdstuk 2** is de relatie tussen psychiatrische symptomen en dagelijkse speeksel alfa-amylase- (sAA) en speekselcortisolspiegels bij patiënten met stemmings-, angst- en somatische symptoom (SAS)-stoornissen en gezonde controles onderzocht. De belangrijkste bevinding was dat sAA-niveaus bij het ontwaken in de depressiegroep gemiddeld hoger lagen dan die in zowel de patiëntengroep met andere SAS-stoornissen als in de gezonde controles. Dit is een nieuwe bevinding. Sterker nog,

de sAA-niveaus bij patiënten met een ernstigere depressie lagen nog hoger dan de sAA-niveaus bij patiënten met een milde of matige depressie. Oftewel, hoe ernstiger de depressie, hoe hoger het sAA-niveau bij het ontwaken. Deze bevinding is het eerste wetenschappelijke bewijs voor de onderscheidende kwaliteit van sAA.

Sociale terugtrekking is een vroeg symptoom van verschillende stressgerelateerde psychiatrische stoornissenbijv.[1,2]. Eerder onderzoek heeft aangetoond dat een toename in sociale terugtrekking kan leiden tot een depressie[3]. Daarnaast heeft eerder onderzoek aangetoond dat verhoogde sociale terugtrekking de relatie tussen speekselcortisol en depressie medieert[4]. In hoofdstuk 3 van dit proefschrift is de relatie tussen speekselcortisol en sAA aan de ene kant, en sociale terugtrekking aan de andere kant bij patiënten met SAS-stoornissen transdiagnostisch onderzocht. Er is geen relatie gevonden tussen sociale terugtrekking en sAA, maar de relatie tussen sociale terugtrekking en speekselcortisol is wel weer gevonden. Daarnaast is gekeken naar het mogelijk mediërend effect van sociale terugtrekking in de relatie tussen speekselcortisol en sAA aan de ene kant en depressie aan de andere kant. Er is, in lijn met eerder onderzoek, een mediërend effect van sociale terugtrekking tussen avond speekselcortisol en depressie gevonden, maar geen mediërend effect van sociale terugtrekking op de relatie tussen sAA en depressie. Deze bevindingen geven aan dat het minder waarschijnlijk is dat ANS-activering betrokken is bij sociale terugtrekking, hoewel het ook zo kan zijn dat de meetinstrumenten die in dit onderzoek zijn gebruikt niet gevoelig genoeg waren om het complexe concept van sociale terugtrekking te meten. Aan de andere kant zijn de eerder gevonden relaties tussen speekselcortisol en sociale terugtrekking in dit onderzoek wel gevonden met gebruik van deze meetinstrumenten, alhoewel de effectgroottes erg klein waren.

De onderzoeken in **Hoofdstukken 2 en 3** zijn op verschillende manieren innovatief. Ten eerste zijn de dagelijkse sAA fluctuaties in meer detail onderzocht dan tot op heden is gedaan. Ten tweede is gebleken dat sAA-niveaus, met name die bij het ontwaken, het potentieel hebben om onderscheid te maken tussen depressieve patiënten en patiënten met andere SAS-aandoeningen. Ten derde is het onwaarschijnlijk dat ANS-activering betrokken is bij sociale terugtrekking. Verder onderzoek is echter nodig om deze resultaten te bevestigen. Daarnaast zijn de gedane studies van cross-sectionele aard, waardoor er geen conclusies getrokken kunnen worden met betrekking tot oorzaak en gevolg. Bovendien maken deze studies gebruik van een aantal speekselmonsters in de loop van de dag, terwijl het verzamelen van meerdere monsters gedurende een langere periode meer inzicht in het ziekteverloop zou hebben opgeleverd. De bevindingen benadrukken echter wel de relevantie om de

(inter-) relaties tussen ANS-activering en stressgerelateerde stoornissen verder te onderzoeken. Ook is van belang dat deze studies opnieuw de voordelen van het meten van ANS- en HPA-as-activering in speeksel laten zien. Speekselafname is namelijk een simpele, relatief goedkope en niet-invasieve methode die ook thuis kan worden uitgevoerd en onder normale dagelijkse omstandigheden. Om deze redenen is het mogelijk om meerdere speekselmonsters te verzamelen buiten een laboratorium.

Deel II: Algemene implicaties met betrekking tot de ziekte van Cushing
De ziekte van Cushing wordt veroorzaakt door een goedaardige tumor op de hypofyse (een kleine klier gelegen onderaan de hersenen), waardoor het lichaam grote hoeveelheden van het stresshormoon cortisol aanmaakt. Het tweede doel van dit proefschrift is om de mogelijk chronische hersenafwijkingen bij langdurig herstelde patiënten met de ziekte van Cushing verder te onderzoeken. Dit is om verschillende redenen belangrijk. Ten eerste vanwege het klinische belang voor deze patiëntenpopulatie zelf. Herstelde Cushing's patiënten ervaren vaak aanhoudende problemen binnen bepaalde cognitieve en psychiatrische domeinen[3-5], zelfs jaren na biochemische remissie (d.w.z. cortisolspiegels die binnen het normale bereik liggen). Ten tweede zijn deze onderzoeksbevindingen van belang voor andere patiënten die lijden aan auto-immuunziekten en die immunosuppressiva krijgen voorgeschreven (zoals glucocorticoïden). Overmatig gebruik van glucocorticoïden kan het syndroom van Cushing veroorzaken, een syndroom dat lijkt op de ziekte van Cushing, maar waarvan de oorzaak exogeen (van buiten) in plaats van endogeen (in het lichaam) is. Bij deze patiëntenpopulatie is soortgelijke chronische problematiek gemeld als bij mensen die hersteld zijn van de ziekte van Cushing. Verder onderzoek naar de mogelijke lange termijn bijwerkingen van doseringen van glucocorticoïdtherapie op (het functioneren van) de hersenen zou aanvullende belangrijke inzichten kunnen opleveren. Ten slotte is eerder gesuggereerd dat de ziekte van Cushing een geschikt naturalistisch model zou kunnen zijn om de (mogelijk blijvende) effecten van endogene blootstelling aan toegenomen cortisol op de hersenen te onderzoeken6. Patiënten met psychiatrische stressgerelateerde stoornissen hebben ook verhogingen van de cortisolspiegels gedurende langere tijd, maar in mindere mate. Door hersenafwijkingen gevonden bij herstelde patiënten met de ziekte van Cushing te vergelijken met hersenafwijkingen gevonden bij patiënten met (herstelde) stressgerelateerde psychiatrische stoornissen, kunnen we de bruikbaarheid van dit voorgestelde model verder onderzoeken.

In **Hoofdstuk 4** zijn de nieuwste bevindingen over de structurele grijze en witte

stof afwijkingen in de hersenen bij patiënten met actieve Cushing en het syndroom van Cushing onderzocht middels een systematische review. Daarnaast is er ook gekeken naar de mate van reversibiliteit van deze veranderingen. Met betrekking tot de structurele afwijkingen van de grijze stof, is een volumevermindering van de hippocampus en een prefrontale regio, waaronder de anterieure cingulate cortex (ACC), gevonden. Wat betreft de reversibiliteit van deze afwijkingen bleek dat het hippocampusvolume gedeeltelijk reversibel was, terwijl de veranderingen in de ACC persisterend leken te zijn. De ACC is betrokken bij cognitieve controle, cognitieve verwerking van angst, emotioneel functioneren en op beloning gedreven besluitvorming[e.g.7]. Schade aan deze regio kan leiden tot vermindering van motivatie, spontaniteit, probleemoplossend vermogen, en tot een verhoogde apathie. Deze domeinen zijn ook vaak afwijkend bij patiënten met stressgerelateerde psychiatrische stoornissen. Deze afwijkingen zouden een deel van de cognitieve en psychiatrische symptomen die vaak worden gezien bij zowel actieve als herstelde patiënten met de ziekte van Cushing kunnen verklaren.
In de review kwamen nog twee belangrijke aspecten naar voren. Het eerste aspect is dat de veranderingen in de ACC een blijvend gevolg lijken te zijn van langdurige blootstelling aan hypercorticolisme. Het tweede aspect is dat er een gebrek is aan goed opgezette studies die gebruik maken van geavanceerde neuroimaging-methoden en analysetechnieken, en ook een gebrek aan studies die de mogelijke onderliggende microbiologische processen van de ziekte van Cushing onderzoeken. Dit zou onderzocht kunnen worden door MRI-data te combineren met bijvoorbeeld data van de Allen Human Brain Atlas (AHBA). De AHBA is een multimodale atlas die genexpressie in het gezonde menselijke brein in kaart brengt[8]. Door deze data te combineren kunnen de mogelijke genen die interacteren met hypercorticolisme worden geïdentificeerd. Hierdoor kan er verder inzicht worden verkrijgen in de processen die kunnen leiden tot de structurele veranderingen in de hersenen.

In **Hoofdstuk 5** zijn de corticale dikte en het corticale oppervlak van de ACC van herstelde patiënten met de ziekte van Cushing en hun gematchte gezonde controles onderzocht. Dit is gedaan met behulp van geavanceerde neuroimaging-methoden en analysetechnieken. Daarnaast is het gehele brein in beeld gebracht. In lijn met eerdere bevindingen was de hypothese dat de herstelde patiënten met ziekte van Cushing afwijkingen in de ACC zouden vertonen in vergelijking met gezonde controles. De drie belangrijkste bevindingen van deze studie zijn dat (i) patiënten inderdaad een verminderde corticale dikte hadden van talrijke corticale gebieden, waaronder de linker caudale ACC, (ii) er geen verschillen zijn gevonden in de corticale oppervlakte, en dat (iii) de corticale dikte van de linker caudale ACC negatief was

geassocieerd met ziekteduur. Dit laatste houdt in dat hoe langer iemand ziek is, hoe dunner de linker caudale ACC. Al met al benadrukken deze bevindingen opnieuw het belang van verder onderzoek naar de ACC.

In **Hoofdstuk 6** is de ACC onderzocht vanuit het perspectief van veerkracht. Het doel was om de neurale correlaten van veerkracht bij traumatische ervaringen vast te stellen. Vanwege hun beroep hebben politieagenten meer kans om traumatische gebeurtenissen mee te maken in vergelijking met andere beroepsgroepen[9]. Toch is er geen bewijs dat politieagenten vaker psychopathologie ontwikkelen ervaren in vergelijking met beroepen die als minder risicovol worden gezien. Politieagenten lijken dus bijzonder veerkrachtig te zijn. In deze studie is een steekproef van Nederlandse politieagenten en rekruten van de politieacademie in drie groepen onderverdeeld, (i) een groep met trauma en psychopathologie, (ii) een groep met trauma zonder psychopathologie, en (iii) rekruten zonder trauma of psychopathologie. Er zijn eerder verbanden gevonden tussen de ACC en psychopathologie[13,14], waarbij dit gebied bij veel veerkrachtige populaties dikker was[15]. Om deze reden is in zowel de ACC als het gehele brein gekeken of er verschillen waren tussen deze bovengenoemde groepen. Er zijn echter geen verschillen in corticale dikte of corticale oppervlakte gevonden tussen de veerkrachtige groep en de andere twee groepen. Dit was verrassend aangezien we veranderingen in de ACC in de groep met trauma en psychopathologie hadden verwacht, net zoals de veranderingen die zijn gevonden bij de herstelde patiënten met de ziekte van Cushing. Een mogelijke verklaring dat er geen verschillen zijn gevonden zou kunnen zijn dat de cortisol spiegels en de ziekteduur bij de kwetsbare groep minder hoog waren en minder lang duurden dan bij de Cushing's patiëntengroep. Daarnaast zijn ook de participanten in de kwetsbare groep ooit voorgeselecteerd op bepaalde veerkrachtspecifieke criteria om te kunnen worden toegelaten tot de politieacademie. Samenvattend suggereren deze bevindingen dat er ofwel geen veerkrachtspecifieke correlaten zijn met betrekking tot corticale dikte en corticale oppervlakte bij de onderzochte groepen, of dat de verschillen tussen deze groepen niet groot genoeg zijn op het gebied van veerkracht om afwijkingen te kunnen identificeren.

In **Hoofdstuk 7** is het planningsvermogen en het werkgeheugen in de herstelde Cushing's patiëntenpopulatie en hun gematchte controles onderzocht. Eerdere studies bij deze patiëntenpopulatie hebben beperkingen op beide cognitieve functies geïdentificeerd. In de studie in dit proefschrift is dit verder onderzocht met behulp van de fMRI Tower of London (ToL)-taak die is afgenomen in een 3T-MRI scanner. De ToL is een vaak gebruikte taak om het planningsvermogen en het werkgeheugen

te meten omdat er veel stappen in het hoofd nagelopen moeten worden. Er werd vanuit gegaan dat planning en het werkgeheugen in deze patiëntengroep afwijkend zou zijn en dat deze patiëntenpopulatie een verhoogde activering in de ACC zou vertonen in vergelijking met hun gematchte controles. Er zijn echter geen verschillen gevonden op het gebied van planning, werkgeheugen, of ACC-activering tussen de twee groepen. In de exploratieve analyse van het gehele brein is wel gevonden dat de herstelde Cushing's patiënten in bepaalde hersenregio's die vaak bij moeilijke processen worden ingezet, meer activiteit vertonen. Dit benadrukt het belang van exploratieve analyses, naast hypothese-gestuurd onderzoek. Samenvattend suggereren de resultaten uit dit onderzoek dat er geen bewijs is voor aanhoudende tekortkomingen bij herstelde Cushing's patiënten op het gebeid van planning en werkgeheugen zoals gemeten op de ToL-taak, maar wel verschillen in hersenactivatie in veel overige hersengebieden. Dit geeft tot op zekere hoogte de flexibiliteit van de hersenen na herstel van de ziekte van Cushing aan. Hoewel verschillende onderzoeken met andere meetinstrumenten dan de ToL afwijkingen hebben gevonden in planning en het werkgeheugen van deze patientenpopulati[e.g.16], kunnen deze vaardigheden ook verbeteren na herstel.

Tot slot, zoals eerder vermeld in Hoofdstuk 4, kunnen MRI-onderzoeken op zichzelf niet genoeg inzicht bieden in de onderliggende biologische processen die kunnen leiden tot veranderingen in de ACC van (herstelde) patiënten met de ziekte van Cushing. In **Hoofdstuk 8** zijn daarom hoge-resolutie MRI-scans gecombineerd met Allen Human Brain Atlas (AHBA) data om de mogelijke microbiologische processen bij de ziekte van Cushing verder te onderzoeken. Dit was een verkennende analyse en er zijn van tevoren geen hypothesen opgesteld. Er is eerst gekeken naar welke genen in de ACC in meer of mindere mate tot uiting kwamen door middel van een differentiële genexpressie-analyse. Hieruit bleek dat de meerderheid van de geïdentificeerde genen immuunsignaleringsgenen zijn. Van deze genen waren de genen die tot onderexpressie kwamen vaak verrijkt voor functionaliteiten die grotendeels te maken hadden met immuunsignalering. Interessant is dat de top tien genen met de meeste onderexpressie allemaal eerder op verschillende manieren in verband zijn gebracht met de ziekte van Alzheimer[17-25]. De belangrijkste bevinding is dat er zeer weinig gedeactiveerde microglia en oligodendrocyten aanwezig zijn in de ACC. Het is van belang dat voldoende van deze celltypes aanwezig zijn omdat gedeactiveerde microglia een aantal ontstekingsremmende cytokinen afgeven. Daarnaast maken ze ook deel uit van het neuroprotectie proces[26], matrixafzetting en weefselremodellering[27]. Oligodendrocyten zijn grotendeels verantwoordelijk voor het remyelinisatieproces[28,29]. Eerder onderzoek heeft aangetoond dat schade aan

oligodendrocyten kan leiden tot een geestelijke of lichamelijke handicap[30]. Daarbij heeft ander onderzoek aangetoond dat langdurige blootstelling aan corticosteroïden geassocieerd is met de remming van het ontwikkelen van oligodendrocytprecursoren in de witte hersenstof[31], en afname van witte stof integriteit in de hersenen van herstelde Cushing patiënten[32]. Dit zou (deels) de aanhoudende veranderingen in de ACC's van herstelde patiënten met de ziekte van Cushing kunnen verklaren, en ook de (mogelijk gerelateerde) cognitieve beperkingen. Alhoewel de ACC's van patiënten met de ziekte van Cushing in deze studie niet rechtstreeks zijn bestudeerd, lijken er wel verschillen te zijn in basale genexpressie bij de Cushing patiënten versus gezonde controles. Ook van belang is dat deze resultaten aangeven welke genen minder waarschijnlijk deel uitmaken van deze initiële kwetsbaarheid.

Samenvattend ondersteunt bovenstaand onderzoek de hypothese dat langdurige overmatige blootstelling aan cortisol, zoals het geval is bij de ziekte van Cushing, blijvende effecten heeft op de hersenen door processen op microbiologisch niveau. Het blijft echter wel onduidelijk hoe lang en met welke hoeveelheid iemand aan cortisol bloot moet worden gesteld om tot deze mogelijk permanente schadelijke effecten te leiden.

Zoals eerder vermeld zou de ziekte van Cushing een geschikt naturalistisch model kunnen zijn om de effecten van overmatige blootstelling aan endogene cortisol op de hersenen te onderzoeken. Patiënten met stressgerelateerde stoornissen hebben ook chronisch verhoogde cortisolspiegels, alhoewel in veel minder mate. Daarnaast is er een overlap in de psychiatrische symptomatologie die zowel patiënten met de ziekte van Cushing als patiënten met psychiatrische stressgerelateerde stoornissen vertonen. Om dit model verder te onderzoeken, is gekeken of er overlap is in de gevonden hersenafwijkingen bij de Cushing's patiëntenpopulatie en patiënten met andere stress-gerelateerde psychiatrische stoornissen.
Eerder onderzoek heeft reeds relaties gevonden tussen structurele hersenafwijkingen bij patiënten met stressgerelateerde stoornissen[bijv.33-43], en in mindere mate bij herstelde patiënten met stressgerelateerde stoornissen[bijv.37-39,43]. Longitudinale studies bij deze patiëntenpopulaties zijn echter schaars[bijv.43]. Hoewel er eerder overlap is gevonden tussen bepaalde hersengebieden (bijv. gebieden die betrokken zijn bij executief en emotioneel functioneren) bij patiënten met stressgerelateerde stoornissen[bijv.33-42], zijn de veranderingen die geïdentificeerd zijn bij de herstelde Cushing-patiënten meer wijdverspreid. Aangezien de niveaus van endogeen cortisol veel hoger zijn bij patiënten met de ziekte van Cushing in vergelijking met patiënten met andere stressgerelateerde stoornissen, lijkt het er dus op dat

deze overmatige blootstelling aan hoge hoeveelheden cortisol zou kunnen leiden tot meer aanhoudende en wijdverspreide hersenveranderingen, ook na herstel, dan het geval is bij andere stressgerelateerde psychiatrische stoornissen. Het is echter belangrijk op te merken dat de patiëntenpopulaties in de bovengenoemde onderzoeken over het algemeen patiënten zijn die milde tot matige klachten rapporteren. Het zou dus zo kunnen zijn dat patiënten die ernstiger klachten hebben (of hebben ervaren) meer overlap vertonen qua hersenafwijkingen met de herstelde Cushing's patiënten.

De gepresenteerde resultaten in dit proefschrift ondersteunen de hypothesen dat (i) het sAA niveau bij het ontwaken onderscheid kan maken tussen bepaalde stressgerelateerde aandoeningen, en dat (ii) blootstelling aan hoge niveaus endogeen cortisol gedurende een lange periode, zoals het geval bij de ziekte van Cushing, kan resulteren in aanhoudende hersenafwijkingen van een aantal belangrijke hersengebieden. De bevindingen in dit proefschrift hebben gezorgd voor dieper inzicht in de etiologie, pathofysiologie, en neurobiologie van stressgerelateerde psychiatrische stoornissen. Deze bevindingen bieden nieuwe inzichten die in de toekomst kunnen leiden tot het sneller en nauwkeuriger identificeren van bepaalde psychiatrische ziektebeelden, het verder verfijnen van instrumenten voor vroege detectie, meer geavanceerde behandelingen en meer succesvolle preventiestrategieën.

Tot slot is het belangrijk om vast te stellen dat een kleine dosis stress goed voor je is. Te weinig stress kan leiden tot onderprikkeling en verveling, en teveel stress kan leiden tot, onder andere, stress-gerelateerde stoornissen en een verslechtering van je gezondheid. Maar de juiste hoeveelheid acute stress kan ervoor zorgen dat je prestaties en gezondheid verbeteren. Kortom, wees niet bang om jezelf te blijven uitdagen.

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Curriculum Vitae

Stéphanie Eleonoor Elisabeth Cornelie Bauduin was born on May 16th 1979 in Amsterdam, The Netherlands and raised in Asia and Canada. She completed the International Baccalaureate program in 1996 at Beverweerd International School. Soon afterwards she moved to Leiden where she became proficient in Dutch and completed her master in English Language and Literature with a minor in Human Resource Management. She began her working career within the field of entrepreneurship and innovation at New Venture, a daughter company of McKinsey & Company and the Ministry of Economic Affairs. In 2010, she began studying Psychology at Leiden University, where her curiosity in the field of research was ignited. This led to her following the Clinical and health psychology research master alongside the Clinical psychology master in 2014, which she completed 'cum laude' in 2016. During this period, she worked as a research assistant at the Leiden University Medical Center (LUMC) Psychiatry department and at Leiden University. She also collected data for several imaging studies and obtained her MRI scan license at the beginning of 2016. Upon finishing her Master's degrees, she began her PhD in the Psychiatry department at the LUMC, focusing on stress-system activation in patients with depression-, anxiety-, and somatic symptom disorders on the one hand, and patients with remitted Cushing's disease on the other hand. She was able to further develop her skills in analyzing both epidemiological and imaging data. At the beginning of 2019 she started to combine her PhD with clinical work. She began as a psychologist at PsyQ's department for bipolar disorders in Rotterdam and in January of 2022 began her training towards becoming a 'gezondheids' or 'health' psychologist. Alongside this, she currently works as a postdoctoral researcher at LUMC's Psychiatry department. In her position as a postdoctoral researcher she collaborates on the PRISM2 project, a study aimed at developing treatments for neuropsychiatric conditions such as Alzheimer's disease and schizophrenia. She hopes to be able to continue combining both research and clinical work for the rest of her career.

List of publications

Bauduin, S. E. E. C., van Noorden, M. S., van der Werff, S. J. A., de Leeuw, M., van Hemert, A. M., van der Wee, N. J. A., & Giltay, E. J. (2018). Elevated salivary alpha-amylase levels at awakening in patients with depression. *Psychoneuroendocrinology, 97*, 69-77.

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