STRESS REGULATION IN THE BRAIN:

ASSOCIATION WITH CORTISOL RELEASE, MODULATION BY EXPOSURE TIME, AND GENDER DIFFERENCES

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FOREWORD

The present work presents three studies exploring the stress regulation in the brain with emphasis on associations with cortisol release, modulation by exposure time, and gender differences. The chapters were composed specifically for this thesis, however, two studies are based on original research articles that are already published (Chapter 4) or currently under peer-review (Chapter 5). They are listed below in order of appearance within the thesis, and are currently not used or designated for use in other dissertations. For improved readability, contents, tables, and figures were numbered continuously and designed consistently, and journal-specific reference styles were standardized and integrated in one combined reference section (see Chapter 7).

The studies were conducted within the framework of the projects “Psychobiologische Stressregulation und Aggression: Untersuchungen zur Dimensionalität von Externalisierung” (DFG, KU 1401/6-1), “Biopsychologische Stressregulation bei Burnout” (DFG, KU 1401/9-1) and “Psychische, endokrine, zentralnervöse und genetische Prädiktoren der Reaktion auf chronischen Stress im Alltag – ein prospektiv-longitudinales Quasi-Experiment“ (DFG, WU 392/9-1) all funded by the German Research Foundation (DFG). Fabian Streit, a co-author, acknowledges support by the German Federal Ministry of Education and Research (BMBF) through the ERA-NET NEURON, "SynSchiz - Linking synaptic dysfunction to disease mechanisms in schizophrenia - a multilevel investigation" (01EW1810) grant. Gina-Isabelle Henze further received funding from the Hanns-Seidel-Foundation (Hanns-Seidel-Stiftung).

Chapter 3

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Chapter 4

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LIST OF ABBREVIATIONS

ACC
: anterior cingulate cortex 12

ACTH
: adrenocorticotropic hormone 10

ANOVA
: analysis of variance 44

AVP
: arginine vasopressin 10

BOLD
: blood oxygenation level dependent 40

CBF
: cerebral blood flow 28

CEN
: central executive network 11

CNS
: central nervous system 5

CRH
: corticotropin releasing hormone 9

DELFIA
: dissociation-enhanced lanthanide fluorescence immune-assay 50

DMN
: default mode network 12

DTI
: diffusion tensor imaging 41

EPI
: echo-planar imaging 40

FC
: functional connectivity 13

FEAT
: FMRI Expert Analysis Tool 41

FILM
: FMRIB’s Improved Linear Model 42

FLAME
: FMRIB's Local Analysis of Mixed-effects 43

fMRI
: functional magnetic resonance imaging 6

FSL
: FMRIB's Software Library 40

FWE
: familywise error 42

FWHM
: Full Width at Half Maximum 42

GLM
: general linear model 41

HCP
: Human Connectome Project 41

HPA
: hypothalamic-pituitary-adrenal 5

MIST
: Montreal Imaging Stress Task 14

MNI
: Montreal Neurological Institute 42

mPFC
: medial prefrontal cortex 5

OCs
: oral contraceptives 26

PANAS
: Positive and Negative Affect Schedule 17

PCC
: posterior cingulate cortex 12

PET
: Positron Emission Tomography 20

PFC
: prefrontal cortex 11

PVN
: paraventricular nucleus 9

ROI
: region of interest 41

RS
: resting state 38

rsFC
: resting state Functional Connectivity 22

sAA
: salivary alpha-amylase 33

SD
: standard deviation 53

SEM
: standard error of the mean 53

SN
: salience network 11

SPM
: Statistical Parametric Mapping 44

TSST
: Trier Social Stress Test 14

VAS
: visual analogue scale 16

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0 SUMMARY

Understanding the interplay between the brain and the hypothalamic-pituitary-adrenal (HPA) axis in response to stress is assumed to be essential to contribute to central questions of stress research, namely how stress can influence disease risk and what are the underlying determinants of stress vulnerability and resilience? Moreover, gender differences in prevalence rates for several stress-related mental disorders exist, highlighting the question to what extent gender differences in stress regulation and stress-related psychopathology can be attributed to gender differences in the brain. Although animal models have contributed extensively to our knowledge on this interplay and gender-related effects, relatively stable findings in animals are not necessarily generalizable to humans.

With the advent of human brain-imaging techniques, successfully studying the brain/HPA axis interplay in response to stress and the impact of gender became feasible. So far, some attempts regarding this issue have already been made but no consistent effect pattern emerged, probably owing to methodological disparities. As this research requires protocols that reliably induce both neural and robust HPA axis responses, the first aim of the present thesis was an improvement of a stress induction protocol for functional magnetic resonance imaging (fMRI) environments called Scan*STRESS*. The studies presented first in this thesis, intended to test the assumption that the application of an improved Scan*STRESS* protocol will reveal more stable responses of different activation systems (cortisol, affect, heart rate, neural) as well as associations between cortisol, psychological and neural responses. Subsequently, the objective of the present thesis was a detailed and comprehensive analysis of gender-specific interactions between cortisol and neural responses. Moreover, this thesis investigated if neural stress responses do change over the course of a relatively long stress protocol (exposure-time effect) to further elucidate habituation or sensitization processes that may also differ between women and men.

In a first step, several aspects of the stress induction protocol have been modified without changing the paradigm itself (described in Chapter 3) and this modified version of the Scan*STRESS* protocol was first applied in a study described in Chapter 4. Briefly, Scan*STRESS* is composed of an alternating block design, presented in two runs, containing two conditions (stress vs. control) prompting the subject to perform arithmetic and rotation tasks while a feedback-giving observation panel is presented via live video stream providing disapproving feedback. To enhance social-evaluative threat as key-

component of the Scan*STRESS* paradigm, one of the panel members informs the subject between the two runs that the performance was below average and makes the urgent request to improve in the second run. The protocol of Scan*STRESS* was improved by implementing a prolonged (45 minutes) relaxing phase prior to stress, by administering a sugary drink to facilitate cortisol reactivity, and by achieving a more abrupt passage from relaxation to stress exposure. Moreover, the observation panel was presented to the subject immediately before paradigm onset and the experimenter announced the negative feedback in between the runs of Scan*STRESS* enhancing the psychosocial stress components. After completion of the fMRI scans, participants remained in the laboratory to fill out questionnaires. Eventually, they received a detailed debriefing.

In the study presented in Chapter 4, changes in brain activation, cortisol levels, affect, and heart rate responses to the improved Scan*STRESS* protocol were assessed in 67 young, healthy participants (36 males, 31 women, all taking oral contraceptives; mean age 23.06 ± 3.14 years). An fMRI analysis approach was implemented considering different analysis levels and thereby different sources of variance were accounted for. As previous findings regarding the brain/HPA axis interplay mostly relied on region of interest (ROI) analyses, the focus of the two studies described in Chapter 4 and 5 was on whole-brain analyses that were subsequently confirmed by *post-hoc* ROI-analyses. Chapter 5 describes an in-depth analysis of gender differences within the same sample regarding the same activation systems (cortisol, affect, heart rate, neural). Moreover, gender-dependent interactions between cortisol and neural responses to stress were investigated by analyzing the association between gender-specific cortisol increases and neural responses.

Stress exposure led to significant increases in cortisol levels, heart rate, and negative affect ratings as well as activations and deactivations in (pre)limbic regions. When individual cortisol increases were used as covariate, stronger responses in a cluster comprising the hippocampus, amygdala, medial prefrontal cortex (mPFC), and cingulate cortex were observed. Moreover, responses within the identical regions predicted negative affect ratings throughout the protocol. Remarkably, an increasing deactivation over the two runs of Scan*STRESS* was found, again, in the same structures. Regarding gender differences, the present data confirmed the well-known gender-specific cortisol response pattern with men exhibiting higher adrenocortical reactions than women. Still, mean increases in cortisol were significant in the female as well as the male subsample, confirming a successful stress induction in both genders. Responses of the hippocampus,

amygdala, cingulate cortex, thalamus, and striatal structures turned out to be differentially associated with cortisol increases in women and men. For men, higher cortisol increases resulted in more activation of these striato-limbic structures whereas in women higher cortisol increases were associated with more deactivation. However, no significant gender differences regarding exposure-time effects, affect, or heart rate measures were detected.

In conclusion, the present thesis introduced an improved Scan*STRESS* procedure and a more sophisticated analysis. Overall, the present findings support the view that especially regarding cortisol responses, the changes applied to the protocol have been effective. Moreover, (pre)limbic structures were consistently found to be associated with cortisol and affective stress responses and furthermore the same structures showed increasing deactivation over stress exposure time. This exposure-time effect might reflect the limbic response to the psychological key-factors of Scan*STRESS*, namely repeated experience of failure and social-evaluative threat. Therefore, investigating possible associations between exposure-time effects in neural stress responses and stress-related interindividual differences (e.g. chronic stress) might be a promising new avenue in stress research. Referring to gender differences, this is the first study revealing neural stress responses in striato-limbic structures to be differentially associated with cortisol increases in women and men. Even though women and men differ in their overall stress reactivity, the present findings do not support the prominent idea of a clear neuroanatomical differentiable 'female-typical' and 'male-typical' response to stress. On the contrary, the data suggest that considering complex interactions and quantitative variables like gender-specific cortisol increases is a more suitable approach to elucidate gender-related differences in central stress regulation. Therefore, the present data document the usability and validity of the Scan*STRESS* paradigm. Moreover, these findings corroborate to a better understanding of interactions between the brain and the HPA axis and to the impact of gender in response to acute psychosocial stress.

CHAPTER 1

1 INTRODUCTION AND OUTLINE OF THE THESIS

To date, several attempts have been made to unravel the mechanisms underlying the central nervous system (CNS) response to acute stress. Endocrine, affective/behavioral, heart rate, and neural responses have been measured most frequently in animals and humans. Although findings derived from animal studies have contributed extensively to our knowledge on specific stress response patterns of various entities and their interactions (Herman et al., 2003; Herman et al., 2016; Herman, Ostrander, Mueller, & Figueiredo, 2005; Hermans, Henckens, Joels, & Fernandez, 2014), these findings are not necessarily transferable to humans. In this context, the hypothalamic-pituitary-adrenal (HPA) axis is of special importance as this neuroendocrine system has proven to be involved in adaptive and maladaptive outcomes modulated by acute and chronic stress exposure (de Kloet, Joëls, & Holsboer, 2005). Hence, direct and indirect projections of limbic structures, such as the hippocampus, amygdala, and medial prefrontal cortex (mPFC) have been discussed to interact with the HPA axis. In animals, the hippocampus and mPFC are thought to have inhibiting effects on HPA axis responses, whereas the amygdala is believed to act excitatory on the HPA axis. In humans, on the contrary, these modes of action have not yet been clearly understood as several studies exist reporting opposite effects of the mentioned structures under stress exposure (Dedovic, Duchesne, Andrews, Engert, & Pruessner, 2009b; Herman et al., 2005; Hermans et al., 2014; Jankord & Herman, 2008; Noack, Nolte, Nieratschker, Habel, & Derntl, 2019; Pruessner et al., 2010). Thus, although studies on stress have provided a wealth of data delineating the effects of acute and chronic stress, much remains to be done to fully understand how the human brain processes stress and how pathology or resilience are developed in the face of adversity (Lupien, McEwen, Gunnar, & Heim, 2009). Moreover, the interplay between limbic inputs and HPA axis activity seems to be the basis for the development of a vulnerable phenotype for mental illness (de Kloet et al., 2005).

A factor that should receive special consideration in this context is the impact of sex and/or gender. Focusing on the brain, findings of average differences between women and men in the structures of specific brain regions have been interpreted as evidence that the typical female brain is different from the typical male brain (Joel et al., 2018). Regarding acute stress reactions, responses in women and men are differentiable on the endocrine and subjective level with men exhibiting higher HPA axis and women reporting greater affective responses when facing stress (Kudielka, Hellhammer, & Wüst, 2009; Kudielka & Kirschbaum, 2005; Zänkert, Bellingrath, Wüst, & Kudielka, 2019). Of

substantial clinical relevance is the clear gender difference in prevalence rates for stress-related mental disorders (Bale & Epperson, 2015; Bangasser & Valentino, 2014; Kiely, Brady, & Byles, 2019; Kudielka & Kirschbaum, 2005) strengthening the idea that also the underlying neural mechanisms of stress processing are sexually dimorphic. Consistently, some investigations according to gender differences in neural responses to psychological stress in humans have led to the view that female and male stress networks are clearly definable (Kogler et al., 2017; Seo, Ahluwalia, Potenza, & Sinha, 2017; Seo et al., 2011; Wang et al., 2007). However, an alternative view is that on average the gender-related differences in the brain are, in fact, rather small and concern characteristics that significantly overlap (Grabowska, 2017; Joel et al., 2015). This is supported by findings describing differential responses for women and men in identical structures (Goldfarb, Seo, & Sinha, 2019; Kogler et al., 2016). Therefore, one aim of the present thesis was to evaluate neural gender differences in response to stress and beyond that, to analyze the differences of women and men regarding the interaction of HPA axis and brain responses to stress.

As a consequence, the present thesis used functional magnetic resonance imaging (fMRI) to measure neural responses to a paradigm inducing acute psychosocial stress, called Scan*STRESS* (Streit et al., 2014). To assess HPA axis reactions, the steroid hormone cortisol was measured repeatedly collecting saliva samples. Also, affective and heart rate responses were captured. Apart from gender differences, the present study pursued to evaluate stress response patterns of different response domains of the human organism and the interplay of these entities. Therefore, interactions of cortisol and neural responses were scrutinized as well as associations between affective and neural reactions. To do this properly, the first step of the present thesis was to evaluate and improve the stress protocol of Scan*STRESS* as to be sure that psychosocial stress was induced in the most effective manner.

Regarding the structure of this thesis the present **Chapter 1** provides the research rationale of the present study. The theoretical background described in **Chapter 2** gives a brief overview of the current state of scientific knowledge. Hence, in a first step, central findings regarding stress regulation in the brain are summarized referring to animal models and human studies. Subsequently, psychosocial stress induction paradigms suited for fMRI environments are introduced focusing on cortisol, affective, heart rate, and neural response patterns that have been evaluated so far. Then, literature on gender differences in psychosocial stress processing is presented, comparing findings from

animal and human studies in the first place. Afterwards, an overview of gender-specific neural response patterns after psychosocial stress induction is given.

Based on animal literature, the present thesis pursued to investigate stress regulation in the human brain. Although psychosocial stress paradigms suited for fMRI environments facilitated to face questions arising in this context, certain responses (i.e. cortisol and/or neural reactions) have not always been consistent and fully convincing, hampering the evaluation of neuroendocrine stress processing (Noack et al., 2019). Therefore, **Chapter 3** includes the improvement of the protocol and analysis strategy for Scan*STRESS*, the psychosocial stress paradigm used in the present study. Subsequently, **Chapter 4** comprises three different research questions: First, an evaluation of the improved Scan*STRESS* protocol is given regarding cortisol, affective, heart rate, and neural responses. Second, the interaction of these different stress activation systems is investigated. Third, as a consequence of the more sophisticated analysis strategy for Scan*STRESS* data described in Chapter 3, the question is asked if neural responses do change over the course of the stress induction by comparing neural responses to the two runs in which Scan*STRESS* is presented to each participant (exposure-time effect). As the results presented in Chapter 4 have already been published in a peer-reviewed journal (Henze et al., 2020b), Chapter 5 refers to findings of this research paper. Thus, **Chapter 5** investigates gender-specific interactions between HPA axis and neural reactions in response to stress exposure (Henze et al., 2020a). Eventually, **Chapter 6** provides a general discussion, aiming at integrating the findings of the present thesis.

CHAPTER 2

2 THEORETICAL BACKGROUND

2.1 Stress regulation in the brain

Studying the brain's response to acute or chronic stress is a central research topic in psychoneuroendocrinology. In this context, it is essential to investigate the relationship between responses of limbic structures and those of the HPA axis, as a dysregulation of both entities is probably a key-feature of affective disorders (Herman et al., 2005). The most important glucocorticoid in humans, cortisol, is the end product of HPA axis responses to each kind of situation threatening homeostasis. In general, the nucleated cells in the human body have glucocorticoid receptors, thus glucocorticoids have a broad variety of effects throughout our system, including metabolic, cardiovascular, and immune responses (McEwen, 1998). Shortly, activation of the HPA axis causes secretion of glucocorticoids driven by neural mechanisms invoking corticotropin releasing hormone (CRH) release from hypothalamic paraventricular nucleus (PVN) neurons. HPA axis effector neurons of the PVN are reached by direct projections of limbic structures including regions expressing both glucocorticoid and mineralocorticoid receptors. Moreover, these structures show extensive overlap and at the same time divergence in their projection pathways. The neural reaction of interacting limbic structures to given stressors is complex and this complex neural pattern is of crucial importance for the control of the HPA axis. In addition, as known from animal studies, the relative involvement of various limbic structures might determine the magnitude of secretory HPA axis responses (Herman et al., 2005).

In the following, results from animal and human studies are presented investigating the specific role of (pre)limbic structures, such as hippocampus, amygdala, and mPFC in cortisol release in response to stress. When discussing the contributions of these structures in regulating the HPA axis, the type of stressor needs to be distinguished (Dedovic et al., 2009b). Different stressor types, such as reactive versus anticipatory stressors, lead to stimulation of the HPA axis through activation changes in various brain regions involved in glucocorticoid regulation (Herman et al., 2003; Herman et al., 2005). Hence, reactive stressors are characterized by increasing the demands on the system through a real sensory stimulus whereas anticipatory stressors are described by social challenges or unfamiliar situations (Dedovic et al., 2009b; Herman et al., 2003). As the present thesis pursues to evaluate the association between limbic and HPA axis responses to acute psychosocial stress induction which might also differ between women and men, the

following chapters focus on research implementing stress induction paradigms characterized by factors that can be summarized as psychosocial. Therefore, uncontrollability, social-evaluative threat, and forced failure (Dickerson & Kemeny, 2004) are inevitable elements of the described procedures.

2.1.1 Animal models

Animal studies have shown that distributed networks including brainstem nuclei and specific limbic system structures exert a regulatory influence on HPA axis function and glucocorticoid regulation (Herman et al., 2003). The HPA axis constitutes a critical adaptive system enhancing survival potential when being confronted with physical or psychological challenges. Glucocorticoid hormones, like cortisol, have proven to be advantageous regarding short-term survival but prolonged or even chronic exposure might cause various dysregulations ranging from metabolic, immune, and psychological impairments (McEwen & Stellar, 1993). Hence, glucocorticoid secretion is required to be a strongly regulated mechanism and therefore administered by efficient feedback inhibition processes (Herman et al., 2005). Direct projections of ascending brain systems or circumventricular organs to the PVN of the hypothalamus generate responses of the HPA axis (Herman et al., 2003). Neurons of the PVN release CRH and arginine vasopressin (AVP) into the portal blood system. Arriving at the anterior pituitary, these effectors stimulate the secretion of adrenocorticotropic hormone (ACTH). From there, it is released into the bloodstream reaching the adrenal cortex where ACTH interacts with adrenocortical receptors and the synthesis of cortisol is initiated. Entering the cardio-circulatory system, cortisol unfolds its effects on multiple gateways (Herman et al., 2016).

Many of the stimuli activating the HPA axis represent a direct threat to homeostasis or survival. Moreover, cortisol is also released in the absence of these entities conducting to prepare the organism to potential homeostatic challenge. These anticipatory responses are attributed to limbic circuits capable of evaluating the potential external threats based on indirect connections to the PVN (Herman et al., 2003). In animal literature, the hippocampus is involved in the circadian glucocorticoid rhythm as well as in the inhibition of HPA axis responses to stress, the latter depending on stimulus type. Nevertheless, under some circumstances, the hippocampus might also have an excitatory effect on HPA axis regulation (Herman et al., 2005). The same dependency on stressor type and, moreover, on anatomically subdivision, can be applied to another limbic structure, namely the amygdala. The amygdala is known as an important regulator of the stress-related glucocorticoid secretion (Jankord & Herman, 2008). It promotes the

activation of the HPA axis when the organism is exposed to either a physical or psychological stressor (Herman et al., 2005). Hence, the influence of the amygdala is highly stressor-specific and depends on the subnuclei addressed. The hypothesis that glucocorticoid receptors of the amygdala potentiate rather than inhibit HPA axis responses to stress may still be valid (Herman et al., 2005) although desensitization processes in the amygdala in response to increasing glucocorticoid levels have been reported (Hermans et al., 2011). Initial findings from animal studies on the involvement of the mPFC in the regulation of the HPA axis and subsequent stress responses suggested a purely inhibitory role of the entire PFC (Herman et al., 2003). However, recent work indicates that specific components of the PFC may play quite different roles in the regulation of cortisol secretion whereby these may also be stressor specific (Herman et al., 2003; Herman et al., 2005; Jankord & Herman, 2008).

In summary, the involvement of limbic structures in HPA axis regulation can be described as a complex system with topographical organization and stimulus characteristics determining how a certain region affects neuroendocrine stress responses. Based on the fact that all limbic structures operate through subcortical intermediaries, effects of these structures seem to be indirect and depending on the functional integrity of the particular area. Moreover, there seems to be considerable overlap between innervation fields of various structures having inhibitory but also excitatory effects. Limbic processing may therefore be summed up at subcortical nodes (Herman et al., 2005). Based on primarily animal data, Hermans et al. (2014) introduced a framework describing stress-related neuromodulators triggering dynamic shifts in (pre)limbic network balance. Aligned with the certain demands, the organism is thereby enabled to reallocate its neural resources. The authors presented a biphasic-reciprocal model of neural reactions in response to stress. This model proposes that stress-related hormones and neurotransmitters enhance the activity of the so-called salience network (SN) during an acute stress phase at the cost of the central executive network (CEN). In this context, the SN is thought to represent a neurocognitive system that integrates the ability to reorient one's attention to potential threats, mobilize energy resources, and react to unsafe situations (Seeley et al., 2007). Apart from the amygdala, the SN comprises the anterior cingulate cortex (ACC), hypothalamus, insula, thalamus, striatum, and brainstem. In the acute stress phase, neural resources are assigned towards the SN, actively suppressing the CEN. In the recovery phase, this effect is reversed by allocating resources to the CEN, suppressing the SN to return to homeostasis. Therefore, when situations of acute threat

require vigilance and rapid action, a shift of neural recourses away from regions involved in executive control functions may be beneficial for short-term survival (Hermans et al., 2014). These functions are supported by different areas of the PFC and parietal regions (Vincent, Kahn, Snyder, Raichle, & Buckner, 2008).

Regarding animal data, the involvement of limbic structures in regulating HPA axis responses to stress has sufficiently been proved. Especially three main structures, namely the hippocampus, amygdala, and mPFC seem to have coordinating influences on stress processing. The following chapter focuses on findings in humans by taking up the idea of the two opponent entities, the SN and the CEN, and expanding this approach by including a third (pre)limbic circuit, the default mode network (DMN), comprising the hippocampus, posterior cingulate cortex (PCC), angular gyrus, precuneus, and mediotemporal areas (Menon, 2011; Van Oort et al., 2017).

2.1.2 Human studies

As stated above, the functional brain networks that were determined as fundamental organizational elements of human brain architecture mostly rely on animal findings. In humans, these brain circuits were identified using fMRI, both under rest and during a wide variety of paradigms including serial subtraction tasks, Stroop color-word interference tasks, script-driven stress stimuli, speech in front of an audience, or psychosocial stressors (Dedovic, D'Aguiar, & Pruessner, 2009a). As brain regions impacted by acute stress do not respond in isolation to a stressor, a system level approach is required to assess the organization of functional and dynamical interacting structures in humans (Van Oort et al., 2017). Supported by previous findings, three neuroanatomical definable systems seem to be associated with HPA axis responsiveness to stress, namely the SN, the CEN, and the DMN (Hermans et al., 2014; Hermans et al., 2011; Seo et al., 2011; Vaisvaser et al., 2013; Van Oort et al., 2017). Although the DMN is not commonly associated with the stress response per se, the activity within the DMN was repeatedly found to be increased in most fMRI studies implementing different stress paradigms (Van Oort et al., 2017). In this context, the triple network model by Menon (2011) integrates into this perspective and provides a framework for understanding how aberrations in the structures of these three entities cause psychiatric states in vulnerable subjects.

Of the three networks mentioned, two showed increased fMRI responses throughout most literature on neural stress processing, namely the SN and the DMN (Van Oort et al., 2017). For example, studies investigating the effects of stressful script driven imagery showed increased activity in the SN and the DMN, but no changes in the CEN. Moreover,

reactions of single inherent structures of these networks were found to be associated with stress-driven changes in cortisol or negative affect values (Hermans et al., 2011). Therefore, stressful and affective content may enhance SN- and DMN-responsiveness to stress exposure, but it seems to have no impact on nodes of the CEN (Van Oort et al., 2017). In accordance with these results, studies implementing stress induction with higher-order cognitive tasks including social-evaluative threat like negative feedback yielded comparable effect patterns. Although the direction of effects varied between studies, the SN and DMN were most frequently stress-responsive whereas the CEN was not. Van Oort et al. (2017) suggested that particularly negative feedback leads to pronounced SN and DMN reactions along with absent or even diminished CEN responses. This is further supported by the fact that higher-order cognitive tasks without such evaluative threat component more often led to responses of CEN-related structures. Hence, the aspect of self-referential processing as key-component of certain paradigms was proposed to provoke DMN responses (Menon, 2011; Van Oort et al., 2017). Moreover, studies evaluating the recovery from stress by analyzing changes in functional connectivity (FC) after stress induction with negative feedback component further support this idea of enhanced stress induced self-evaluative processing by reporting increased SN-DMN connectivity patterns up to two hours after stress onset (Quaedflieg et al., 2015; Vaisvaser et al., 2013; Veer et al., 2011).

A first review focusing on studies that have examined the effects of purely psychological stress on neural processes in neuroimaging environments by Dedovic et al. (2009a) yielded that only studies using serial subtraction (Wang et al., 2005) or psychosocial stress component-based paradigms (Pruessner et al., 2008) were able to induce a significant cortisol stress response. Until then, most consistent findings of note were increased activations in frontal areas, especially in the ACC, and deactivations of limbic structures like the hippocampus. Though, this picture has become more diverse over the past decade. Therefore, the following chapter focuses on findings of studies implementing two different psychosocial stress paradigms, namely the Montreal Imaging Stress Task (MIST) (Dedovic et al., 2005) and – a paradigm of our own group – Scan*STRESS* (Streit et al., 2014).

2.2 Psychosocial stress induction in functional magnetic resonance imaging (fMRI) environments

The best-known paradigm to induce psychosocial stress in the laboratory is the Trier Social Stress Test (TSST) (Kirschbaum, Pirke, & Hellhammer, 1993). Consisting of public speaking and oral mental arithmetic in front of an investigator panel in professional attire, the TSST comprises stress-eliciting components like cognitive load in combination with social evaluation (Zänkert et al., 2019). With respect to cortisol changes in response to the TSST, studies reported robust increases in approximately 70.0 - 80.0 % of the participants (Dickerson & Kemeny, 2004; Kirschbaum et al., 1993; Skoluda et al., 2015; Zänkert et al., 2019). Comparing the TSST with other commonly used laboratory stressors (e.g. Stroop color-word interference task, bicycle ergometry, Cold Pressor Test) and a resting control condition, the TSST evoked the highest increases in perceived stress levels as well as in HPA axis responses. Based on a meta-analysis covering 208 laboratory stress studies, motivated performance tasks reliably elicited ACTH and cortisol responses if they were uncontrollable or characterized by social-evaluative threat. Therefore, tasks including both elements, like the TSST, were associated with the largest hormonal changes and the longest recovery times (Dickerson & Kemeny, 2004).

The direct assessment of changes in brain activation in response to stress has been limited for a long time, as fMRI research emerged in the past three decades and a lack of appropriate protocols to induce and measure stress in fMRI endured. The MIST (Dedovic et al., 2005) was the first successful paradigm that aimed at inducing and measuring psychosocial stress in fMRI environments. Based on the Trier Mental Challenge Test (Kirschbaum, Diedrich, Gehrke, Wüst, & Hellhammer, 1991), another laboratory stressor consisting of computerized mental arithmetic with negative feedback, the MIST allows the investigation of interindividual differences in stress responsivity (Dedovic et al., 2009b; Pruessner et al., 2008). Studies that implemented the MIST are presented in the following chapter according to neural effect patterns with emphasis on the involvement of (pre)limbic structures. In addition, responses in cortisol, affect measures, and heart rate are reported. Thereafter, the same is done for studies using Scan*STRESS* as psychosocial stress induction paradigm which is based on the TSST (Streit et al., 2014). As there are more similarities in the brain's stress response between studies using the same stress paradigm than between studies using different stress induction procedures (Van Oort et al., 2017), findings are presented separately.

2.2.1 The Montreal Imaging Stress Task (MIST)

The MIST combines the stress-eliciting components of high cognitive demand with negative social evaluation. It is composed of a series of computerized mental arithmetic tasks with an induced failure algorithm. Participants are asked to solve a sequence of mental arithmetic tasks under time pressure while receiving a feedback on their current performance via a text field, visualizing statements like 'correct', 'incorrect' or 'timeout', and the participants performance relative to the average performance of all other participants on a mock performance bar. During the stress condition, a progress bar is visible, enforcing a time limit. This time limit is depending on previous performance and adapts so that participants typically achieve a performance range of 45.0 - 50.0 % correct answers. Moreover, the social-evaluative threat component as built into the program is also reinforced by the investigator providing negative feedback between scanning sessions. In the control condition, no time limit for solving the arithmetic tasks is present and participants are told that their performance is not being recorded and evaluated. In the original design, the MIST was presented as a block design with a duration of 14 minutes (Dedovic et al., 2005; Pruessner et al., 2008) although also an event-related design exists (Dedovic et al., 2009c). So far, the MIST is the most frequently used fMRI stress paradigm. A PubMed search yielded 51 studies implementing the MIST with respect to a variety of research questions. For the present chapter, only studies in healthy subjects that implemented the original block design are presented, focusing on cortisol, affective, and heart rate responses as well as activations and deactivations of (pre)limbic structures as mentioned above. In this context, structures are described as activated when they showed more neural response to the stress compared to the control conditions (contrast: stress > control), whereas deactivations describe the opposite (i.e., more activation in control compared to stress conditions; contrast: control > stress).

The MIST has been implemented in a variety of contexts in dozens of studies and most of them described an increase of cortisol levels after exposure (Albert, Pruessner, & Newhouse, 2015; Boehringer et al., 2015; Dedovic et al., 2005; Dong et al., 2020; Geva, Pruessner, & Defrin, 2014, 2017; Lederbogen et al., 2011; Li et al., 2019; Pruessner et al., 2008; Shermohammed et al., 2017; Sun et al., 2020a; Sun et al., 2020b; Tomova et al., 2017; Voellmin et al., 2015; Zhong et al., 2019). However, studies do also exist that failed to register cortisol changes (Gheorghe, Panouillères, & Walsh, 2018; Hoegh, Poulsen, Petrini, & Graven-Nielsen, 2020; Khalili-Mahani, Dedovic, Engert, Pruessner, & Pruessner, 2010) or even reported a decline in cortisol values when comparing baseline

measures (before stress onset) and post-stress measures (after MIST induction) (Chung et al., 2016a; Chung et al., 2016b; Corbett, Weinberg, & Duarte, 2017; Gossett et al., 2018; Kogler, Gur, & Derntl, 2015; Kogler et al., 2017; Orem et al., 2019). Moreover, some studies using group difference analyses according to their experimental design (e.g., stress condition vs. control condition; presence or absence of a trait) reported cortisol responses for only one group (Corbett et al., 2017; Gheorghe et al., 2018; Grimm et al., 2014; Zschucke, Renneberg, Dimeo, Wustenberg, & Strohle, 2015). Furthermore, some studies reported cortisol increases for only the first run of the MIST (Dagher, Tannenbaum, Hayashi, Pruessner, & McBride, 2009) or only a certain proportion of participants, the so-called cortisol responders (Corbett et al., 2017; Gheorghe et al., 2018; Khalili-Mahani et al., 2010). Based on a criterion formulated by Miller, Plessow, Kirschbaum, and Stalder (2013) a cortisol responder shows an increase of cortisol concentrations of at least 1.5 nmol/L rise in response to the experimental procedure. Regarding the MIST, only a handful of studies mentioned responder/non-responder ratios (Corbett et al., 2017; Gheorghe et al., 2018; Khalili-Mahani et al., 2010; Pruessner et al., 2008) and the amount of non-responders varied between 47.5 and 65.0 % in healthy subjects (Noack et al., 2019). Hence, it has to be outlined, that three of the four studies reporting on responder rates did not simply dichotomize between those participants that yielded cortisol increases exceeding 1.5 nmol/L and those who did not (Pruessner et al., 2008). Instead, they used a tertile split based upon their change in cortisol levels following the MIST (post MIST - pre MIST) and separated their participants into three groups. The top third of the participants were classified as responders, the bottom third as non-responders and the middle third was removed from analysis (Corbett et al., 2017; Gheorghe et al., 2018; Khalili-Mahani et al., 2010). Therefore, further analyses of these studies, for instance regarding associations with other variables like neural or subjective stress responses, have to be interpreted with caution as they are at most valid for cortisol responders. Overall, the cortisol stress response initiated by the MIST has not always been fully convincing (Noack et al., 2019).

Regarding the subjective emotional stress response, usually, studies found increases in perceived stress levels and negative affect measures as well as decreases in positive affect ratings. In this context, most of the studies used a visual analogue scale (VAS) asking ‘How stressed do you feel?’ resulting in answers ranging from 0 (absence of stress) to 10 (maximal stress) (Allenby et al., 2020; Boehringer et al., 2015; Chung et al., 2016a; Dong et al., 2020; Geva et al., 2014, 2017; Gheorghe et al., 2018; Kogler et al., 2015;

Lederbogen et al., 2011; Li et al., 2019; Sun et al., 2020a; Sun et al., 2020b; Voellmin et al., 2015; Zhong et al., 2019; Zschucke et al., 2015). When applying the Positive and Negative Affect Schedule (PANAS) scale (Watson, Clark, & Tellegen, 1988) the only study reporting an increase instead of an decrease in positive affect ratings after psychosocial stress induction was the one by Kogler et al. (2015). All other studies using the PANAS scale reported on – as expected – declines in positive affect and increases in negative affect ratings in response to stress (Chung et al., 2016a; Chung et al., 2016b; Kogler et al., 2015; Kogler et al., 2017; Zschucke et al., 2015). Other studies, using none of the above measurements, also registered elevated stress levels after the MIST (Ashare et al., 2016; Brugnera et al., 2018; Hoegh et al., 2020; Shermohammed et al., 2017; Tomova et al., 2017).

As marker of the autonomic stress response, heart rate elevations comparing the stress and control condition of the MIST were analyzed in a small number of studies. All studies reported on elevated heart rate responses when contrasting stress and control blocks (Boehringer et al., 2015; Geva et al., 2014, 2017; Gossett et al., 2018; Lederbogen et al., 2011; Leicht-Deobald et al., 2018; Orem et al., 2019; Shermohammed et al., 2017; Voellmin et al., 2015). Moreover, a study by Brugnera et al. (2018) comparing heart rate responses to different stress tasks revealed higher responses to the MIST compared to two verbal stress tasks (free speech, Stroop color-word interference task).

As a summary, Table 1 gives an overview of the presented results according to cortisol, affect (perceived stress and negative affect), and heart rate responses to the MIST. Total sample sizes are presented in the second column that are sometimes accompanied by further sample sizes in parentheses indicating that group difference analyses were conducted or only a subsample was exposed to the MIST. Hence, responses in cortisol, affect (perceived stress and negative affect), and heart rate measures increased in some cases only in a subgroup of the total sample.

Table 1.
Overview of studies measuring cortisol, affect (perceived stress and negative affect), and heart rate responses after psychosocial stress induction implementing the MIST.

study	sample size	gender ratio		cortisol	affect	heart rate
		women	men			
Albert et al. (2015)	28	28	0	↑		
Allenby et al. (2020)	75	35	40		↑	
Ashare et al. (2016)	39	17	22		↑	
Boehringer et al. (2015)	25	14	11	↑	↑	↑
Brugnera et al. (2018)	60	31	29		↑	↑
Chung et al. (2016a)	31	31	0	↓	↑	
Chung et al. (2016b)	46	30	16	↓	↑	
Corbett et al. (2017)	78 (39)*	0	78	↓		
				↑ **n* =13 responders		
Dagher et al. (2009)	15	7	8	↑ (only to the first run)		
Dedovic et al. (2005)	22	0	22	↑		
Dong et al. (2020)	148	85	63	↑	↑	
Geva et al. (2014)	29	0	29	↑	↑	↑
Geva et al. (2017)	25	0	25	↑	↑	↑
Gheorghe et al. (2018)	48 (25)*	27	21	↔	↑	
				↑**n* = 7 responders		
Gossett et al. (2018)	57	21	36	↓		↑

study	sample size	gender ratio		cortisol	affect	heart rate
		women	men			
Grimm et al. (2014)	32 (17)*	0	32	↑*		
Hoegh et al. (2020)	25	0	25	↔	↑	
Khalili-Mahani et al. (2010)	23	0	23	↔		
				↑ *n* = 9 responders		
Kogler et al. (2015)	43	23	20	↓	↑	
Kogler et al. (2017)	80	40	40	↓	↑	
Lederbogen et al. (2011)	32	16	16	↑	↑	↑
Leicht-Deobald et al. (2018)	31	14	17			↑
Li et al. (2019)	152	77	75	↑	↑	
Orem et al. (2019)	239	113	126	↓		↑
Pruessner et al. (2008)	40	20	20	↑		
				n = 21 responders		
Shermohammed et al. (2017)	56 (29)*	27	29	↑*	↑*	↑*
Sun et al. (2020a)	307	153	154	↑	↑	
Sun et al. (2020b)	101	0	101	↑	↑	
Tomova et al. (2017)	76 (35)*	0	76	↑*	↑*	
Voellmin et al. (2015)	104	104	0	↑	↑	↑
Zhong et al. (2019)	96	57	39	↑	↑	
Zschucke et al. (2015)	36 (18)*	0	40	↑*	↑	

↑ significant increase; ↓significant decrease; ↔ no significant change; * significant for subgroup.

The most-quoted MIST-finding is the hippocampus deactivation reported by Pruessner et al. (2008). The degree of deactivation was thereby positively associated with the amount of cortisol released, suggesting a linear relationship between hippocampal deactivation and HPA axis activation. In this context, it has to be noted that this study implemented the MIST within two different methodologies, Positron Emission Tomography (PET) and fMRI, and that an overall deactivation was only found according to the PET data. Regarding the fMRI study, the hippocampus deactivation was limited to those subjects responding to the task with a cortisol increase. Hence, this prominent finding is limited to cortisol responders and it should be kept in mind that the total fMRI sample consisted of 40 participants, of which only 21 were responders (and 19 non-responders, respectively). Therefore, this study was the first to confirm predictions from animal studies as it found evidence for inhibitory effects of the hippocampus on HPA axis responses to stress. However, other findings were not in accordance with what would be expected from animal models. For example, Pruessner et al. (2008) also reported on deactivations of the amygdala and activations of the mPFC although the qualitative opposite (activations of the amygdala, deactivations of the mPFC) would be expected for each structure. It can be summarized that early reports on neural patterns of stress responses to the MIST suggested deactivations of limbic structures but this picture has become more diverse until today (Dedovic et al., 2009b; Noack et al., 2019; Pruessner et al., 2008).

Regarding the response of certain limbic structures in response to stress induction, exceedingly few studies reported on qualitative (activation vs. deactivation) and quantitative (e.g., high vs. low) results. For the hippocampus, four studies confirmed deactivations (Albert et al., 2015; Dagher et al., 2009; Sun et al., 2020a; Sun et al., 2020b), while also four studies reported on activations for the total sample (Boehringer et al., 2015; Chung et al., 2016a; Chung et al., 2016b; Kogler et al., 2015) or a subsample (Grimm et al., 2014; Leicht-Deobald et al., 2018). When using correlational approaches for analyzing the interaction between cortisol and hippocampus responses, three studies found a negative association between cortisol and hippocampus activation (Lederbogen et al., 2011; Sun et al., 2020b; Zhong et al., 2019). Moreover, a study by Khalili-Mahani et al. (2010) described a positive linear relationship between the amount of hippocampus deactivation and cortisol response as reported by Pruessner et al. (2008). Thus, more hippocampus deactivation appears to be related to greater cortisol stress responses. In addition, the study by Khalili-Mahani et al. (2010) reported a group difference describing

the positive association between cortisol and hippocampus deactivation to be greater in extent but not in magnitude for cortisol responders compared to non-responders. For the amygdala, five studies found activations (Boehringer et al., 2015; Chung et al., 2016a; Chung et al., 2016b; Kogler et al., 2015; Orem et al., 2019) and three studies reported on deactivations (Dagher et al., 2009; Lederbogen et al., 2011; Pruessner et al., 2008), whereby the study of Lederbogen et al. (2011) even introduced a negative association between cortisol responses and activations. Hence, higher responses in the stress condition compared to the control condition led to less cortisol responses and vice versa. A similar mixed effect pattern emerged according to the mPFC with findings varying by subdivision (ventromedial PFC, dorsolateral PFC) including deactivations (Dagher et al., 2009; Kogler et al., 2017; Sun et al., 2020a; Sun et al., 2020b) and activations (Orem et al., 2019; Pruessner et al., 2008). So far, only one study reported an association between HPA axis response and decreased mPFC activity during the MIST (Albert et al., 2015). Moreover, activations within mPFC and cingulate cortex were positively related to stress ratings (Orem et al., 2019).

The cingulate cortex and its subdivisions (especially the ACC) were also shown to be deactivated (Albert et al., 2015; Dagher et al., 2009; Kogler et al., 2017), activated (Boehringer et al., 2015; Lederbogen et al., 2011; Pruessner et al., 2008), or both (Sun et al., 2020a; Sun et al., 2020b) in response to stress. One study reported on a negative association of dorsal ACC activation and cortisol release (Li et al., 2019), while another study reported a positive association between cingulate activations and stress ratings (Orem et al., 2019). Regarding striatal structures (ncl. accumbens, ncl. caudatus, putamen), studies predominantly reported on deactivations during MIST exposure (Dagher et al., 2009; Khalili-Mahani et al., 2010; Kogler et al., 2017; Pruessner et al., 2008).

So far, some studies using the MIST confirmed the findings arising from animal models whereas others did not. According to different (pre)limbic structures, consistent effect patterns failed to appear for unknown reasons. In the following, results regarding the implementation of Scan*STRESS* are presented in a similar way confirming this mixed data situation in humans.

2.2.2 Scan*STRESS*

The Scan*STRESS* paradigm is a recently developed stress induction protocol for fMRI environments implemented by our own group (Streit et al., 2014). Scan*STRESS* is based on the TSST asking the participant to solve challenging cognitive tasks (mental rotation

and serial subtraction) under time pressure while being monitored by an investigator panel visible via life video stream to the participant lying in the scanner. Hence, the social-evaluative threat component is enforced through the presence of the panel. Moreover, participants are told that their mimics and behavior are monitored via live video of their faces. Comparable to the TSST, the panel gives standardized feedback. Here, via live video stream, the responses 'Error' or 'Work faster' are displayed to the subject when the panel pushes a buzzer. Task difficulty and speed are adapted to the performance of each participant ensuring frequent failure and uncontrollability. Like the MIST, Scan*STRESS* was originally invented as block design with alternating stress and control blocks presented in two runs, lasting 24 minutes in total. In the control conditions easier tasks (figure and number matching) are presented and there is neither feedback nor observation and no time pressure exerted. Between the two runs, participants receive a negative feedback about their individual performance and the urgent request to improve in the second run. A comprehensive description and visualization of the experimental procedure of Scan*STRESS* is given in Chapter 3.

To date, Scan*STRESS* has been implemented in eight studies, including two studies investigating changes in resting state functional connectivity (rsFC) in response to stress (Dimitrov et al., 2018; Nowak et al., 2020). Recently, a short-version of Scan*STRESS* was established by Sandner et al. (2020) called Scan*STRESS*-C (compact). However, in the present section, findings are limited to studies implementing the original block design of Scan*STRESS*. Again, activations are referred to as higher responses in the stress compared to the control conditions (contrast: stress > control) and vice versa for deactivations (contrast: control > stress).

So far, five studies utilized Scan*STRESS* (Akdeniz et al., 2014; Dahm et al., 2017; Lederbogen et al., 2011; Streit et al., 2017; Streit et al., 2014) as psychosocial stress paradigm of which three reported on cortisol stress responses (Akdeniz et al., 2014; Dahm et al., 2017; Streit et al., 2014). Although the first study evaluating HPA axis reactions to Scan*STRESS* failed to show an increase in the whole sample, significant increases were at least found for a subsample characterized as cortisol responders (Streit et al., 2014). Here, after applying the criterion of 1.5 nmol/L increase as cortisol response (Miller et al., 2013), 22 of 42 subjects were classified as responders (52.0 %) while the study of Dahm et al. (2017) showed a cortisol responder rate of 69.0 % (57 responders, 26 non-responders). Akdeniz et al. (2014) also reported on significant cortisol elevations, which were even positively associated with activations in the ACC. Moreover, Scan*STRESS* led

to increases in self-reported stress levels and significant heart rate elevations in the stress compared to the control blocks. Furthermore, a positive association between ACC activations and heart rate acceleration in the second run was reported. Streit et al. (2014) also described significant differences in heart rate responses when comparing the two conditions and in addition, these differences between heart rates in stress versus control conditions were more pronounced in cortisol responders than in non-responders. Table 2 gives an overview of cortisol, affect, and heart rate responses in the studies implementing the original version of Scan*STRESS* whereby Streit et al. (2017) analyzed a sample derived from two previous studies (Akdeniz et al., 2014; Streit et al., 2014).

Table 2.
Overview of studies implementing Scan*STRESS* and available measures of cortisol, affect, and heart rate responses after psychosocial stress induction.

study	sample size	gender ratio		cortisol	affect	heart rate
		women	men			
Akdeniz et al. (2014)	80	41	39	↑	↑	↑
Dahm et al. (2017)	86	50	36	↑ *n* = 55 responders		
Lederbogen et al. (2011)	23	7	16			
Streit et al. (2014)	42	20	22	↔ ↑ *n* = 22 responders		↑
Streit et al. (2017)	32	32	0			

↑ significant increase; ↓significant decrease; ↔ no significant change.

According to neural responses of (pre)limbic structures, most of the studies showed activations in the hippocampus and the amygdala (Akdeniz et al., 2014; Streit et al., 2014) rather than deactivations or both (Dahm et al., 2017). The mPFC was once found to be deactivated (Akdeniz et al., 2014) whereas the cingulate cortex – depending on the subregion – was found to be activated as well as deactivated (Akdeniz et al., 2014; Dahm et al., 2017; Streit et al., 2014). Striatal structures were consistently found to show higher

responses to the stress compared with the control blocks (i.e., activations) (Akdeniz et al., 2014; Dahm et al., 2017; Streit et al., 2014). Moreover, the study by Dahm et al. (2017) investigated if neural responses to Scan*STRESS* change after social-evaluative negative feedback is given between the two runs. Thus, increasing activations and deactivations were found describing that subregions of the ACC, bilateral insula, and amygdala responded differently to the first compared with the second run of Scan*STRESS*.

To conclude, it can be noted that in these studies no consistent effect pattern regarding neural responses of (pre)limbic structures to psychosocial stress induction emerged, neither according to the implementation of the MIST nor to Scan*STRESS*. Moreover, cortisol stress responses have likewise not always been fully convincing, underlining that the obstacles of inducing stress responses in fMRI environments may have not been overcome. For the present study, it was therefore assumed that stable and replicable neural patterns depend on robust psychosocial stress induction. For this, in turn, robust cortisol responses can be viewed as a reliable indicator. Therefore, an evaluation of Scan*STRESS* is pursued in the first place of the present thesis facilitating to investigate cortisol, affective, heart rate, and neural responses as well as interactions of these different activation systems. Moreover, the impact of gender will be analyzed. Therefore, the next chapter summarizes findings according to gender differences in stress processing.

2.3 Gender differences in psychosocial stress processing

The current knowledge on HPA axis and limbic involvement regarding several mental, cardiovascular, endocrine, and immune disorders with more or less clear gender differences in prevalence rates derived from both animal and human studies (Bangasser & Valentino, 2014; Cahill, 2006; Grabowska, 2017; Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999). However, some effect patterns based on sex differences in animals are not necessarily transferable to gender differences in humans. In the following, animal and human findings are compared according to sex-/gender-specific findings in cortisol, affective, and heart rate responses to stress. As the present study investigated neural responses to psychosocial stress induction, the subsequent section summarizes studies implementing paradigms which can – in the broad sense – be characterized as psychosocial stress paradigms. Here, gender differences in (pre)limbic response patterns are focused on.

2.3.1 Comparing animal and human findings: the impact of sex/gender

When comparing animal and human findings, especially regarding female- and male-typical effect patterns, the terminology used has to be specified. As *gender* relates to a rather social term, *sex* emphasizes a biological term addressing particularly genetic and anatomical disparities. For the present thesis, *sex* is used in terms of real biological differences between the female and male organism and therefore mostly in the context of animal findings, whereas *gender* is utilized when referring to human studies.

Moreover, one aspect that should be considered when comparing animal and human findings in response to stress, is the intensity of stressors used in animal versus human studies. Although some stressors in animal research, like restraint, may also be applicable to humans, the impact of stress-eliciting components is different. For instance, while humans are aware that they are able to abort an experimental procedure at any time, animals are not. Moreover, most of the stressors used in animal research include situations that are completely new to the animal and therefore may also appear life threatening.

In general, female rodents exhibit greater basal ACTH and corticosterone levels and secrete higher concentrations of corticosterone in response to stress (Haleem, Kennett, & Curzon, 1988; Kant et al., 1983; Kitay, 1961, 1963; Oyola & Handa, 2017; Rincón-Cortés, Herman, Lupien, Maguire, & Shansky, 2019; Yoshimura et al., 2003). For example, when female and male rats were exposed to a psychological stressor like restraint for about 30 minutes, corticosterone levels were higher in female rats when terminating restraint (Goel, Workman, Lee, Innala, & Viau, 2014). Moreover, this effect pattern occurred in response to a variety of stressors including physical stressors like foot shock (Heinsbroek, Van Haaren, Feenstra, Endert, & Van de Poll, 1991) or forced running and psychological stressors (e.g. restraint, noise) (Goel et al., 2014). In humans, however, although women and men show comparable total cortisol levels under basal conditions, one of the most prominent findings is the significantly larger ACTH and cortisol response in healthy adult men after the induction of short-term laboratory stressors like the TSST (Kirschbaum, Wüst, Faig, & Hellhammer, 1992; Liu et al., 2017; Nicolson, Storms, Ponds, & Sulon, 1997; Seeman, Singer, Wilkinson, & McEwen, 2001; Stroud, Salovey, & Epel, 2002; Zänkert et al., 2019). To date, several reviews and meta-analyses therefore concluded that sex/gender is a prominent source of variability for stress-related HPA axis responses (Kudielka, Buske-Kirschbaum, Hellhammer, & Kirschbaum, 2004b; Kudielka et al., 2009; Kudielka & Kirschbaum, 2005; Zänkert et al., 2019).

In this context, the intake of oral contraceptives (OCs) as well as the menstrual cycle in women have to be considered as these factors seem to be crucial in female stress responses. Early studies reported that women medicated with OCs show blunted salivary cortisol responses to acute stress exposure (Kudielka et al., 2009; Kudielka & Kirschbaum, 2005). Applying the TSST in a study sample comprising men and women in different menstrual cycle phases (follicular phase, luteal phase) and those taking OCs revealed significant response differences. While salivary cortisol responses of men and women in the luteal phase were comparable, both women in the follicular phase and OC-taking women showed lower cortisol reactions (Kirschbaum et al., 1999). Although some studies replicated these findings (Kudielka et al., 2009; Zänkert et al., 2019), there also exists data contradicting HPA axis response differences across menstrual cycle phases (follicular, ovulatory, luteal) (Duchesne & Pruessner, 2013; Herbison et al., 2016). Therefore, available evidence for the impact of hormonal contraceptives and menstrual cycle phases on gender-specific response differences after psychosocial stress induction is still heterogeneous. According to a recent review by our own group (Zänkert et al., 2019) it is strongly suggested to collect data and provide information about the use of OCs and menstrual cycle phases in female subjects when evaluating HPA axis responses to stress. Referring to the United Nations (2019), OCs are still used by the majority of married or in relationship living women in Europe. Therefore, for the present thesis, we decided to include only women taking OCs as controlling for menstrual cycle phases might have hampered the data collection according to available volunteers and organizational requirements (i.e. timing of the study appointment has to fit the timing of menstrual cycle phase).

When measuring behavioral differences in response to stressors in animals or affective responses by self-report in humans, typically female subjects show more pronounced stress responsiveness compared to male subjects (McEwen & Milner, 2017; Rincón-Cortés et al., 2019). For example, female rats show greater immobility (i.e. passiveness) during the forced swim test than male rats (Dalla et al., 2008; Drossopoulou et al., 2004; Rincón-Cortés & Grace, 2017). Although depending on stressor-type, most of the stressors implemented in animal studies yielded higher behavioral stress responses in female compared to male rodents (e.g., isolation, social instability) (Beery & Kaufer, 2015). Likewise, in humans, perceived stress ratings and negative affect ratings after psychosocial stress induction have repeatedly been found to be higher in women. Moreover, women reported significantly more feelings of tension and stress-induced

anxiety than men did (Buske-Kirschbaum et al., 2003; Helbig & Backhaus, 2017; Kelly, Forsyth, & Karekla, 2006; Kelly, Tyrka, Anderson, Price, & Carpenter, 2008; Kudielka et al., 1998; Merz & Wolf, 2015). Hence, the female tendency to experience and report negative emotions at a greater frequency and intensity compared to men may be confirmed (Kelly et al., 2008). Moreover, the only study implementing the MIST that was able to show this typical effect pattern for perceived stress levels after stress induction (i.e. women > men) was the one by Brugnera et al. (2018).

Regarding heart rate responses, animal studies on sex differences have yielded mixed results, primarily depending on stressor type (Azar, Sharp, & Lawson, 2005; Crestani, 2016; Vieira et al., 2018). While some studies reported on overall lower heart rate measures in females compared to males, acute heart rate responses were found to be more pronounced in females and recovered more rapidly. On the other hand, some studies showed contrary findings, i.e., higher baseline responses and lower stress-induced heart rate responses in female than in male rodents (Anishchenko, Glushkovskaya-Semyachkina, Berdnikova, & Sindyakova, 2007; Azar et al., 2005; Weinstock, Razin, Schorer-Apelbaum, Men, & McCarty, 1998). Moreover, others failed to detect any sex-related differences according to heart rate measures and an impact of estrous cycle phases cannot be ruled out (Crestani, 2016; Eikelis & Van Den Buuse, 2000). Similarly, in humans, there has been a lack of clear evidence for gender differences in measures of autonomic responding to acute stress. Some studies demonstrated no differences regarding physiological reactivity (Kelly et al., 2006; Kelly et al., 2008; Kirschbaum et al., 1999), while some reported on gender differences (Emery, Stoney, Thayer, Williams, & Bodine, 2018; Koenig & Thayer, 2016; Seo et al., 2017; Steptoe, Fieldman, Evans, & Perry, 1996; Stoney, Davis, & Matthews, 1987) that partly depended on menstrual cycle phases (Childs, Dlugos, & De Wit, 2010; Kudielka, Buske-Kirschbaum, Hellhammer, & Kirschbaum, 2004a). Moreover, age-related gender differences in heart rate responses have been observed in response to laboratory social stressors (Kudielka et al., 2004a).

Considering the aforementioned findings regarding sex/gender differences in endocrine, affective, and cardiac responses to stress, the present study pursued to shed more light on gender-specific reaction patterns in response to psychosocial stress induction. Given the composition of the female subsample consisting of only OC-taking women, clear gender differences in acute cortisol responses should occur as described above. Similar effect patterns should be expected for affective stress responses, i.e., lower perceived stress levels in men after Scan*STRESS* completion compared to women.

Moreover, differences in heart rate reactions might be more unlikely to be detected. In the following, as the present study refers to neural stress reactions in response to psychosocial stress induction, an overview of the current literature on gender-related neural response differences is given.

2.3.2 Neural gender differences in response to psychosocial stress

The first study investigating gender differences in neural responses to a paradigm including psychosocial components was the one by Wang et al. (2007). In this perfusion based fMRI study psychological stress was elicited using mental arithmetic tasks under varying time pressure. Perceived stress in men was associated with cerebral blood flow (CBF) increases in primarily frontal structures, whereas in women with CBF elevations in limbic structures comprising the striatum, insula, and cingulate cortex. In addition, the correlation between frontal activations and cortisol responses were stronger in men than the correlation between striato-limbic CBF increases and cortisol measures in women, respectively. As central outlook of their study, the authors suggested only a small degree of overlap between the stress networks of women and men. This hypothesis was built on results from conjunction analyses revealing anatomical overlap between female and male neural stress processing when adapting the threshold level (i.e., from $p < .01$ to $p < .05$).

To date, four studies in total have investigated neural response differences between women and men after psychosocial stress induction using a block design version of the MIST (Chung et al., 2016b; Kogler et al., 2015; Kogler et al., 2017) or Scan*STRESS* (Dahm et al., 2017). While Dahm et al. (2017) detected no gender differences in neural reactions, studies implementing the MIST reported on striato-limbic response differences, varying in direction (women > men, men > women) between studies. Moreover, the studies by Chung et al. (2016b) and Kogler et al. (2015) can only be included into this overview in a limited extent as they conducted additional experimental manipulations (i.e. androstadienone versus placebo treatment, cognitive regulation). Therefore, only the study by Kogler et al. (2017) serves as basis for the present thesis. The direct comparison of female and male neural responses thereby resulted in stronger activation of a cluster comprising parts of the hippocampus and PCC when contrasting men > women. The opposite contrast (women > men), however, revealed no significant activations.

Referring to the aforementioned conclusion of Wang et al. (2007), three other studies by the same group, implementing script-driven stress manipulation or aversive images, suggested that neural gender differences are not based on anatomical differentiable circuits of the female brain and the male brain addressed during stress (Goldfarb et al.,

2019; Seo et al., 2017; Seo et al., 2011). Especially the most recent study on neural gender differences by Goldfarb et al. (2019) underlined that the same striato-limbic structures respond differently in women and men. While men showed more activation in prefrontal areas and more deactivation in striato-limbic structures, the opposite was found for women (i.e. more activation in striato-limbic, deactivation in prefrontal areas). Although the paradigms implemented in these studies did not meet the requirements to be characterized as psychosocial stress induction paradigm, these results are relevant for the present thesis. When taking up the aforementioned idea that stable and replicable neural response patterns depend on robust psychosocial stress induction, which can be regarded as a reliable marker of robust cortisol responses, gender differences in neural stress processing might also only be detectable when gender-specific cortisol responses are present. So far, gender-related cortisol response differences after psychosocial stress induction in fMRI environments have not been reported. Hence, in the present study, individual cortisol response differences of women and men – if present – are taken into account when analyzing neural activations and deactivations during Scan*STRESS*.

CHAPTER 3

Henze, G.-I., & Wüst, S. (2020). Improvement of the protocol and analysis strategy of Scan*STRESS* – a psychosocial stress paradigm for scanner environments. Unpublished method development.

Gina-Isabelle Henze and Stefan Wüst designed the new protocol for Scan*STRESS*. Gina-Isabelle Henze developed the new analysis strategy for Scan*STRESS* data. Gina-Isabelle Henze drafted the manuscript and Stefan Wüst provided critical revisions.

3 IMPROVEMENT OF THE PROTOCOL AND ANALYSIS STRATEGY OF Scan*STRESS* – A PSYCHOSOCIAL STRESS PARADIGM FOR SCANNER ENVIRONMENTS

3.1 Abstract

The advent of human brain-imaging techniques enabled to successfully study the interplay of the brain and the HPA axis in response to stress. So far, some attempts have been made to study this relationship, but no consistent effect pattern emerged probably owing to methodological disparities.

The present chapter evaluates a stress induction protocol for fMRI environments called Scan*STRESS* that proved to be a promising psychosocial stress paradigm. However, in order to further enhance its capability to reliably induce cortisol, affective, heart rate, and neural responses, an improved protocol of Scan*STRESS* is introduced. This protocol includes the implementation of a prolonged relaxing phase prior to stress onset, the administration of a sugary drink to facilitate cortisol reactivity, and the enhancement of psychosocial stress components by achieving a more abrupt passage from relaxation to stress exposure.

In addition, a more sophisticated analysis strategy for Scan*STRESS* data using the software FSL is described. The description of each hierarchical analysis step to account for different sources of variance is focused on and different statistical models are introduced to face the research questions of the present thesis.

3.2 Introduction

Neuroimaging paradigms have been developed to understand the neural processes of the stress response in general as well as the mechanisms leading to increased vulnerability and, on the other hand, to pronounced resilience. To date, dozens of studies implemented stress paradigms suited for fMRI environments to investigate stress regulation in the brain in combination with different variables. In particular, psychophysiological stress indices and cortisol concentrations were measured most frequently. However, especially cortisol responses were not always fully convincing and cortisol responder rates varied between studies (Noack et al., 2019). Moreover, some studies reported a decline instead of an increase in cortisol values after psychosocial stress induction (Chung et al., 2016a; Chung et al., 2016b; Corbett et al., 2017; Gossett et al., 2018; Kogler et al., 2015; Kogler et al., 2017; Orem et al., 2019). These inconsistencies regarding cortisol stress responses in fMRI environments need further consideration and may be caused by specific characteristics of the fMRI environment itself (Gossett et al., 2018). In the following, information about the evaluation of a psychosocial fMRI stress induction paradigm conducted by our group, called Scan*STRESS* (Streit et al., 2014), is provided. Subsequently a detailed description of the improved experimental protocol of Scan*STRESS* is given and an optimized analysis strategy for Scan*STRESS* data is introduced.

3.3 Evaluation and improvement of Scan*STRESS*

3.3.1 The impact of a relaxing phase prior to stress onset

To investigate the interaction of neural and HPA axis responses to stress it is essential to carefully design a stress induction paradigm suited for fMRI environments. Although a study by Muehlhan, Lueken, Wittchen, and Kirschbaum (2011) revealed that in the majority of subjects (i.e. 87.2 %) the scanner environment does not lead to cortisol stress responses per se, subjects participating in fMRI examinations regularly report anxiety and stress related reactions (McGlynn, Smitherman, Hammel, & Lazarte, 2007). While previous research suggests that prior exposure to the scanner environment reduces stress reactivity to fMRI methodology during subsequent imaging sessions (Tessner, Walker, Hochman, & Hamann, 2006), others did not report differences in baseline cortisol levels between scanner-experienced and scanner-naïve participants (Gossett et al., 2018). In an attempt to minimize potential artifacts, some studies have familiarized their participants

with the fMRI environment by using mock scanners (Gianaros, Onyewuenyi, Sheu, Christie, & Critchley, 2012), testing them on separate days (Dahm et al., 2017), or by recruiting only scanner-experienced participants (Cousijn et al., 2010; Cousijn, Rijpkema, Qin, van Wingen, & Fernández, 2012; Streit et al., 2014).

In general, it is important to establish stable baseline cortisol values (i.e., cortisol levels prior to stress onset). In this context, a recent study by Gossett et al. (2018) investigated whether anticipatory stress (indexed by cortisol) was greater when participants were facing the MIST in the fMRI compared to being exposed to the TSST in a standard behavioral laboratory. Baseline cortisol measures were significantly greater in the MIST condition indicating that anticipatory stress was greater before testing in the fMRI setting than in a standard laboratory. Furthermore, the authors reported that the mere anticipation of being immersed into the scanner elicited similar HPA axis responses as the TSST by comparing pre-stress cortisol levels in the MIST condition to post-stress levels in the TSST condition. Moreover, a reliable decrease of cortisol levels from baseline to post-stress in the MIST condition was observed, suggesting that higher baseline levels in the fMRI environment might have disrupted the cortisol response to the MIST (Gossett et al., 2018). However, these findings are not in line with the aforementioned results by Muehlhan et al. (2011) reporting a decrease in cortisol levels over the course of an experimental fMRI procedure including a visual detection task. Only five of 39 participants showed a significant fMRI-related cortisol-rise. Nevertheless, they refer to other variables like salivary alpha-amylase (sAA) and subjective mood showing a significant fMRI-related increase and therefore conclude that fMRI environments may elicit subjective and neuroendocrine stress reactions. Hence, when implementing stress paradigms in the fMRI it cannot be ruled out that the potentially stress-eliciting properties of fMRI methodology lead to cortisol elevations prior to stress onset that confound the response to the stress paradigm itself. Thus, cortisol responses to a subsequent stressor may be relatively weak, or even diminished by HPA axis' negative feedback loop (Keller-Wood, 2015). Therefore, an adequate relaxation phase prior to stress onset may enhance cortisol responses to a psychosocial fMRI stress paradigm like Scan*STRESS*. So far, relaxing phases prior to stress were only reported in two MIST studies with a duration of 20 and 60 minutes (Gossett et al., 2018; Shermohammed et al., 2017). A review by Noack et al. (2019) on stress induction paradigms suited for fMRI environments reported that evaluating the impact of relaxing or acclimatization phases (to familiarize with the scanner) thoroughly is hampered as most of the studies omitted

to report on the exact procedure. The authors pointed out that implementing acclimatization periods (approximately 45 minutes) leading to sufficient baseline conditions prior to stress onset (i.e., low cortisol levels) may avoid to mistakenly classify participants as cortisol non-responders.

Owing to methodological risks associated with fMRI studies, volunteers have to complete thorough safety screenings and consent processes prior to participation. These safety precautions in combination with the potentially novelty of scanner environments may also provoke stress reactions at least in some subjects. Moreover, the strict guidelines of fMRI safety coupled with the urgent request not to move during the procedure may cause fear of negative evaluation by the experimenter (McGlynn et al., 2007). Therefore, a detailed description and comprehensive clarification about the general scanning procedure may help to prevent concerns prior to scanning, such as fear of the unknown procedure, harm by the machine, and claustrophobia (McGlynn et al., 2007; Thorpe, Salkovskis, & Dittner, 2008). Such a clarification should be implemented as soon as possible in an fMRI stress induction protocol, for instance, right before the onset of an adequate relaxing phase or when receiving general information about the study days before the fMRI appointment. As a consequence, participants should be able to sufficiently anticipate the upcoming methodological procedure and high anxious subjects may get the chance to reconsult the experimenter or terminate the testing precociously. Most importantly, potential stress responses to the scanner – whether they concern safety precautions or the methodology itself – and those to a stress induction paradigm should no longer mix up.

3.3.2 Sugar administration to facilitate cortisol reactivity

As circulating cortisol levels vary strongly during the course of the day (Clow, Thorn, Evans, & Hucklebridge, 2004; Fries, Dettenborn, & Kirschbaum, 2009; Weitzman et al., 1971), experimental designs in stress research have to take time-of-day-effects regarding HPA axis responsiveness into account. To minimize confounding circadian influences, fMRI stress induction paradigms should be performed during the afternoon (Noack et al., 2019). Moreover, another factor influencing cortisol responses to stress exposure, also varying throughout the day, is the nutritional state of participants (Strahler, Skoluda, Kappert, & Nater, 2017). In this context, the influence of glucose on cortisol stress responses was first studied in males who fasted for eight to eleven hours and ingested either 100 g glucose or a placebo before being confronted with the TSST or a control

setting (Kirschbaum et al., 1997). Results showed that glucose administration per se did not affect cortisol levels, but that acute stress induced a larger cortisol response in glucose-treated subjects compared to controls. Additionally, energy administration through protein or fat consumption was not found to amplify cortisol responses (Gonzalez-Bono, Rohleder, Hellhammer, Salvador, & Kirschbaum, 2002). To date, the underlying mechanisms are poorly understood, but an impact of hunger and saturation of regulating neuropeptides has been discussed (Rohleder & Kirschbaum, 2007). Based on this finding, it has been recommended to avoid major differences in blood sugar levels between participants facing the performance in psychosocial stress paradigms to minimize confounding effects of the variability in energy availability on cortisol stress responses (Gonzalez-Bono et al., 2002).

A recent study by our group investigated the effect of different sugar-containing drinks (200 ml grape juice, a 75 g glucose, or a 75 g maltodextrin drink) on acute cortisol stress responses of women and men (Zänkert, Kudielka, & Wüst, 2020). Subjects were instructed to refrain from eating major meals three hours before testing. The sugary drinks were administered at the beginning of a resting period lasting 50 minutes until entering the stressful situation (implemented by the TSST). In response to the TSST, participants showed significantly higher cortisol levels after administration of grape juice or a glucose drink but not after a maltodextrin drink. As a consequence – particularly regarding the evaluation of the Scan*STRESS* protocol – the administration of a sugary drink may facilitate cortisol stress reactivity. Moreover, comparable nutritional baseline conditions for each subject should be established and scanning sessions should start at standardized times in the afternoon.

3.3.3 Enhancing psychosocial stress components by achieving a more abrupt passage from relaxation to stress onset

If all the aforementioned aspects are accounted for in the protocol of Scan*STRESS*, it should be possible to measure a cortisol stress response that is almost exclusively driven by the paradigm itself. Nevertheless, the impact of the key-components of Scan*STRESS* as psychosocial stressor on the cortisol response should also be enhanced. A review by Dickerson and Kemeny (2004) reported that motivated performance tasks elicited cortisol responses if they were uncontrollable or characterized by social-evaluative threat (task performance could be negatively judged by others), when methodological factors and other stressor characteristics were controlled for. Therefore, it is of central importance to

disentangle possible reactions to the scanner environment, as stated above, from reactions elicited by Scan*STRESS*. As a consequence, the time needed to bring the participant into the scanner tube should be minimized as much as possible. Hence, trained technical assistants and a well prepared scanner environment (i.e., required equipment in reach) are essential. In addition, it is important to address the questions of the participants beforehand by giving a clear description about the methodology. Moreover, subjects should at first be confronted with the stress-eliciting elements of the paradigm when listening to the introduction given by the observation panel which is presented via live video stream. Hence, they see the panel for the first time when lying in the scanner enhancing uncontrollability and social-evaluative threat as characteristics that have proven to be associated with the largest cortisol and adrenocorticotropic hormone changes (Dickerson & Kemeny, 2004). Furthermore, the negative feedback that is given in between the two runs of Scan*STRESS* by the observation panel should be announced by the experimenter to strongly highlight the stress components embodied by the panel.

3.3.4 Description of the improved Scan*STRESS* protocol

In the following, the experimental procedure of Scan*STRESS* is presented in detail, focusing on the implementation of the three major modifications to the original protocol (Streit et al., 2014): integration of a relaxing phase prior to stress onset, administration of a sugary drink, and achievement of a more abrupt passage from relaxation to stress exposure to reinforce the psychosocial stress components elicited by the paradigm itself. As the HPA axis' response to stress unfolds over several minutes and has a pronounced impact on subsequent stress reactions (de Kloet et al., 2005; Dedovic et al., 2009a), especially the timing is important. Hence, compared to previous studies using stress protocols suited for fMRI, a more closely monitoring of the HPA axis activity was operationalized by collecting ten saliva samples throughout the experimental procedure (Noack et al., 2019). Therefore, the present thesis pursued to measure individual stress reaction profiles and to disentangle responses to different experimental manipulations in the Scan*STRESS* protocol.

The stress induction via Scan*STRESS* includes several psychosocial components such as pressure to perform, time pressure, forced failure, social-evaluative threat, uncontrollability, and unpredictability. Thus, it was established following the TSST (Streit et al., 2014). Figure 1A describes the experimental procedure of the Scan*STRESS* protocol including cortisol, psychological, and heart rate measurements as implemented

in the present thesis. Saliva samples for cortisol assessment were collected at ten time points (t = -75, -15, -1, +15, +30, +50, +65, +80, +95, +110 minutes) using 'Cortisol Salivettes' (Sarstedt, Nuembrecht, Germany). To collect samples when the participant was lying in the scanner at minutes -1 to +65, the experimenter, wearing medical gloves, gave the salivette swab to the participant. Throughout the procedure, mood state was compiled at the same ten time points using the German version of the PANAS scale (Watson et al., 1988). During Scan*STRESS*, heart rate recordings were obtained with an MRI-compatible finger oximeter (Model 7500 FO; Nonin Medical, Plymouth, USA) on the index finger, with a sampling rate of the highest heart beat within four seconds.

Scan*STRESS* was implemented in Presentation® software (Version 12.9, www.neurobs.com). It consists of two different tasks presented in a block design via monitor. The first task of the stress condition prompts the participant to match a three-dimensional figure to its rotated equivalent from three options presented below the target figure [source of stimulus material: Peters and Battista (2008)]. Analogous to the TSST (Kirschbaum et al., 1993) the second task asks the participant to continuously subtract a number (here 13) from a four-digit number. The correct answer is presented below together with three other response options. In case of an error, participants have to start subtracting all over again. In the stress blocks, the participant has to perform under time pressure presented by a countdown timer, signaling the remaining time. Both task speed as well as difficulty are adapted by the participant's individual performance to ensure frequent failure. In the control conditions, tasks are less demanding, performed without time pressure, and social evaluation (number matching without subtraction and figure matching without rotation). Answers were given with a five-button hand-shaped response box. The block design of Scan*STRESS* (two runs of 680 seconds duration each) includes repeated 60 seconds task blocks (control or stress) preceded by five seconds announcement and followed by 20 seconds rest period. In sum, the paradigm comprises 16 epochs of 60 seconds each with alternating stress and control blocks presented in two runs (see Figure 1B).

In the present thesis, Scan*STRESS* was applied as previously reported (Streit et al., 2014) but with a slightly modified protocol. Participants arrived at the laboratory 75 minutes prior to stress onset (see Figure 1A). In the beginning, they received detailed descriptions about methodological aspects of the scanner procedure and the instruction that the aim of the study was the "investigation of brain activation during maximal mental performance". Thus, they were asked "to show maximal effort" when tested in the fMRI.

After collection of the first saliva sample, a prolonged (45 minutes) relaxing phase was implemented. During this phase, participants watched a neutral movie (nature documentary) while resting in an armchair. Forty-five minutes prior to stress, participants received a sugary drink (75 g glucose in 200 mL herbal tea) to facilitate cortisol reactivity (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997; Zänkert et al., 2020). During relaxation, the participants completed a brief training session of the Scan*STRESS* control blocks (approximately 25 minutes prior to stress onset). To achieve a more abrupt passage from relaxation to stress exposure (starting directly after time point -15 minutes), the duration of the participant's transition into the scanner tube was optimized (< 10 minutes) and the observation panel was presented to the participant immediately before paradigm onset. Hence, compared to the protocol implemented in the study of Streit et al. (2014), no introduction to the panel took place before the measurement to further reduce possible anticipation reactions. After immersion into the scanner tube (time point -1 minute) and subsequent pre-measurements (localizer etc.), participants were confronted with the demanding tasks immediately after receiving the instructions of the panel via live video stream and audio system. The observation panel consisted of a female and a male researcher in professional attire, sitting in the control room. Additionally, the panel informed the participant that behavior, mimics, and answers are monitored via live video transmission of the participant's face. Also, a live video transmission of the panel was presented during the scanning procedure to induce social-evaluative threat. During the stress blocks, the panel explicitly gazed at the monitors. The observers gave standardized disapproving visual feedback after wrong or slow answers by pressing buttons on a "buzzer" visible on the video transmission. Depending on the individual performance and the button pressed, the participants either received the message "Error!" or the demand "Work faster!". In the control blocks, the panel behaved passively, gave no feedback, and turned away from the camera (but was still visible for the participant). A diagonal grey cross signaling that no observation takes place overlaid the video picture. See Figure 1C for a representation of the participant's monitor while being exposed to the paradigm. After the first run (time point +15 minutes), a saliva sample was collected, and the experimenter announced that the panel was unsatisfied with the participant's performance. Thereafter, one of the panel members notified the participant that the performance was below average and that the participant has to improve in the second run, otherwise the fMRI data would be useless. After completion of the fMRI scans (65 minutes in total, time point +65 minutes) that also included a multiband resting state (RS)

sequence (results not reported in the present manuscript) as well as anatomical measurements, participants remained in the laboratory to fill out questionnaires (time point +65 – +110 minutes). Finally, they received a detailed debriefing.

Food consumption or drinking (except water) was not allowed during the whole procedure. Regarding the time prior to arriving at the laboratory, participants were asked not to consume major meals 90 minutes before protocol onset (i.e., three hours prior to stress onset). Participants arrived at standardized times (12:00 PM, 1:20 PM, or 2:20 PM) and scan onset was not before 1:20 PM.

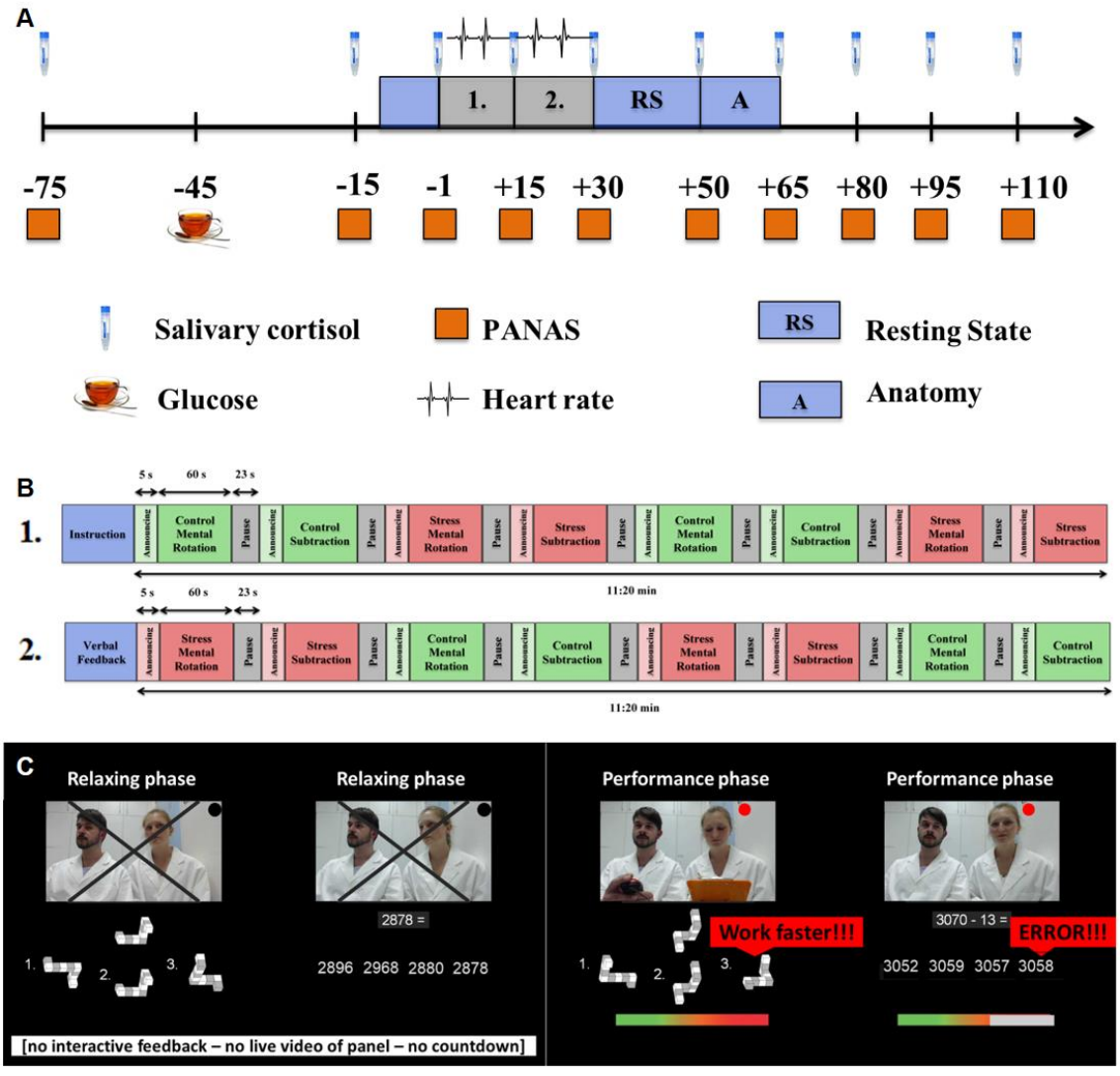

Figure 1. A) Visualization of the experimental procedure including cortisol, psychological, and heart rate measures. B) Design of Scan*STRESS* with two runs, preceded by an instruction phase and interrupted by negative verbal feedback of the panel. C) Screenshot of the two different conditions (control and stress) and the two different tasks for each condition (mental rotation and subtraction).

We recently developed Scan*STRESS* and the first publications showed that it works well and enables relevant effects to be found (Akdeniz et al., 2014; Dahm et al., 2017; Lederbogen et al., 2011; Streit et al., 2017; Streit et al., 2014). Moreover, we assume that Scan*STRESS* takes key psychological elements for generating moderate stress better into account than other paradigms. In addition, it represents a good compromise of psychobiological stress-inducing components, ethical aspects, and technical as well as methodological requirements of an fMRI examination. Moreover, the original block design enables to contrast stress and control conditions and therefore allows statements about activations (stress > control) as well as deactivations (control > stress). Hence, for the present thesis, we did not change the Scan*STRESS* paradigm itself.

3.4 Description of the analysis strategy for Scan*STRESS*

3.4.1 Data acquisition and software package FSL

Participants were scanned in a Siemens MAGNETOM Prisma 3T MRI scanner (Siemens Healthcare, Erlangen, Germany) equipped with a 64-channel head coil. As Scan*STRESS* includes two runs, a series of blood oxygenation level-dependent (BOLD) gradient echo-planar imaging (EPI) images with the following parameters were acquired twice: repetition time 2000 ms, echo time 30 ms, 90° flip angle, 64 x 64 matrix, 192 mm field of view, 37 3 mm axial slices with 1 mm gap. Data were analyzed using Version 6.0 of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl, Oxford, UK) (Jenkinson, Beckmann, Behrens, Woolrich, & Smith, 2012; Smith et al., 2004; Woolrich et al., 2009). A recent study by Bowring, Maumet, and Nichols (2019) outlined that the impact of methodological choices for task-based fMRI has been investigated extensively. While qualitatively similar, different software packages exhibit variability in *T*-statistic values and locations of significant activation. Especially weak effects may not be generalizable across packages. Moreover, they pointed out that varying processing conditions like changing the software version used or switching the workstation from which a software is run manifests deviations in the final results. Therefore, it is important to minimize possible artifacts driven by methodological aspects like software package, version, or workstation used and therefore the same modalities were implemented for each analysis and each subject, respectively. In the course of the data acquisition for the present study changes in rsFC data were also of interest wherefore a rsFC sequence was implemented after the accomplishment of Scan*STRESS* and on a separate testing session, also including

a diffusion tensor imaging (DTI) measurement (results not presented in the present manuscript). As these rsFC and DTI sequences emerged in the progress of the Human Connectome Project (HCP) (Van Essen et al., 2012) using FSL as standard software, FSL was chosen likewise for analyzing the present task-based data.

fMRI data processing was carried out using FEAT version 6.0 (FMRI Expert Analysis Tool) (Woolrich, Behrens, Beckmann, Jenkinson, & Smith, 2004; Woolrich, Ripley, Brady, & Smith, 2001). Data modeling of FEAT is based on general linear modelling, also known as multiple regression. FEAT allows to describe the experimental procedure of a paradigm by creating a statistical model fitting individual data. The general linear model (GLM) method implemented in FEAT can be separated in first-level (time-series) and higher-level analysis. A hierarchical approach can be utilized with a GLM at each level of the hierarchy introducing distinct random effects variance components (Woolrich et al., 2004). In the present study, GLMs were carried out on three levels: for each subject, one GLM was computed for each run (first level) to account for scanner drifting. Subsequently, a fixed-effects analysis (second level) was obtained to measure mean responses of each subject. On a third level, group analyses (mixed effects) were conducted to study the overall neural stress response and to investigate further research questions as stated above and below.

3.4.2 Hierarchical analysis strategy

In the following, a detailed description of the analysis steps regarding the whole-brain analyses of the Scan*STRESS* data is given that realized the analysis of the aforementioned study aims. No further description focusing on region of interest (ROI) analyses is given. Instead, in the following chapters, information about ROI-analyses is provided where relevant.

To account for the two runs of Scan*STRESS*, first-level analysis GLMs were defined for each subject regarding both runs separately. For the data of both runs, the first five EPI images volumes were discarded to allow for T1 equilibration. Registration to high resolution structural and standard space images was carried out using FLIRT (Jenkinson, Bannister, Brady, & Smith, 2002; Jenkinson & Smith, 2001). Registration from high resolution structural to standard space was then further refined using FNIRT nonlinear registration (Andersson, Jenkinson, & Smith, 2007). The following pre-statistics processing were applied: motion correction using MCFLIRT (Jenkinson et al., 2002), slice-timing correction using Fourier-space time-series phase-shifting, non-brain tissue

removal using BET (Smith, 2002), spatial smoothing using a Gaussian kernel of Full Width at Half Maximum (FWHM) 8.0 mm, grand-mean intensity normalization of the entire 4D dataset to Montreal Neurological Institute (MNI) 152 space by a single multiplicative factor, highpass temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma = 120.0 s). The GLM method used in first-level data is FILM (FMRIB's Improved Linear Model) (Woolrich et al., 2001) using a robust and accurate nonparametric estimation of time-series autocorrelation to prewhiten each voxel's time-series improving estimation efficiency. The *z* (Gaussianized *t*/*F*) statistic images on the first-level analysis for each run were thresholded nonparametrically using clusters determined by $z > 3.1$ and a (corrected) cluster significance threshold of $p < .05$ (Worsley, 2001). The GLM for each run contained regressors for control and stress conditions and the respective announcement phases. In sum, twelve regressors resulted: six conditions (stress arithmetic subtraction, stress figure rotation, control numbers, control figures, announcement of stress and announcement of control) and six motion regressors. Contrast images of the stress versus control condition were analyzed in one-sample *t*-tests as well as multiple regression analyses. For the main task effects (stress > control, control > stress) correction was performed over the whole-brain, with each contrast thresholded at familywise error (FWE) $p < .025$ (two-tailed combined test, FWE $p < .05$).

For combining these first-level analyses of each subject, a higher level analysis (second level, $z > 3.1$) was used that is capable of analyzing across sessions or across subjects. Here, the aim was to measure the mean response of each subject to the main task effects (stress > control, control > stress) using fixed-effects higher modelling. Compared to mixed-effects higher modelling, fixed-effects modelling is more sensitive to activation and restricted in the inferences that can be drawn from its results. Fixed-effects analysis ignores cross-subject (or cross session) variance and therefore, observed activation is with respect to the two runs of Scan*STRESS* for each subject not representative of the wider population. Therefore, only the within-subjects variance is taken into account. Hierarchically, this second-level analysis was implemented to analyze across the two runs of the paradigm and then mixed-effects higher modelling was performed on a third level. In contrast, mixed-effects modelling does model the between-subjects variability and therefore allows inferences to be made about the wider population from which the participants were drawn (Woolrich et al., 2004). In general, for higher level analyses FEAT uses FLAME (FMRIB's Local Analysis of Mixed-effects).

On a third level, a group analysis (mixed effects, $z > 3.1$) was conducted to study the overall neural stress response. For the main task effects (stress > control, control > stress) correction was performed over the whole-brain, with each contrast thresholded at FWE $p < .025$ (two-tailed combined test, FWE $p < .05$). As stated above, one of the aims of the present thesis was to investigate the association between cortisol and neural stress responses. Hence, a single-group average with additional covariate analysis was performed, measuring positive linear relationships between a behavioral variable (e.g., reaction times, age) and BOLD activation. Here, this GLM used individual cortisol increases (grand mean centered) in response to Scan*STRESS* (stress > control, $z > 2.3$, FWE $p < .05$) as a covariate. Moreover, as stress-driven neural gender differences were also of interest, an unpaired two-group difference analysis ($z > 2.3$) was applied. Thus, two group memberships were specified (women vs. men), and different variances for different groups of subjects were estimated to investigate gender-specific (men > women, women > men) neural responses for the main task effects (stress > control, control > stress). As an extension, an unpaired two-group analysis with continuous covariate interaction (grand mean centered, $z > 2.3$) was performed to examine if the linear relationships between neural stress responses (stress > control, control > stress) and cortisol increases (continuous covariate) differ between women and men (men > women, women > men). This model considers mean cortisol stress response differences between women and men. Corrections were performed over the whole-brain with each contrast thresholded at FWE $p < .025$ (two-tailed combined test, FWE $p < .05$).

3.4.3 Exposure-time effects

As an exploratory analysis, the present thesis pursued to investigate if activation changes occur when comparing neural responses to the first compared with responses to the second run of Scan*STRESS*. Hence, the question was raised if neural stress responses do change over the course of the relatively long stress protocol. To examine these potential exposure-time effects, another GLM as group analysis ($z > 3.1$) was conducted directly after first-level analysis including *run* as regressor. This allowed to identify regions responding differently to the two Scan*STRESS* runs in the whole sample by relying on the comparison of each subject's response to the first and second run. Moreover, gender-specific activation changes were also of interest and therefore a whole-brain two-way mixed-effects analysis of variance (ANOVA) was performed (two groups (women, men),

two runs per subject, $z > 3.1$, FWE $p < .05$). As stated above, information about ROI-analyses in this context are presented where relevant.

In general, it could be argued that these potential exposure-time effects simply reflect limited reliability of the paradigm. However, Smith et al. (2005) reanalyzed 33 supposedly identical fMRI sessions from the same subject to investigate the nature of session variability (McGonigle et al., 2000). Moreover, they compared the different preprocessing, registration, and time-series statistical options available in FEAT implemented in FSL and another software for the analysis of fMRI data, called SPM (Statistical Parametric Mapping). The authors showed that owing to the preprocessing modules comprised in FEAT, less additional session variability was induced by the FSL based software, suggesting higher accuracy. As similar results were reported in Bianciardi, Cerasa, and Hagberg (2003), utilizing FSL for the present research questions should be beneficial.

CHAPTER 4

Henze, G.-I., Konzok, J., Kreuzpointner, L., Bärtl, C., Peter, H., Giglberger, M., Streit, F., Kudielka, B. M., Kirsch, P. & Wüst, S. (2020). Increasing deactivation of limbic structures over psychosocial stress exposure time. *Biological Psychiatry: Cognitive Neuroscience and Neuroimaging*. https://doi.org/10.1016/j.bpsc.2020.04.002

Gina-Isabelle Henze and Stefan Wüst developed the study concept and study design. Gina-Isabelle Henze and Julian Konzok performed data collection. Gina-Isabelle Henze performed data analysis and drafted the manuscript. Julian Konzok, Ludwig Kreuzpointner, Christoph Bärtl, Hannah Peter, Marina Giglberger, Fabian Streit, Brigitte M. Kudielka, Peter Kirsch & Stefan Wüst provided critical revisions.

4 INCREASING DEACTIVATION OF LIMBIC STRUCTURES OVER PSYCHOSOCIAL STRESS EXPOSURE TIME

4.1 Abstract

Understanding the interplay between CNS and HPA axis responses to stress in humans is assumed to be essential to contribute to the central question of stress research, namely how stress can increase disease risk. Therefore, the present study used a neuroimaging stress paradigm to investigate the interplay of three stress response domains. Furthermore, we asked if the brain's stress response changes over exposure-time.

In an fMRI study, changes in brain activation, cortisol levels, affect, and heart rate in response to an improved Scan*STRESS* protocol were assessed in 67 young, healthy participants (31 women).

Stress exposure led to significant increases in cortisol levels, heart rate, and negative affect ratings as well as to activations and deactivations in (pre)limbic regions. When cortisol increase was used as a covariate, stronger responses in hippocampus, amygdala, mPFC, and cingulate cortex were observed. Responses within the same regions predicted negative affect ratings. Remarkably, an increasing deactivation over the two Scan*STRESS* runs was found, again, in the same structures. A reanalysis of an independent sample confirmed this finding.

For the first time, reactions in a cluster of (pre)limbic structures were consistently found to be associated with changes in cortisol and negative affect. The same neural structures showed increasing deactivations over stress exposure-time. We speculate that investigating possible associations between exposure-time effects in neural stress responses and stress-related interindividual differences (e.g., chronic stress) might be a promising new avenue in stress research.

4.2 Introduction

Animal models have contributed extensively to our knowledge of the interplay of the CNS and the HPA axis in response to stress. For example, limbic brain structures including the hippocampus, amygdala, and PFC, were found to integrate the appraisal of potential stressors and HPA axis activation (Herman et al., 2005). However, to further elucidate the processes underlying a dysregulation of this complex interplay and to better understand how stress can increase disease risk in vulnerable individuals, studying stress regulation in the human brain is essential.

Successfully studying the CNS/HPA axis interplay requires protocols that reliably induce both neural and robust HPA axis responses. The first paradigm that was shown to convincingly induce psychosocial stress in an fMRI environment is the MIST (Dedovic et al., 2005). A recent development by our group is the Scan*STRESS* paradigm (Streit et al., 2014), predominantly aiming at inducing social-evaluative threat and uncontrollability as stress-inducing psychological components (Dickerson & Kemeny, 2004; Kirschbaum et al., 1993). Both paradigms were employed successfully in several studies, and significant mean cortisol increases as well as heart rate increases were found (Noack et al., 2019). However, the development of imaging stress paradigms is still in its infancy, and both paradigms are not flawless. Observed HPA axis responses were not always fully convincing in studies that used the MIST (Chung et al., 2016a; Chung et al., 2016b; Gossett et al., 2018; Kogler et al., 2015; Kogler et al., 2017) or Scan*STRESS* (Lederbogen et al., 2011; Streit et al., 2014), and neural activation patterns did show some variability across studies. Moreover, while increases in self-reported stress ratings have been found (Akdeniz et al., 2014; Chung et al., 2016a; Chung et al., 2016b; Kogler et al., 2015; Kogler et al., 2017; Lederbogen et al., 2011; Shermohammed et al., 2017; Wheelock et al., 2016), only one study revealed associations with neural responses (Orem et al., 2019).

Key pathways involved in stress processing that have been clearly identified in animal models are the SN and the CEN (Hermans et al., 2014). Moreover, animal studies provided evidence for inhibitory effects of the hippocampus and mPFC, along with activating effects of the amygdala on HPA axis activity (Jankord & Herman, 2008). Also, in humans, the SN, including, among others, the insula, dorsal ACC, amygdala, and temporal pole, has been shown to be involved in stress processing (Akdeniz et al., 2014; Henckens et al., 2012; Khalili-Mahani et al., 2010; Pruessner et al., 2008). However, there

are also significant differences between animal models and human stress research. For instance, several studies in humans failed to find a distinct CEN activation in response to psychosocial stress (Akdeniz et al., 2014; Boehringer et al., 2015; Khalili-Mahani et al., 2010; Lord, Steiner, Soares, Carew, & Hall, 2012; Pruessner et al., 2008). Van Oort et al. (2017) suggested that this difference could partly be explained by the stress-eliciting components of the paradigms; negative feedback, as a typical component in human stress paradigms, may not result in a pronounced CEN activation. Instead, key-regions of the DMN are addressed under psychosocial stress, namely the mPFC, PCC, and angular gyrus (Quaedflieg et al., 2015; Vaisvaser et al., 2013; Vaisvaser et al., 2016; Van Oort et al., 2017; Veer et al., 2012). Remarkably, other regions involved in stress processing, like the parahippocampal gyrus and hippocampus, are strongly related to the DMN (Van Oort et al., 2017).

Additionally, relatively stable findings in animal models (Hermans et al., 2014) are not necessarily stable in humans. For example, while an involvement of (pre)limbic areas in stress regulation was repeatedly found in animals, some human studies detected consistent activations (Dedovic et al., 2009c; Kogler et al., 2015; Zschucke et al., 2015), whereas others reported deactivations (Chung et al., 2016b; Dedovic et al., 2005; Pruessner et al., 2008; Wheelock et al., 2016), or both patterns (Akdeniz et al., 2014; Boehringer et al., 2015; Chung et al., 2016a; Dahm et al., 2017; Inagaki et al., 2016; Lederbogen et al., 2011; Streit et al., 2014). Moreover, these response patterns were found to be positively but also negatively associated with cortisol (Akdeniz et al., 2014; Boehringer et al., 2015; Dedovic et al., 2014; Khalili-Mahani et al., 2010; Lederbogen et al., 2011; Pruessner et al., 2008). In sum, it can be speculated that the regulatory role of (pre)limbic brain circuits in stress integration of humans is modulated by various factors, including differential processing of complex psychological stress components owing to distinct interindividual differences, as well as stressor type or context variables.

The present study had two aims. First, we intended to test the assumption that the application of an improved stress induction protocol will reveal more stable associations between the CNS and HPA axis, as well as reveal more psychological responses to acute stress, than was previously reported. Among others, this improvement aimed at inducing a more robust HPA axis activation. Second, we asked if neural stress responses do change over the course of our relatively long stress protocol, which is characterized by repeated forced failure and a potentially accumulating experience of social-evaluative threat. Assuming that such an exposure-time effect exists, it may indicate habituation or

sensitization processes, and interindividual differences in this shift could possibly be a relevant marker of central stress regulation. This is certainly a speculative hypothesis, but the first evidence in support of this idea comes from rsFC studies that reported stress-driven changes in FC over time (Quaedflieg et al., 2015; Vaisvaser et al., 2013; Veer et al., 2012). Moreover, the existence of an exposure-time effect was also suggested by exploratory findings of task-related fMRI studies (Dahm et al., 2017; Sinha, Lacadie, Constable, & Seo, 2016).

4.3 Methods and materials

4.3.1 Participants

Participants were recruited via flyers and social media internet platforms. Sixty-seven young, healthy, scanner-naïve volunteers (31 women, 36 men, mean age 23.06 ± 3.14 years, mean body mass index: 22.69 ± 2.93 kg/m^2) participated in the present study. Owing to HPA axis activity differences depending on menstrual cycle phase and hormonal contraceptives use (Kudielka & Kirschbaum, 2005; Zänkert et al., 2019), only women using OCs were tested. Furthermore, individuals who met any of the following criteria were excluded: self-reported history of or current psychiatric, neurological, or endocrine disorders; treatment with psychotropic medications or any other medication affecting CNS or endocrine functions; daily tobacco (> 5 cigarettes per day) or alcohol use; incompatibility with fMRI scanning (e.g., metal parts, pregnancy); regular night-shift work; or a current stressful episode. All participants provided written informed consent and received a monetary compensation. The study was approved by the local ethics committee of the University of Regensburg.

4.3.2 General procedure and statistical analysis of cortisol, affect, and heart rate data

To induce psychosocial stress in the fMRI environment, the Scan*STRESS* paradigm was applied (Streit et al., 2014); see Chapter 3 for details. Briefly, Scan*STRESS* is composed of a block design, presented in two runs, containing two conditions (stress vs. control) prompting the subject to perform arithmetic and rotation tasks while the feedback-giving observation panel is presented via live video stream. However, the protocol was slightly modified without changing the paradigm itself. First, we implemented a prolonged (45 minutes) relaxing phase prior to stress. During this phase, a neutral movie was presented. Second, 45 minutes prior to stress, participants received a sugary drink (75 g glucose in

200 ml herbal tea) to facilitate cortisol reactivity (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997; Zänkert et al., 2020). Third, to achieve a more abrupt passage from relaxation to stress exposure, the duration of the participant's transition into the scanner tube was optimized (< 10 minutes), and the observation panel was presented to the subject immediately before paradigm onset. During relaxation, the participants completed a brief training session of the Scan*STRESS* control blocks. After immersion into the scanner tube and subsequent premeasurements (localizer, etc.), participants were confronted with the demanding tasks immediately after receiving the instructions of the panel via live video stream and audio system. After the first run, a saliva sample was collected, and the experimenter announced that the panel was unsatisfied with the participant's performance. After completion of the fMRI scans (65 minutes in total) that also included a multiband RS sequence (results not reported in the present thesis) as well as anatomical measurements, participants remained in the laboratory to fill out questionnaires. Last, they received a detailed debriefing. Test sessions took place between 1:00 and 6:00 PM.

Saliva samples for cortisol assessment were collected at ten time points (t = -75, -15, -1, +15, +30, +50, +65, +80, +95, +110 minutes) using the Cortisol Salivette (Sarstedt, Nuembrecht, Germany). To collect samples at minutes -1 to +65, the experimenter, wearing medical gloves, gave the salivette swab to the participant lying in the scanner. Throughout the procedure, mood state was compiled at the same ten time points using the German version of the PANAS scale (Watson et al., 1988). Saliva samples were assayed in duplicate using a time-resolved fluorescence immunoassay with fluorometric end-point detection (DELFIA [dissociation-enhanced lanthanide fluorescence immune-assay]) at the biochemical laboratory at the University of Trier (Dressendörfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992); see Supplemental Methods for details. The intra-assay coefficient of variation was between 4.0 % and 6.7 %, inter-assay coefficients of variation were between 7.1 % and 9.0 %. During Scan*STRESS*, heart rate recordings were obtained with an MRI-compatible finger oximeter (Model 7500 FO; Nonin Medical, Plymouth, USA) on the index finger, with a sampling rate of the highest heart beat within four seconds.

Data were analyzed in IBM SPSS Statistics version 25 (IBM Corp., Armonk, NY) using repeated-measures ANOVAs with cortisol (nmol/L), heart rate (beats/min), and positive and negative affect (test score) as within-subjects factors. Cortisol responder rates were computed, with an increase of at least 1.5 nmol/L rise being defined as

response (Miller et al., 2013). A cortisol increase was defined as the difference between the individual cortisol peak (sample +30, +50, or +65) and the pre-stress level (sample -1). Mean heart rates were calculated separately for each control and stress block. Greenhouse-Geisser corrections were applied where appropriate, and only adjusted results are reported.

4.3.3 fMRI acquisition and data analysis

Participants were scanned in a Siemens MAGNETOM Prisma 3T MRI scanner (Siemens Healthcare, Erlangen, Germany) equipped with a 64-channel head coil. A series of BOLD gradient EPI images was acquired with the following parameters: repetition time 2000 ms, echo time 30 ms, 90° flip angle, 64 x 64 matrix, 192 mm field of view, 37 3 mm axial slices with 1 mm gap. Data were analyzed using FSL 6.0. The first five EPI volumes were discarded to allow for T1 equilibration. fMRI data processing was carried out using FEAT version 6.0 (see Chapter 3 for details). The *z* (Gaussianized *t*/*F*) statistic images were thresholded nonparametrically using clusters determined by either $z > 3.1$ or $z > 2.3$.

For each subject, GLMs were defined containing regressors for control and stress conditions and the respective announcement phases. GLMs were carried out on three levels: for each subject, one GLM was computed for each run (first level) to account for scanner drifting. Subsequently, a fixed-effects analysis (second level) was obtained to measure mean responses. On a third level, a group analysis (mixed effects) was conducted to study the overall neural stress response, as well as a GLM with individual cortisol increases as a covariate. To examine exposure-time effects, another GLM as group analysis was conducted (after first level) including *run* as regressor.

In sum, twelve regressors resulted: six conditions (stress arithmetic subtraction, stress figure rotation, control numbers, control figures, announcement of stress, and announcement of control) and six motion regressors. Contrast images of the stress versus control condition were analyzed in one-sample *t*-tests as well as multiple regression analyses. For the main task effects (stress > control, control > stress) correction was performed over the whole brain, with each contrast thresholded at FWE $p < .025$ (two-tailed combined test, FWE $p < .05$). The GLM including cortisol increases as covariate (grand mean centered) was conducted for the main task effect, stress > control, thresholded at FWE $p < .05$. Associations with cortisol increase were analyzed within *a-priori* defined anatomical ROIs using masks from the Harvard-Oxford Atlas, resulting in ten masks (Benjamini-Hochberg corrections were applied (Nichols et al., 2017)):

hippocampus (bi- and unilateral), parahippocampal gyrus, amygdala (bi- and unilateral), mPFC, cingulate cortex (ACC and PCC). ROI-analyses were performed using fslmaths and featquery. Moreover, we added mean β-values of these ROIs as additional covariate within repeated-measures ANOVAs with affect ratings (test score) as within-subjects factors (performed in SPSS). To account for exposure-time effects in limbic areas, repeated measures ANOVAs were computed using mean β-values of the first and second run of Scan*STRESS* as within-subjects factors.

In order to replicate our findings in an independent sample, we reanalyzed data from a previous Scan*STRESS* study (Streit et al., 2014) by applying the same analytical approach as described above.

4.4 Results

4.4.1 Manipulation check: cortisol, psychological, heart rate, and neural measures

In response to stress exposure, mean cortisol levels showed a significant rise ($F_{3,171}$ = 10.38, $p < .001$, $\eta^2 = .136$). As expected, men showed higher mean responses ($F_{3,162}$ = 3.33, $p = .028$, $\eta^2 = .045$; see Figure 2A) and higher responder rates than women (men: 74.0 %; women: 26.0 %). Mean increases were also significant in the female subsample ($F_{2,73} = 3.47$, $p = .028$, $\eta^2 = .104$). Additionally, a significant rise in negative affect scores ($F_{4,255} = 47.53$, $p < .001$, $\eta^2 = .422$) and a decrease in positive affect scores ($F_{5,340} = 37.79$, $p < .001$, $\eta^2 = .368$) were observed during Scan*STRESS* (see Figure 2B). As shown in Figure 2C, participants showed increased heart rates during the stress blocks compared to the control blocks in both runs (run 1 [$F_{3,201} = 27.58$, $p < .001$, $\eta^2 = .304$], run 2 [$F_{3,160}$ = 41.48, $p < .001$, $\eta^2 = .417$]). The whole-brain analysis (two-tailed combined FWE-corrected $p < .05$; see Figure 2D) revealed a distributed network of activations and deactivations including (pre)limbic regions such as cingulate cortex (ACC and PCC), thalamus, insula, and mPFC. Peak voxels are reported separately in the Supplemental Results Table 6.

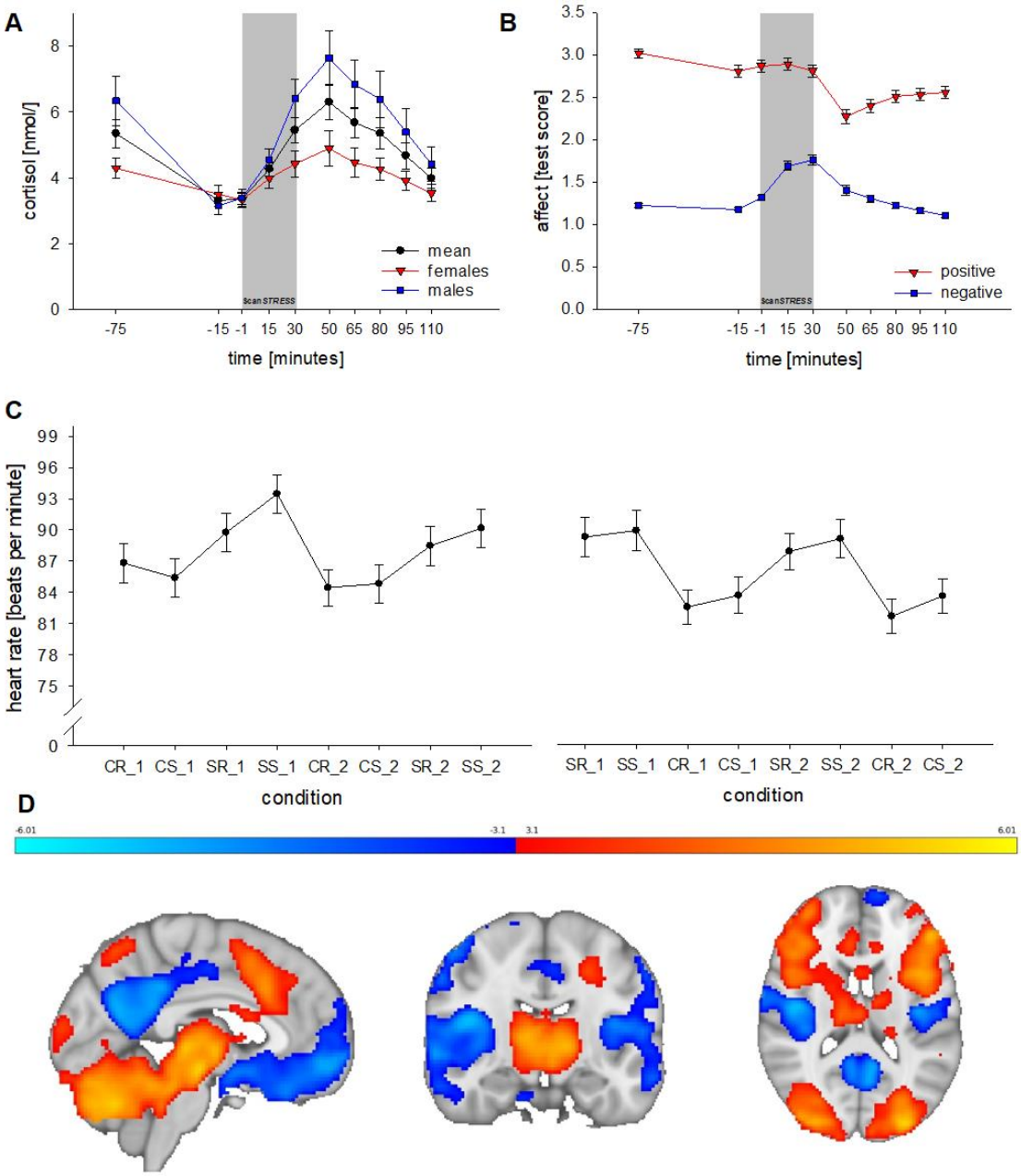

Figure 2. (A) Salivary cortisol responses to Scan*STRESS* in women, men, and the total study sample (± *SEM*). (B) PANAS scale scores throughout the experimental procedure (± *SEM*). (C) Mean heart rate for each control block and stress block over the two runs (± *SEM*). (D) Activations (red to yellow) and deactivations (blue) in response to psychosocial stress induction. CR, control rotation; CS, control subtraction; SR, stress rotation; SS, stress subtraction.

Table 3 depicts mean β-values ± *SD* derived from the contrast stress > control of the *a-priori* defined ROIs hippocampus (bi- and unilateral), parahippocampal gyrus, amygdala (bi- and unilateral), mPFC, and cingulate cortex (ACC and PCC).

Table 3.
β-values of the main task effect of stress > control for hippocampus (bi- and unilateral), parahippocampal gyrus, amygdala (bi- and unilateral), mPFC, and cingulate cortex (ACC and PCC) derived from masks using the Harvard-Oxford Atlas.

		β	
region	subregion	mean	*SD*
hippocampus	bilateral	-.05	.23
	left	-.06	.19
	right	-.05	.23
parahippocampal gyrus		-.12	.34
amygdala	bilateral	-.03	.25
	left	-.02	.27
	right	-.04	.27
mPFC		-.35	.51
cingulate cortex	ACC	.09	.29
	PCC	-.14	.27

4.4.2 Association of cortisol increase and negative affect with neural responses

To account for the association between CNS and HPA axis responsivity to acute psychosocial stress, the individual cortisol increase (grand mean centered) was used as a covariate (FWE-corrected $p < .05$). Cortisol increases significantly predicted neural responses (stress > control) in the left hippocampus, parahippocampal gyrus, amygdala, and insula, as well as in mPFC and PCC (Figure 3A; peak voxels can be seen in Table 7 in the Supplemental Results). In *post-hoc* ROI-analyses, Benjamini-Hochberg corrections were applied to account for increases in false discovery rate. A positive linear relationship between individual cortisol increases and the neural stress response emerged in hippocampus (bilateral [$r = .327$, $p < .01$], left [$r = .303$, $p < .05$], right [$r = .249$, $p < .05$; not significant after correction]), parahippocampal gyrus ($r = .359$, $p < .01$), amygdala (bilateral [$r = .295$, $p < .05$], left [$r = .367$, $p < .001$]), mPFC ($r = .46$, $p < .01$), and PCC ($r = .295$, $p < .05$); values for each ROI are shown in Supplemental Results Table 8.

Furthermore, negative affect ratings rose significantly when mean β-values of hippocampus ($F_{4,248} = 2.77$, $p = .029$, $\eta^2 = .042$), amygdala ($F_{4,245} = 2.61$, $p = .038$, $\eta^2 = .039$), and PCC ($F_{4,252} = 2.96$, $p = .021$, $\eta^2 = .044$) were considered as covariates.

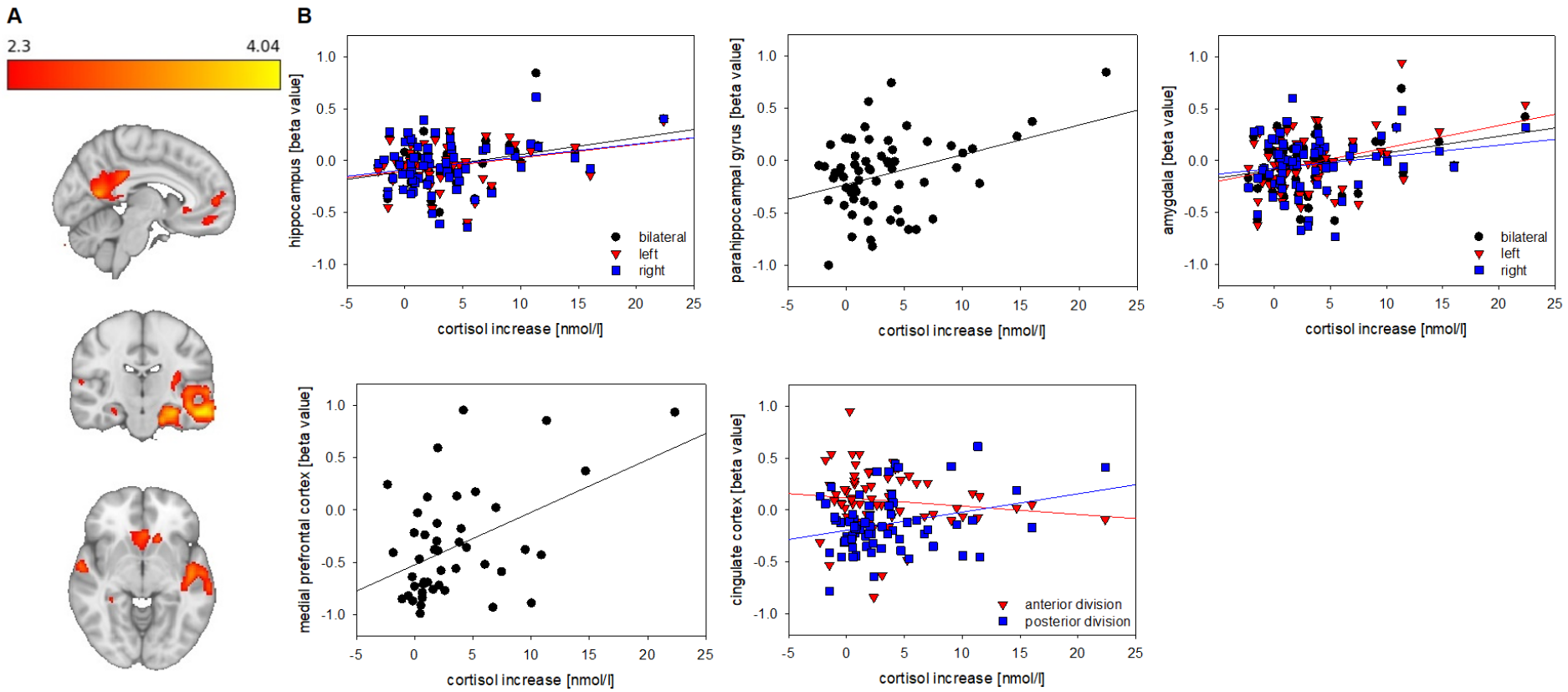

Figure 3. **(**A) Significant cluster of the GLM for the main task effect of stress > control, with cortisol increase (grand mean centered) as an additional covariate. (B) Correlations of cortisol increase and β-values of the main task effect of stress > control in the hippocampus, parahippocampal gyrus, amygdala, mPFC, ACC, and PCC derived from masks using the Harvard-Oxford Atlas.

4.4.3 Increasing deactivation of limbic structures

Areas that showed differential responses to the first run compared with the second run are displayed in Figure 4A, namely hippocampus, parahippocampal gyrus, amygdala, mPFC, and cingulate cortex (ACC and PCC; see Table 9 in the Supplemental Results for peak voxels). A *post-hoc* ROI-analysis (Figure 4B) revealed increasing deactivations over the two runs for the same regions: hippocampus (bilateral [$F_{1,62}$ = 14.38, p < .001, η^2 = .188], left [$F_{1,62}$ = 14.05, p < .001, η^2 = .185], right [$F_{1,62}$ = 22.04, p < .001, η^2 = .262]), parahippocampal gyrus ($F_{1,61}$ = 11.69, p = .001, η^2 = .161), amygdala (bilateral [$F_{1,58}$ = 19.31, p < .001, η^2 = .250], left [$F_{1,59}$ = 25.78, p < .001, η^2 = .304], right [$F_{1,55}$ = 12.12, p < .001, η^2 = .181]), and mPFC ($F_{1,31}$ = 8.13, p = .008, η^2 = .208).

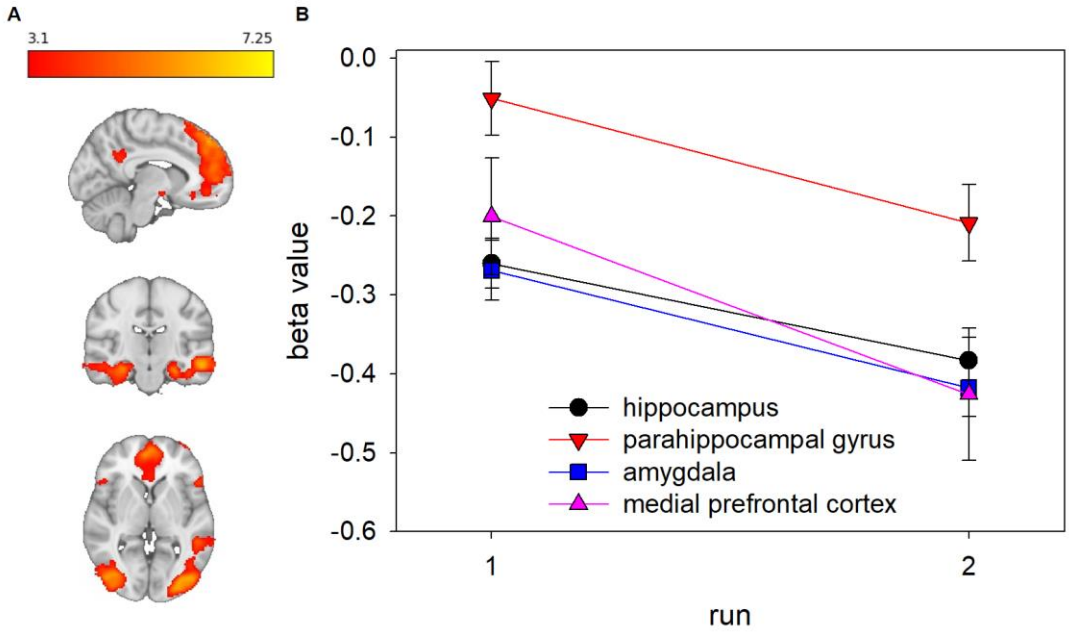

Figure 4. (A) Main effect *run* for areas that showed differential responses to the first and second run of Scan*STRESS*. (B) Mean β-values of activation changes in hippocampus, parahippocampal gyrus, amygdala, and mPFC over the two runs.

Additionally, data recently published by our group (Streit et al., 2014) were reanalyzed by applying the same analysis approach. This analysis confirmed the present findings. A whole-brain analysis revealed that the hippocampus, parahippocampal gyrus, amygdala, mPFC, and cingulate cortex (ACC and PCC) responded differently to the first compared with the second run (see Figure 5A). In *post-hoc* ROI-analyses (Figure 5B), a main effect for run was confirmed for hippocampus ($F_{1,39}$ = 6.76, p = .013, η^2 = .148), amygdala ($F_{1,39}$ = 6.67, p = .014, η^2 = .146), and ACC ($F_{1,33}$ = 5.58, p = .024, η^2 = .145). Further information on peak voxels can be found in the Supplemental Results Table 10.

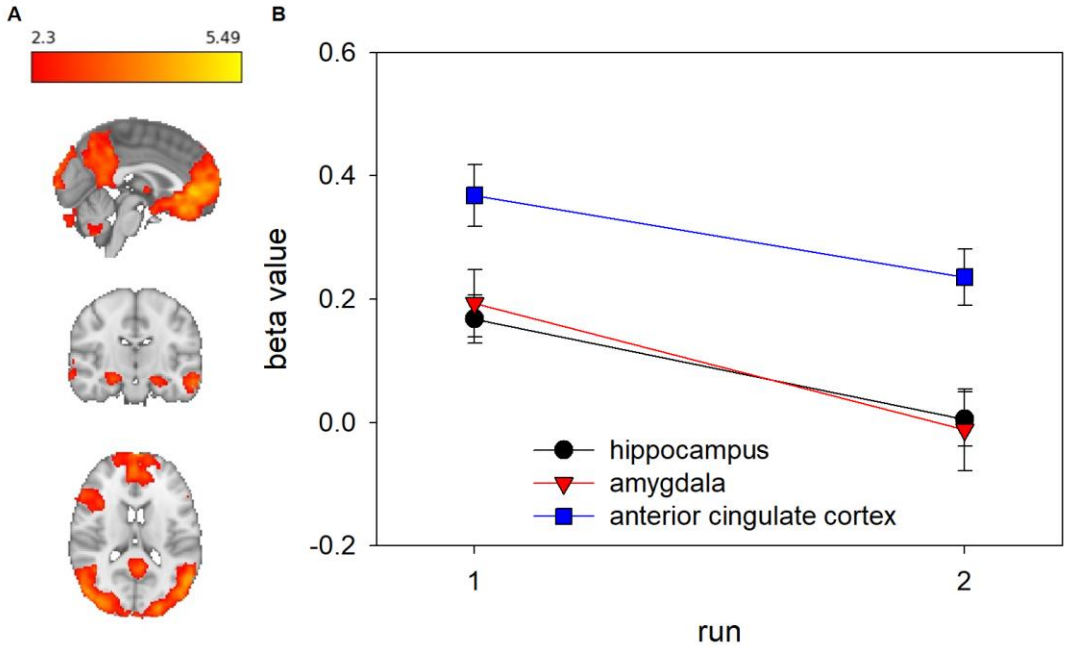

Figure 5. A) Main effect *run* for areas that showed differential responses to the first and second run of Scan*STRESS* for the sample of Streit et al. (2014). B) Mean β-values of activation changes in hippocampus, amygdala, and ACC over the two runs for the sample of Streit et al. (2014).

4.5 Discussion

4.5.1 Improvement of the stress induction protocol

To facilitate the investigation of the CNS/HPA axis interplay, the present study aimed at optimizing Scan*STRESS*. Without modifying the paradigm itself, a longer pre-stress relaxing phase was established, glucose was administered, and a more abrupt stress onset was established to minimize anticipation processes. Overall, our findings support the view that, regarding cortisol responses, these changes have been effective. First, the relaxation phase had the expected consequence, with cortisol levels decreasing from time points -75 to -1 (Figure 2A). Subsequently, mean cortisol levels increased significantly in response to stress exposure. Remarkably, this effect was also significant in women. On average, women showed lower responses than men. This gender difference is a well-established finding in psychosocial stress research. It is particularly pronounced when, as in the present study, women used OCs (Kudielka & Kirschbaum, 2005; Zänkert et al., 2019). Therefore, we conclude that in the present study a more robust cortisol response could indeed be achieved, e.g., compared to previous studies that have used Scan*STRESS*

(Akdeniz et al., 2014; Dahm et al., 2017; Lederbogen et al., 2011; Streit et al., 2017; Streit et al., 2014).

We generally assume that basic characteristics of an fMRI block design, with frequent interruptions of the stress exposure by control blocks, interfere with even more pronounced HPA axis responses. This speculation is supported by findings of relatively distinct cortisol responses to paradigms without such frequent interruptions (Wang et al., 2005). However, while not using an alternating block design may facilitate more pronounced cortisol responses, the detection power of such approaches is significantly limited (Noack et al., 2019; Quaedflieg, Meyer, & Smeets, 2013).

Consistent with Van Oort et al. (2017), the whole-brain analysis revealed that the SN as well as the DMN and related limbic circuits were addressed by stress exposure, whereas the negative feedback component of Scan*STRESS* may explain the lack of responses in the CEN.

The significant increase in negative affect and higher heart rate levels in stress compared with control blocks further document a successful stress induction. The decrease in positive affect with lowest levels after RS measurement and not after Scan*STRESS*, deserves discussion. This scale contains items like *interested*, *excited*, *alert*, and *active*. Thus, it appears not surprising that lowest ratings were found after 18 minutes of RS measurement when participants were asked to lay still, with eyes open, looking at a fixation cross.

4.5.2 Association of cortisol increase and negative affect with neural responses

As outlined above, (partially inconsistent) associations between brain activation changes and cortisol stress responses have been previously found, and first evidence for a correlation between neural and self-reported perceived stress responses has also been observed. However, to the best of our knowledge, the present study is the first to report a consistent effect pattern over three different response domains (Levine & Ursin, 1991): on the one hand, cortisol increases were found to be associated with neural responses in specific structures, and on the other hand, responses in the identical structures were significantly related to subjective stress ratings. Three regions reached significance throughout all analyses, namely the hippocampus, amygdala, and PCC. In detail, we found plausible associations between individual cortisol increases and neural responses – both on whole-brain level and in ROI-analyses – further documenting the importance of (pre)limbic areas, such as hippocampus, amygdala, mPFC, and cingulate cortex, for the

integration of CNS and neuroendocrine stress processing. These regions also seem to be relevant when the relationship between psychological and neural stress responses are scrutinized, as activation patterns in these structures were associated with negative affect ratings throughout the procedure. So far, associations of activation changes (in the mPFC and cingulate cortex) and affect changes in response to psychosocial stress were only found in one MIST study (Orem et al., 2019). McKlveen, Myers, and Herman (2015) emphasized the potential role of the mPFC as the coordinator of behavioral and physiological stress responses across temporal and contextual domains. This assumption is supported by the fact that this area reached significance within all present analyses. In addition, parts of the cingulate cortex reached significance throughout the reported analysis, emphasizing its role as a node in limbic pathways.

Positive as well as negative associations between cortisol responses and responses in single (pre)limbic structures have been previously reported (Akdeniz et al., 2014; Boehringer et al., 2015; Khalili-Mahani et al., 2010; Lederbogen et al., 2011; Pruessner et al., 2008). However, the present findings offer, to a certain extent, a more integrative perspective. Our whole-brain analysis with cortisol increase as an additional covariate revealed a significant cluster comprising the left hippocampus, parahippocampal gyrus, amygdala, insula, mPFC, and PCC. ROI-analyses showed a positive linear relationship between individual cortisol increase and neural stress response for hippocampus, parahippocampal gyrus, amygdala, mPFC, and PCC. Inconsistencies regarding these associations can possibly be explained by methodological differences between studies. Among others, the cortisol increase has been included as a continuous predictor instead of enforcing a dichotomous variable (cortisol responders vs. non-responders) as in earlier work (Dedovic et al., 2009c; Khalili-Mahani et al., 2010; Wheelock et al., 2016), thus potentially increasing statistical power. Moreover, our analysis was based on a relatively large sample and, owing to the robust cortisol responses, on a sample that represented a wider and thus possibly more valid range of neuroendocrine interplay in response to acute stress. Integrating our findings with animal models, it can be stated that our results overall confirm the association between cortisol stress responses and consistent responses of a cluster formed by (pre)limbic structures. However, regarding specific structures, the present findings are partly not in line with those in animals. On the one hand, in contrast to animal models, we detected an amygdala deactivation (see Table 3), but a similar effect was previously found in humans (Dedovic et al., 2009c; Pruessner et al., 2008). On the other hand, the correlation between amygdala and cortisol responses was positive and

thus in line with animal models. For hippocampus, parahippocampal gyrus, mPFC, and PCC, the opposite pattern emerged, with stress-related deactivations (see Table 3) being consistent with animal models (Jankord & Herman, 2008), while again positive correlations with cortisol increases have been found (not consistent with animal studies, see Figure 3). Regarding the hippocampus, previous studies in human subjects indeed reported deactivations to be positively associated with cortisol increases (e.g., Pruessner et al. (2008), consistent with animal models), but for mean stress-related responses, activations as well as deactivations have been found [reviewed in Noack et al. (2019)]. These results document differences between animal models and human stress research, but it appears questionable if they document true species differences in central stress regulation. As outlined above, variability in stress-related response patterns can also be observed within human stress research (Noack et al., 2019). Furthermore, differences between animal and human studies might also be due to systematic methodological differences, including lower stress intensities, which can be applied in humans.

4.5.3 Exposure-time effect

The most prominent finding of the present study is the exposure-time effect on neural stress responses within limbic areas. While whole-brain analysis showed that the limbic cluster responded differently to the first and second Scan*STRESS* run, this effect proved to be an increased deactivation in a subsequent ROI-analysis. Remarkably, this temporal effect, again, emerged in the same regions that were shown to be significantly associated with cortisol and subjective stress responses in our study. Moreover, reanalyzing the data of an independent sample (Streit et al., 2014) confirmed both the exposure-time effect as well as the involvement of virtually the same regions. It could be argued that this difference between runs simply reflects limited reliability of our paradigm, but this appears unlikely. Reanalyzing a previous study (McGonigle et al., 2000), Smith et al. (2005) investigated the variability in fMRI activation patterns when testing a single subject repeatedly with the same paradigms over two months. It was shown that intersession variability is negligible relative to within-session variability, particularly when – as in the present study – different sources of variance are accounted for, when distinct analysis levels are considered, and when analyses are computed with FSL. Hence, although the task itself was the same across Scan*STRESS* runs, the perception of the stressful components has probably changed over time, and likewise the psychological situation, leading to the observed differences. It is tempting to speculate that this

exposure-time effect reflects the limbic response to the repeated experience of failure and social-evaluative threat (Dickerson & Kemeny, 2004). As the response pattern changed from a modest to a rather distinct deactivation, it appears unlikely that these changes represent the neural correlate of a habituation process (Sinha et al., 2016). An influence of cumulating psychosocial stress leading to a sensitization might be more plausible. Limbic structures have been shown to be involved in processes contributing to chronic stress vulnerability (Jovanovic, Perski, Berglund, & Savic, 2011) and to adaptive coping or stress resilience (van der Werff, van den Berg, Pannekoek, Elzinga, & van der Wee, 2013).

Altogether, we introduced an improved Scan*STRESS* procedure and a more sophisticated analysis. For the first time, the present study found that neural reactions in one cluster, comprising hippocampus, amygdala, mPFC, and cingulate cortex, are consistently associated with cortisol increases as well as changes in negative affect. We propose that usability and validity of this paradigm are now sufficiently documented. Thus, it can be recommended for usage in (sub)clinical samples to study the interplay and possible dysregulation of neural, endocrine, and affective responses in chronically stressed individuals or patients with stress-related disorders. However, analogous to the research done with the TSST, a relatively robust stress induction may not be reasonable for all samples [e.g., children or patients with anxiety disorders (Fehlner et al., 2020)].

Remarkably, limbic structures showed an increased deactivation over stress exposure. Assuming that this exposure-time effect may represent a correlate of sensitization processes after ongoing stress exposure is certainly a highly speculative hypothesis. Nevertheless, probing the possible link between exposure-time effects in neural stress responses and, e.g., interindividual differences related to stress vulnerability and resilience, might be a promising new avenue in stress research.

CHAPTER 5

Henze, G.-I., Konzok, J., Kreuzpointner, L., Bärtl, C., Giglberger, M., Peter, H., Streit, F., Kudielka, B. M., Kirsch, P. & Wüst, S. (2020). Gender-specific interaction between cortisol and limbic responses to psychosocial stress. Manuscript under review in *Social Cognitive and Affective Neuroscience*.

Gina-Isabelle Henze and Stefan Wüst developed the study concept and study design. Gina-Isabelle Henze and Julian Konzok performed data collection. Gina-Isabelle Henze performed data analysis and drafted the manuscript. Julian Konzok, Ludwig Kreuzpointner, Christoph Bärtl, Marina Giglberger, Hannah Peter, Fabian Streit, Brigitte M. Kudielka, Peter Kirsch & Stefan Wüst provided critical revisions.

5 GENDER-SPECIFIC INTERACTION BETWEEN CORTISOL AND STRIATO-LIMBIC RESPONSES TO PSYCHOSOCIAL STRESS

5.1 Abstract

Although women and men differ in psychological and endocrine stress responses as well as prevalence rates of stress-related disorders, knowledge on gender differences regarding stress regulation in the brain is scarce.

Therefore, we performed an in-depth analysis of data from 67 healthy participants (31 women, taking OCs), who were exposed to the Scan*STRESS* paradigm in an fMRI study. Changes in cortisol, affect, heart rate, and neural activation in response to psychosocial stress were examined in women and men as well as potential gender-specific interactions between stress response domains.

Stress exposure led to significant cortisol increases with men exhibiting higher levels than women. Dependent on gender, cortisol elevations were differently associated with stress-related responses in striato-limbic structures: Higher increases were associated with activations in men but with deactivations in women. Regarding affect or heart rate responses, no gender differences emerged.

Although women and men differ in their overall stress reactivity, our findings do not support the idea of definable and distinct neural networks as base of this gender difference. Instead, we found differential stress reactions for women and men in identical structures. We propose considering quantitative predictors like gender-specific cortisol increases when exploring neural response differences of women and men.

5.2 Introduction

On average, women and men show various differences in variables related to the CNS, including neuroanatomical, autonomic, and psychological variables; consistently, a gender-specific genetic architecture was found for several CNS-related phenotypes (David et al., 2018; McCarthy, Nugent, & Lenz, 2017). With the advent of human brain-imaging techniques, gender differences in the brain have been further elucidated, covering anatomical variables (gray matter volume, cortical thickness) as well as neural correlates in affect and cognitive functions (verbal and spatial abilities) (Cahill, 2006; Choleris, Galea, Sohrabji, & Frick, 2018; Grabowska, 2017).These findings are paralleled by the clear gender difference in prevalence rates for stress-related mental disorders (Bangasser & Valentino, 2014; Kudielka & Kirschbaum, 2005). However, the distribution of variables like FC or neuroanatomical variables were reported to be widely overlapping in women and men (Grabowska, 2017; Joel et al., 2015; Joel & Fausto-Sterling, 2016; Joel, Garcia-Falgueras, & Swaab, 2020; Joel et al., 2018). Although these findings have been called into question for methodological reasons (Chekroud, Ward, Rosenberg, & Holmes, 2016; Del Giudice et al., 2015; Del Giudice et al., 2016; Rosenblatt, 2016), they challenge the idea of clear gender dimorphisms. Therefore, it is a plausible and relevant question, to what extent gender differences in stress regulation and stress-related psychopathology can be attributed to gender differences in the brain's response to stress exposure.

Women tend to report more perceived stress, anxiety, and tension during and after acute stress exposure than men (Buske-Kirschbaum et al., 2003; Helbig & Backhaus, 2017; Kelly et al., 2006; Kelly et al., 2008; Kirschbaum et al., 1999; Kirschbaum et al., 1996; Merz & Wolf, 2015). Interestingly, these self-report based differences are consistent with findings in animal models as female rodents show more passive and stress-related behavior in response to stress (Beery & Kaufer, 2015; McEwen & Milner, 2017; Mueller & Bale, 2008; Rincón-Cortés & Grace, 2017; Rincón-Cortés et al., 2019). Moreover, mean corticosterone stress responses are higher in female than in male rodents (Goel et al., 2014; Haleem et al., 1988; Heinsbroek et al., 1991; Kant et al., 1983; Oyola & Handa, 2017; Yoshimura et al., 2003). On the other hand, these sex-differences in HPA axis reactivity in rodents are not consistent with findings in humans, as healthy men show significantly larger adrenocorticotropic hormone and cortisol responses than women to acute psychosocial stress induction (Kirschbaum et al., 1992; Kudielka et al., 2009;

Kudielka & Kirschbaum, 2005; Liu et al., 2017; Nicolson et al., 1997; Seeman et al., 2001; Stroud et al., 2002; Zänkert et al., 2019). The modulating impact of menstrual cycle phases and OCs on HPA axis responses explains these effects only in part. Regarding heart rate responses to stress findings are more inconclusive with some studies reporting gender differences (Emery et al., 2018; Koenig & Thayer, 2016; Seo et al., 2017; Stoney et al., 1987) that partly depended on menstrual cycle phases (Childs et al., 2010; Kudielka et al., 2004a), while others failed to find differences (Kelly et al., 2008; Kirschbaum et al., 1999).

So far, a few attempts have been made to evaluate neural gender differences by implementing distinct stress paradigms in fMRI environments. A perfusion-based fMRI study reported a gender-specific neural activation model featuring primarily striato-limbic activation in women and asymmetric frontal blood flow in men. Moreover, the correlation between these gender-specific activation patterns and salivary cortisol was higher in men (Wang et al., 2007). Regarding gender differences in neural stress processing, (pre)limbic structures seem to be of particular relevance since dissociations between women and men for these regions have been reported (Goldfarb et al., 2019; Kogler et al., 2015; Seo et al., 2017; Seo et al., 2011). Focusing on the amygdala, rsFC studies emphasized gender-specific responses in limbic circuits. Furthermore, associations between FC and cortisol were also found to differ significantly between genders (Henckens, van Wingen, Joels, & Fernandez, 2010; Kogler et al., 2016; Vaisvaser et al., 2013; Veer et al., 2012; Vogel et al., 2015). To date, investigations into gender differences regarding neural processing of psychosocial stress remain scarce and they yielded mixed results (Noack et al., 2019). While some evidence for stress-induced neural response differences between women and men exists (Chung et al., 2016b; Dahm et al., 2017; Kogler et al., 2015; Kogler et al., 2017), no consistent gender-specific neural response-pattern emerged.

The heterogeneity of previous findings can probably – at least in part – be explained by methodological disparities resulting from different stress induction paradigms. Studies varied in dependent variables (endocrine, subjective, cardiovascular) as well as in stress intensity, and thereby in the magnitude of stress responses (Noack et al., 2019). A recent study by our group (Henze et al., 2020b) aimed at elucidating the interaction of distinct stress activation systems in response to an improved psychosocial fMRI stress protocol (Streit et al., 2014). We found significant cortisol, subjective, heart rate, and neural reactions in response to Scan*STRESS* (Henze et al., 2020b). Moreover, neural stress

reactions in (pre)limbic structures were associated with individual changes in cortisol and negative affect ratings. Given the robust cortisol responses and the consistent interactions between different stress response domains within a relatively large cohort, it appeared promising to further elaborate the role of gender within the same sample. Therefore, the objective of the present study was a detailed and comprehensive analysis of gender-related stress response differences in distinct response domains including neural and cortisol responses as well as changes in heart rate and affect. We applied a statistical model using individual cortisol increases as a continuous predictor to examine stress-related gender differences in the brain in more quantitative rather than qualitative ways (David et al., 2018; Grabowska, 2017; Joel et al., 2015; Joel & Fausto-Sterling, 2016; Joel et al., 2020; Joel et al., 2018). This model assumed that differences in neural stress processing between women and men might emerge when gender-related cortisol response differences are taken into account.

5.3 Methods and materials

5.3.1 Participants

Sixty-seven young, healthy, scanner-naïve volunteers (mean age 23.06 ± 3.14 years) participated in the present study. Stress-induced cortisol, affect, heart rate, and neural responses of the present sample have been previously reported (Henze et al., 2020b). It consisted of 31 women (mean age 22.10 ± 2.12 years) and 36 men (mean age 23.89 ± 3.64 years). Owing to HPA axis activity differences depending on menstrual cycle phase and OC-use (Kudielka & Kirschbaum, 2005; Zänkert et al., 2019), only women using OCs were tested. Participants were recruited via flyers and social media internet platforms. Individuals who met any of the following criteria were excluded: self-reported history of or current psychiatric, neurological, or endocrine disorders; treatment with psychotropic medications or any other medication affecting CNS or endocrine functions; daily tobacco (> 5 cigarettes per day) or alcohol use; incompatibility with fMRI scanning (e.g., metal parts, pregnancy); regular night-shift work; or a current stressful episode. All participants provided written informed consent and received a monetary compensation. The study was approved by the local ethics committee of the University of Regensburg.

5.3.2 General procedure and statistical analysis of cortisol, affect, and heart rate data

To induce psychosocial stress in the fMRI environment the Scan*STRESS* paradigm was applied (Streit et al., 2014); see Chapter 3 for details. Briefly, Scan*STRESS* is composed of a block design, presented in two runs, containing two conditions (stress vs. control) prompting the participants to perform arithmetic and rotation tasks while a feedback-giving observation panel is presented via live video stream providing disapproving feedback. Moreover, between the two runs, the participants are notified that their performance was below average and they have to improve in the second run. For the present study, the protocol was slightly modified without changing the paradigm itself. We implemented a prolonged (45 minutes) relaxing phase prior to stress, administered a sugary drink (75 g glucose in 200 ml herbal tea) to facilitate cortisol reactivity (Gonzalez-Bono et al., 2002; Zänkert et al., 2020), and we achieved a more abrupt passage (< 10 minutes) from relaxation to stress exposure [details see (Henze et al., 2020b)]. Test sessions took place between 1:00 and 6:00 PM.

Saliva samples for cortisol assessment were collected at ten time points (t = -75, -15, -1, +15, +30, +50, +65, +80, +95, +110 minutes) using 'Cortisol Salivettes' (Sarstedt, Nuembrecht, Germany). To collect samples at minutes -1 to +65, the experimenter, wearing medical gloves, gave the salivette swab to the participant lying in the scanner. Mood state was compiled at the same ten time points using the German version of the PANAS scale (Watson, Clark, & Tellegen, 1988). Saliva samples were stored at -20°C until analysis. Samples were assayed in duplicate using a time-resolved fluorescence immunoassay with fluorometric end-point detection (DELFIA) at the biochemical laboratory of the University of Trier (Dressendörfer et al., 1992); see Supplemental Methods for details. The intra-assay coefficient of variation was between 4.0 % and 6.7 %; inter-assay coefficients of variation were between 7.1 % and 9.0 %. During Scan*STRESS*, heart rate recordings were obtained with an MRI-compatible finger oximeter (Model 7500 FO; Nonin Medical, Plymouth, USA) on the index finger, with a sampling rate of the highest heart beat within four seconds.

Data were analyzed in IBM SPSS Statistics version 25 (IBM, Corp., Armonk, NY) using repeated-measures ANOVAs regarding cortisol (nmol/L), positive and negative affect (test score), and heart rate (beats/min) with *time* as within-subjects factor and *gender* as between-subjects factor. A cortisol increase was defined as the difference between the individual cortisol peak (sample +30, +50, +65) and the pre-stress cortisol

level (sample -1). Mean heart rates were calculated separately for each control and stress block. Greenhouse-Geisser corrections were applied where appropriate, and only adjusted results are reported.

5.3.3 fMRI acquisition and data analysis

Participants were scanned in a Siemens MAGNETOM Prisma 3T MRI (Siemens Healthcare, Erlangen, Germany) equipped with a 64-channel head coil. A series of BOLD gradient EPI images was acquired with the following parameters: repetition time 2000 ms, echo time 30 ms, 90° flip angle, 64 x 64 matrix, 192 mm field of view, 37 3 mm axial slices with 1 mm gap. Data were analyzed using FSL 6.0. The first five EPI volumes were discarded to allow for T1 equilibration. fMRI data processing was carried out using FEAT version 6.0 (see Chapter 3 for details). The *z* (Gaussianized *t*/*F*) statistic images were thresholded nonparametrically using clusters determined by either $z > 3.1$ or $z > 2.3$.

For each subject, GLMs were defined containing regressors for control and stress conditions and the respective announcement phases. In sum, twelve regressors resulted: six conditions (stress arithmetic subtraction, stress figure rotation, control numbers, control figures, announcement of stress, and announcement of control) and six motion regressors. GLMs were carried out on three levels: for each subject, one GLM was computed for each run (first level, $z > 3.1$) to account for scanner drifting. Subsequently, a fixed-effects analysis (second level, $z > 3.1$) was obtained to measure mean responses. On a third level, unpaired two-group analyses (mixed effects, $z > 2.3$) were conducted to study gender differences: First, an unpaired two-group difference analysis was conducted to study gender-specific (men > women, women > men) neural responses for the main task effects (stress > control, control > stress). Secondly, we performed an unpaired two-group analysis with continuous covariate interaction (grand mean centered) to examine if the linear relationships between neural stress responses (stress > control, control > stress) and cortisol increases (continuous covariate) differ between women and men (men > women, women > men). This model considers mean cortisol stress response differences between women and men. Corrections were performed over the whole brain with each contrast thresholded at FWE $p < .025$ (two-tailed combined test, FWE $p < .05$).

Association analysis with *cortisol increase* were computed within *a-priori* defined striato-limbic anatomical ROIs using masks from the Harvard-Oxford Atlas. We included the following eight masks, as the respective regions have been reported to respond to stress in a gender-specific manner (Chung et al., 2016b; Dahm et al., 2017; Goldfarb et

al., 2019; Kogler et al., 2015; Kogler et al., 2017; Noack et al., 2019; Seo et al., 2017; Seo et al., 2011; Wang et al., 2007): hippocampus, parahippocampal gyrus, amygdala, cingulate cortex, thalamus, ncl. caudatus, ncl. accumbens, and putamen. We applied Benjamini-Hochberg corrections (Nichols et al., 2017) to account for increases in false discovery rate and report uncorrected as well as corrected *p*-values. ROI-analyses were performed using fslmaths and featquery. Mean β-values (extracted from second level analysis) were exported to SPSS and *post-hoc* one-way ANOVAs were computed with *gender* as fixed factor and *cortisol increase* as covariate.

5.4 Results

5.4.1 Gender differences in cortisol, psychological, and heart rate responses

For cortisol measures, we detected a significant *time* x *gender* interaction ($F_{3,162} = 3.33$, $p = .028$, $\eta^2 = .045$ (see Figure 6) as well as significant main effects for *time* ($F_{3,162} = 9.85$, $p < .001$, $\eta^2 = .132$) and *gender* ($F_{1,65} = 6.69$, $p = .012$, $\eta^2 = .093$). While men showed significantly higher cortisol levels than women briefly after they had entered the lab (-75 minutes), levels subsequently decreased and both groups showed similar cortisol concentrations immediately prior to stress onset (-15 minutes). In response to stress exposure men showed significantly higher cortisol responses than women. Results of calculated *post-hoc t*-tests regarding each time point and cortisol increases are shown in Supplemental Results Table 11. When analyzing the two subsamples separately, the main effect *time* reached significance in women ($F_{2,73} = 3.47$, $p = .028$, $\eta^2 = .104$) and men ($F_{2,82} = 8.01$, $p < .001$, $\eta^2 = .188$).

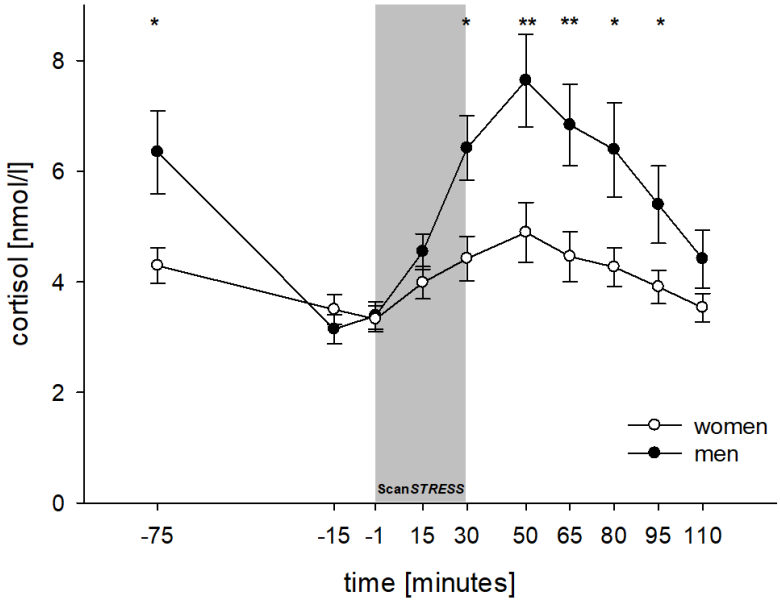

Figure 6. Salivary cortisol responses to Scan*STRESS* in women and men (± *SEM*). ** $p \leq .01$ and * $p \leq .05$ indicate significant results of *post-hoc* unpaired *t*-tests for each time point.

Consistent to our previous analysis (Henze et al., 2020b), we found significant main effects for *time* in affect measures and mean heart rate levels (*p*s $\leq .001$, $\eta^2 > .299$). Positive affect scores decreased and negative affect scores increased during Scan*STRESS*. Participants showed elevated heart rates during the stress blocks compared to the control blocks in both runs. We detected neither significant interactions of *time* x *gender* nor main effects *gender* regarding affect or heart rate measures (*p*s $\geq .153$, $\eta^2 < .020$). Given the absence of gender differences in affect and heart rate reactions, no gender-specific associations with neural responses were analyzed.

5.4.2 Gender-specific associations of cortisol and neural responses

A whole-brain unpaired two-group difference analysis revealed no significant gender-specific cluster for activations (stress > control) nor deactivations (control > stress); two-tailed combined FWE-corrected $p < .05$. However, when cortisol increases were used as covariate in a whole-brain unpaired two-group difference analysis with continuous covariate interaction (grand mean centered, two-tailed combined FWE-corrected $p < .05$), a gender-specific cluster reached significance (see Figure 7). In detail, we detected a gender-specific relationship between cortisol increases and neural responses in the hippocampus, parahippocampal gyrus, cingulate cortex, thalamus, and ncl. caudatus.

When men were compared to women in the total sample, higher cortisol increases were found to be related to more activation within this cluster (see Figure 7A). In the female subsample alone, higher cortisol increases were associated with more deactivation in the cingulate cortex, thalamus, and ncl. caudatus (see Figure 7B), while in the male subsample higher cortisol increases were associated with more activation in the hippocampus, parahippocampal gyrus, amygdala, cingulate cortex, prefrontal areas, ncl. caudatus, and ncl. accumbens (see Figure 7C). Peak voxels are reported in the Supplemental Results Table 12-14.

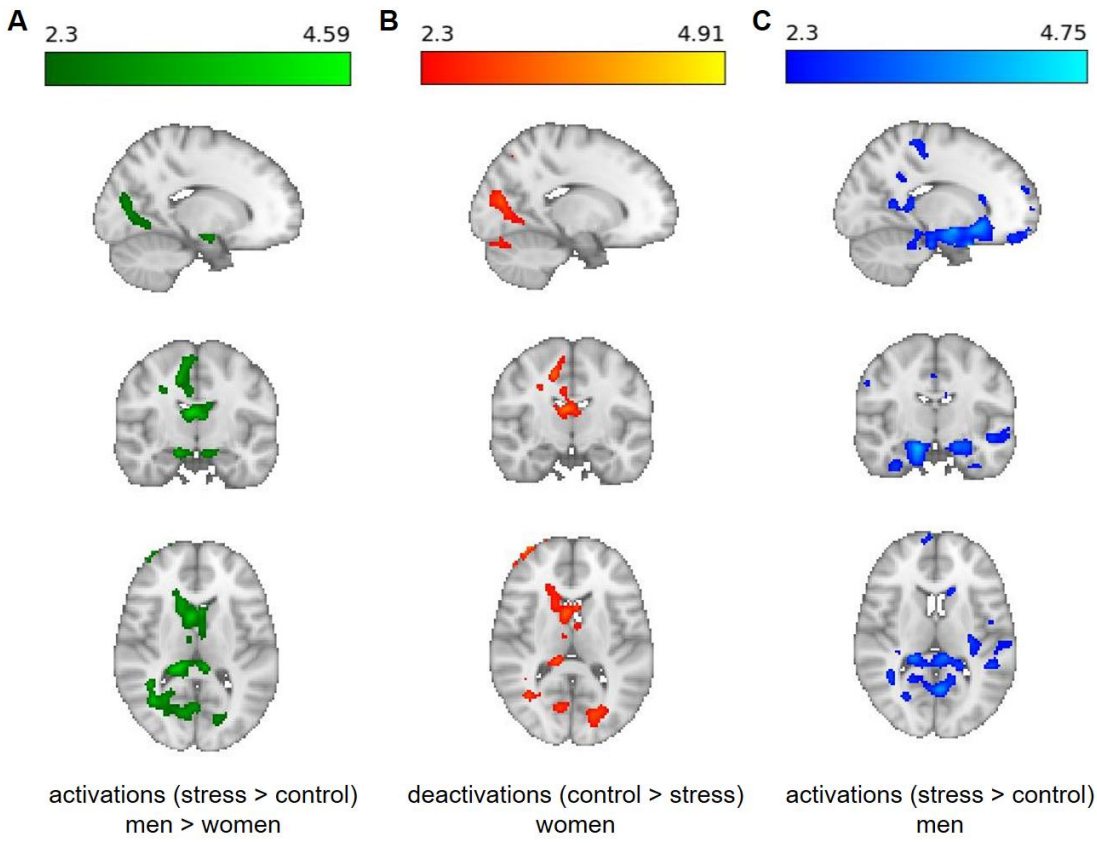

Figure 7. (A) Gender-specific cluster in an unpaired two-group difference analysis with continuous covariate interaction (grand mean centered, two-tailed combined FWE-corrected for whole-brain, threshold $p < .05$) describing a gender-specific relationship (men > women) between cortisol increases and neural responses (stress > control) in the hippocampus, parahippocampal gyrus, cingulate cortex, thalamus, and ncl. caudatus. (B) In women, higher cortisol increases were associated with more deactivation (control > stress) within a cluster including the cingulate cortex, thalamus, and ncl. caudatus. (C) In men, higher cortisol increases were associated with more activation (stress > control) in a cluster comprising the hippocampus, parahippocampal gyrus, amygdala, cingulate cortex, prefrontal areas, ncl. caudatus, and ncl. accumbens.

Table 4 depicts the results from *post-hoc* ROI-analyses including uncorrected and Benjamini-Hochberg corrected *p*-values. We found significant interactions of *gender* x *cortisol increase* for the amygdala, ncl. caudatus, and ncl. accumbens. Men showed positive associations between β-values and *cortisol increases* while women showed negative associations (see Figure 8). Main effects of *gender* were found for the hippocampus, parahippocampal gyrus, amygdala, cingulate cortex, ncl. caudatus, ncl. accumbens, and putamen. *Post-hoc* analyses in the two subsamples separately are displayed in Table 5. In women, we found negative associations between *cortisol increases* and β-values for the thalamus and ncl. caudatus. In men, positive associations emerged for the hippocampus, parahippocampal gyrus, amygdala, cingulate cortex, ncl. caudatus, and ncl. accumbens.

Table 4.
Results from *post-hoc* one-way ANOVAs with *gender* as fixed factor and *cortisol increase* as covariate for the hippocampus, parahippocampal gyrus, amygdala, cingulate cortex, thalamus, ncl. caudatus, ncl. accumbens, and putamen including uncorrected and Benjamini-Hochberg corrected *p*-values (n = 8 ROIs).

ROI	effect	uncorrected values				corrected *p*-value
		df	*F*	*p*-value	η^2	
hippocampus	*gender x cortisol increase*	1, 63	2.04	.158	.031	≤ .04375
	gender	1, 63	6.57	.013	.094	≤ .01875
	cortisol increase	1, 63	3.93	.052	.059	≤ .00625
parahippocampal gyrus	*gender x cortisol increase*	1, 63	3.10	.084	.049	≤ .03125
	gender	1, 63	4.82	.032	.074	≤ .0375
	cortisol increase	1, 63	3.01	.088	.048	≤ .01875
amygdala	*gender x cortisol increase*	1, 63	4.42	.039	.066	≤ .01875
	gender	1, 63	9.46	.003	.131	≤ .0125
	cortisol increase	1, 63	2.10	.152	.032	≤ .025
cingulate cortex	*gender x cortisol increase*	1, 63	3.35	.072	.051	≤ .025
	gender	1, 63	5.52	.022	.081	≤ .025
	cortisol increase	1, 63	0.01	.924	.000	≤ .05

ROI	effect	uncorrected values				corrected *p*-value
		df	*F*	*p*-value	η^2	
thalamus	*gender x cortisol increase*	1, 63	2.98	.089	.046	≤ .0375
	gender	1, 63	2.19	.144	.034	≤ .05
	cortisol increase	1, 63	3.38	.071	.052	≤ .0125
ncl. caudatus	*gender x cortisol increase*	1, 63	6.55	.013	.094	≤ .0125
	gender	1, 63	4.96	.030	.073	≤ .03125
	cortisol increase	1, 63	0.35	.557	.005	≤ .0375
ncl. accumbens	*gender x cortisol increase*	1, 63	8.66	.005	.121	≤ .00625
	gender	1, 63	10.12	.002	.138	≤ .00625
	cortisol increase	1, 63	.075	.391	.012	≤ .03125
putamen	*gender x cortisol increase*	1, 63	1.41	.240	.022	≤ .05
	gender	1, 63	4.17	.045	.062	≤ .04375
	cortisol increase	1, 63	0.07	.798	.001	≤ .04375

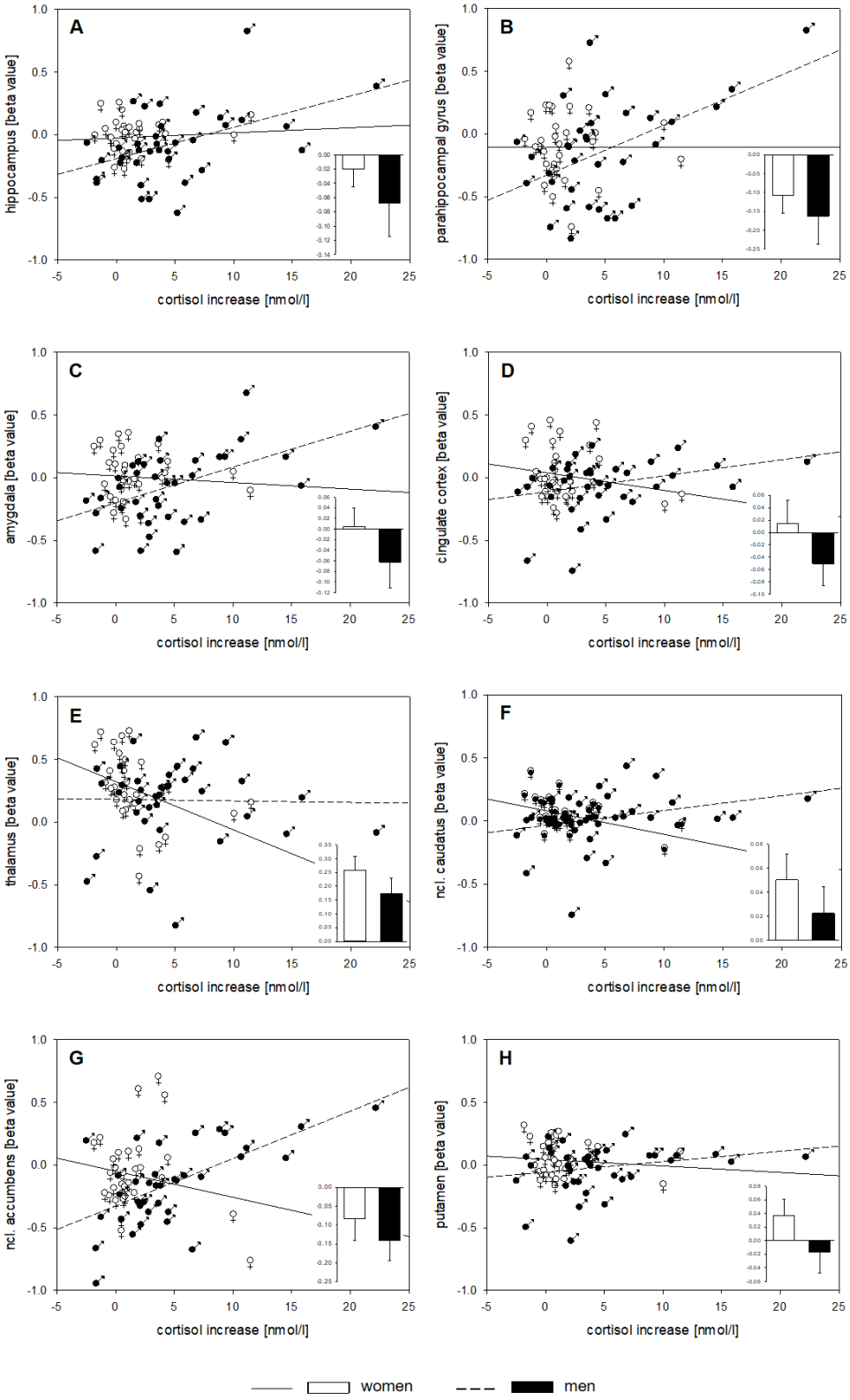

Figure 8. Gender-specific mean neural responses (± *SEM*, stress > control) and correlations of *cortisol increases* with β-values of the main task effect stress > control in the (A) hippocampus, (B) parahippocampal gyrus, (C) amygdala, (D) cingulate cortex, (E) thalamus, (F) ncl. caudatus, (G) ncl. accumbens, and (H) putamen derived from masks using the Harvard-Oxford Atlas.

Table 5.
Results from correlation analyses between mean β-values of the hippocampus, parahippocampal gyrus, amygdala, cingulate cortex, thalamus, ncl. caudatus, ncl. accumbens, and putamen with *cortisol increase* in the two subsamples separately including uncorrected and Benjamini-Hochberg corrected *p*-values (n = 8 ROIs).

ROI	correlation with *cortisol increase*					
	women (n = 31)			men (n = 36)		
	uncorrected values		corrected *p*-value	uncorrected values		corrected *p*-value
	r	*p*-value		*r*	*p*-value	
hippocampus	.087	.321	≤ .0375	.466	.002	≤ .025
parahippocampal gyrus	-.003	.494	≤ .05	.510	.001	≤ .0125
amygdala	-.079	.336	≤ .04375	.516	.001	≤ .0125
cingulate cortex	-.196	.145	≤ .01875	.311	.032	≤ .03125
thalamus	-.395	.014	≤ .0125	-.020	.456	≤ .05
ncl. caudatus	-.449	.006	≤ .00625	.290	.043	≤ .0375
ncl. accumbens	-.193	.149	≤ .025	.621	.001	≤ .00625
putamen	-.116	.266	≤ .03125	.234	.085	≤ .04375

5.4.3 Explorative analysis of gender differences in exposure-time effects

As the research questions of the present study arose from findings of our aforementioned study (Henze et al., 2020b), we also addressed the question if women and men show distinct neural reactions in response to the two runs of Scan*STRESS*. We speculated that *gender* might modulate the previously reported exposure-time effect of (pre)limbic structures, i.e., differences between neural responses in the first run and those in the second run. A whole-brain two-way mixed-effects ANOVA (two groups (women, men), two runs per subject, $z > 3.1$, FWE-corrected $p < .05$) did not reveal a significant *run* x *group* interaction. We found similar clusters in women and men comprising the hippocampus, parahippocampal gyrus, amygdala, PFC, and cingulate cortex to respond differently to the two runs. Figure 9 illustrates these activation changes in both genders; peak voxels are reported in the Supplemental Results Tables 15 and 16. Consistent to our previous analysis (Henze et al., 2020b), *post-hoc* ROI-analyses (repeated measures ANOVAs, *run* as within-subjects factor, *gender* as between-subjects factor) revealed main effects of *run* for the hippocampus, parahippocampal gyrus, and amygdala ($ps \leq .001$, $\eta^2 > .160$). While we did not find significant interactions of *run* x *gender* ($ps \geq .367$, $\eta^2 < .014$); for thalamus, a significant main effect *gender* ($F_{1,61} = 5.22$, $p = .026$, $\eta^2 = .079$) was detected, indicating mean response differences of women and men in the first run (women: $M = -.01$, $SD = .22$; men: $M = -.10$, $SD = .26$) compared to the second run (women: $M = .01$, $SD = .21$; men: $M = -.15$, $SD = .27$) of Scan*STRESS*.

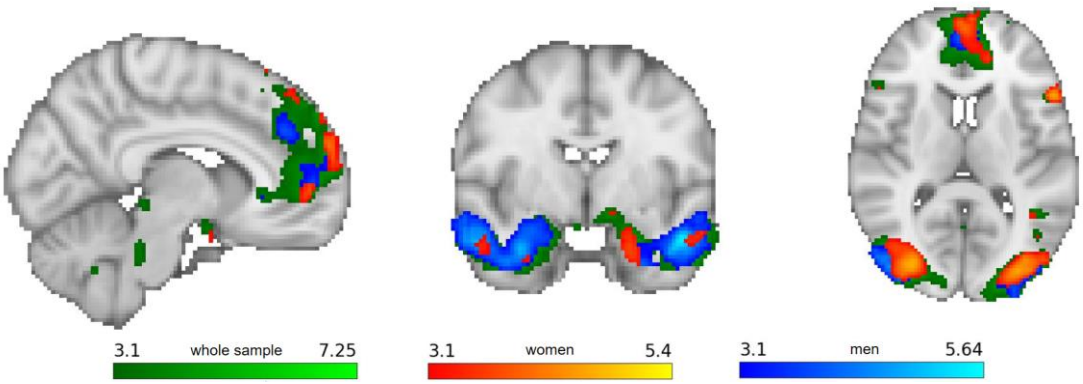

Figure 9. Activation changes over the two runs of Scan*STRESS* of the female (red to yellow) and male (blue to light blue) subsample compared to the total sample as reference [green to light green; Henze et al. (2020b)].

5.5 DISCUSSION

5.5.1 Gender-specific associations of cortisol and neural responses

The present data confirmed the well-known gender-specific cortisol stress response pattern, with men exhibiting higher responses than women (Goel et al., 2014; Kudielka & Kirschbaum, 2005; Liu et al., 2017; Nicolson et al., 1997; Seeman et al., 2001; Zänkert et al., 2019). Higher cortisol levels in men occurred already 75 minutes prior to stress onset, suggesting a more pronounced anticipation response (Kirschbaum et al., 1992; Kudielka & Kirschbaum, 2005). After the relaxation phase, both genders reached similar mean levels (see Figure 6). It should be noted again that all female participants used OCs and that women tested in the luteal phase of their menstrual cycles were repeatedly found to show higher cortisol responses to stress (Kirschbaum et al., 1999; Rohleder, Schommer, Hellhammer, Engel, & Kirschbaum, 2001; Rohleder, Wolf, Piel, & Kirschbaum, 2003; Uhart, Chong, Oswald, Lin, & Wand, 2006; Wolf, Schommer, Hellhammer, McEwen, & Kirschbaum, 2001).

While empirical evidence for consistent interactions of distinct stress domains remains scarce (Campbell & Ehlert, 2012; Cohen et al., 2000; Henze et al., 2020b), this is, to the best of our knowledge, the first study focusing on gender-related differences between associations of cortisol and task-related neural responses to psychosocial stress. Whereas previous findings on gender-specific neural stress responses suggest pronounced striato-limbic activation in women and stronger frontal activation in men (Goldfarb et al., 2019; Kogler et al., 2015; Seo et al., 2017; Seo et al., 2011; Wang et al., 2007), our data revealed no such clear neuroanatomical distinction. Instead, we found different associations for women and men between cortisol reactions and responses of identical striato-limbic structures. A whole-brain analysis in the total sample documented higher cortisol increases in men to be associated with more activation in the hippocampus, parahippocampal gyrus, cingulate cortex, thalamus, and ncl. caudatus, compared to women. In the female subsample, higher cortisol increases were related to deactivation in cingulate cortex, thalamus, and ncl. caudatus. In the male subsample, higher cortisol increases were related to activation in the hippocampus, parahippocampal gyrus, amygdala, cingulate cortex, prefrontal areas, ncl. caudatus, and ncl. accumbens. In contrast to previous findings, proposing a small degree of overlap between the stress networks of women and men (Kogler et al., 2017; Seo et al., 2017; Seo et al., 2011; Wang et al., 2007), our data corroborates differential responses for women and men in identical structures (Goldfarb et al., 2019; Kogler et al., 2016). ROI-analyses confirmed interactions of *gender* x *cortisol increase* for the amygdala, ncl. caudatus, and ncl. accumbens, underpinning positive associations in men

and negative associations in women, with the latter describing lower cortisol increases to be associated with more activation. Thus far, only RS data exists, including two male-only samples (Veer et al., 2011; Vogel et al., 2015) and one mixed sample (Kogler et al., 2016), reporting a positive effect pattern for men and a negative one for women.

The only structure that reached significance in whole-brain as well as every ROI-based correlation analysis, also in both subsamples separately, is the ncl. caudatus. As part of the striatum, this area showed gender-specific FC patterns partly depending on menstrual cycle phase (Hidalgo-Lopez et al., 2020; Yoest, Quigley, & Becker, 2018). Moreover, evidence for bigger amounts of gray matter in female brains within this structure exists (Luders, Gaser, Narr, & Toga, 2009). Another stress-relevant area within the striatum is the ncl. accumbens (McEwen, Nasca, & Gray, 2016). As reward-related area, an expressed desire of revenge in men was found to be correlated with increased activity while in women this was associated with deactivations (Dumais, Chernyak, Nickerson, & Janes, 2018; Singer et al., 2006). In this context, two aspects should be considered: first, when applying Scan*STRESS*, participants are instructed to show maximal effort and that the study aims at investigating brain activations during maximal mental performance. Second, after the first run of Scan*STRESS*, participants are exposed to a standardized negative feedback regarding their performance combined with the urgent request to try harder. Therefore, these factors of psychosocial stress might have led to pronounced reactions of the ncl. accumbens in particular and to an overall striatal response. Moreover, another Scan*STRESS* study that found associations between striatal activation and perceived group discrimination in ethnic minority individuals strengthens the evidence for an involvement of the striatum and inherent structures (Akdeniz et al., 2014). While this view is also supported by a recent study that used a psychosocial stress paradigm (Kogler et al., 2015), another study did not report gender differences in putamen responses during stress perception (Wang et al., 2007). Therefore, it is tempting to speculate that the striatal network modulates gender-specific interactions in response to the repeated experience of failure and social-evaluative threat as induced by Scan*STRESS*. The finding of altered left amygdala FC to striatal regions correlating positively with cortisol in men but negatively in women (Kogler et al., 2016) emphasizes this hypothesis. Moreover, a previously reported analysis of our present data on the association between cortisol and neural stress responses, independent of gender, revealed no significance for striatal structures. This is consistent with the assumption of a gender-specific modulating striatal effect on stress responses (Henze et al., 2020b).

Among others, the hippocampus has been considered as decisive HPA axis related structure ever since (Herman et al., 2005; Hermans et al., 2014; Jankord & Herman, 2008) and one of

the most prominent findings describes deactivations in response to psychosocial stress along with negative associations with cortisol (Pruessner et al., 2008). However, data exists showing the opposite (Henze et al., 2020b; Noack et al., 2019). Here, we found a positive correlation of activations and cortisol in men confirming gender-related differences after stress induction for the hippocampus (Seo et al., 2011; Yagi & Galea, 2019). Moreover, our data showed a comparable pattern regarding the parahippocampal gyrus for men, while in women no significant association with cortisol emerged for hippocampus and parahippocampal gyrus, respectively.

Concerning the amygdala and the cingulate cortex, the dissociation between women and men is more obvious. Especially for the amygdala, its activating impact on HPA axis responses to stress has been reported frequently (Henze et al., 2020b; Herman et al., 2005; Jankord & Herman, 2008; Noack et al., 2019). Previous work showed an association between stress and amygdala activation only in women (Kogler et al., 2015; Wang et al., 2007). We found that activations were associated with higher cortisol increases in men and lower values in women, confirming a gender-specific effect pattern regarding amygdala FC in association with cortisol (Kogler et al., 2016). Moreover, this study also revealed a negative association of altered left amygdala FC to the ACC in women while the opposite was reported in a male-only sample (Veer et al., 2012).

A negative association with cortisol was found for the thalamus in women while in men no significant relationship emerged, confirming previous findings (Wang et al., 2007). The thalamus is thought to actively and dynamically gate salient inputs, minimizing the importance of currently irrelevant ones (Wolff & Vann, 2019). A recent study showed altered thalamic network centrality in response to acute psychosocial stress within a male-only sample (Reinelt et al., 2019). Moreover, previous studies have shown stress-driven changes in thalamic activation in both genders (Noack et al., 2019). With reference to the aforementioned hypothesis on striatal involvement in gender-specific cortisol responses, the thalamus as adjacent structure may act as additional coordinator. Nevertheless, there exists just as much evidence for pronounced thalamo-striatal stress reactions in women (Wang et al., 2007) as in men (Seo et al., 2017; Seo et al., 2011).

5.5.2 Explorative analysis of gender differences in exposure-time effects

As an exploratory analysis, we also addressed the question whether dynamic changes during neural stress responses, previously reported for (pre)limbic structures (Henze et al., 2020b), differ between women and men. Assuming that this exposure-time effect may represent a

correlate of sensitization processes of ongoing stress exposure, gender differences might corroborate to a better understanding of interindividual differences related to stress vulnerability and resilience. First evidence derived from animal studies revealing chronic stress to cause damages to the hippocampus in male rats and monkeys but less, if at all, in females (McEwen, 2000). Moreover, human studies showed women to respond differently to chronic stress and repeated stress induction (Goldfarb et al., 2019; McEwen & Milner, 2017). However, we did not detect any significant gender-specific activation nor deactivation changes when comparing responses of the first with the second run of Scan*STRESS*. Although increasing deactivations emerged for both subsamples, our results may on a descriptive level suggest different extents for women and men regarding the targeted clusters (see Figure 9).

5.5.3 Heart rate and psychological responses

As previously reported (Henze et al., 2020b), we found a significant decline in positive affect ratings and an increase in reported negative affect. However, we detected no significant gender differences. Moreover, women and men exhibited similar heart rate responses during stress. In this regard, again, the composition of the present sample has to be considered. Earlier research supports the idea of a pronounced impact of menstrual cycle phases and/or OC-use on the presence or absence of gender differences (Hidalgo-Lopez et al., 2020; Sharma et al., 2020; Yoest et al., 2018), especially regarding psychological measures (Albert et al., 2015; Childs et al., 2010; Lewis et al., 2019). Moreover, we generally assume that basic characteristics of an fMRI block design, with frequent interruptions of the stress exposure by control blocks, interfere with even more pronounced responses. Hence, the overall lower stress intensity, achievable by scanner paradigms compared to laboratory stressors, has to be considered. Furthermore, the absence of gender differences in a particular outcome should not lead to the misconception that the neural substrates underlying these mechanisms are necessarily identical for women and men (Cahill, 2006; Goldfarb et al., 2019).

5.5.4 Limitation and conclusion

The current study suggests gender-specific cortisol reactions to be differentially associated with striato-limbic responses to psychosocial stress (Seo et al., 2017). However, as our study sample included only OC-taking women, we have to emphasize that the present data may only contribute to a better understanding of differences in the association of neural stress responses and cortisol increases of women taking OCs versus men. It could well be appropriate to limit our conclusions to the (large) subgroup of women taking hormonal contraceptives as it was previously found in female-only studies that OC-use and menstrual cycle phase can influence

the brain's response to negative stimuli (Petersen & Cahill, 2015) and psychosocial stress (Albert et al., 2015; Chung et al., 2016a). Furthermore, at least cortisol increases are known to be modulated not only by OCs but also by the specific phase of the menstrual cycle (Kirschbaum et al., 1999; Zänkert et al., 2019). However, as simple group-level analyses contrasting female and male neural responses to psychosocial stress paradigms failed to reveal consistent differences [in the present as well as in previous studies: Chung et al. (2016b); Dahm et al. (2017); Kogler et al. (2015); Kogler et al. (2017)] it appears unlikely that corresponding differences between OC-taking and naturally cycling women are extremely large. To date, studies on the impact of gender on the interaction between cortisol and neural stress responses in OC-taking women, women in the luteal and follicular phase as well as in men do not exist.

Therefore, even though women and men differ in their overall stress reactivity and regarding prevalence rates of certain stress-related pathologies, our findings do not support the view of a clear neuroanatomically differentiable 'female-typical' and 'male-typical' response to stress. Instead, our data provides further evidence for the idea that considering complex interactions and quantitative rather than qualitative variables is a more suitable approach to elucidate gender-related differences in central stress regulation (Shalev, Admon, Berman, & Joel, 2020).

CHAPTER 6

6 GENERAL DISCUSSION

The present chapter intends to provide a general discussion regarding the evaluation and improvement of the Scan*STRESS* paradigm presented in Chapter 3 and the two studies presented in Chapter 4 and Chapter 5 in the context of the current state of research (presented in Chapter 2) and the research rationale (presented in Chapter 1).

6.1 Summary of main findings

The present thesis aimed at elucidating stress regulation in the brain with emphasis on the association with cortisol release, the modulation by exposure time, and gender differences. In order to achieve these study aims, the stress induction paradigm used – Scan*STRESS* – was improved and a more sophisticated analysis strategy for Scan*STRESS* data was developed.

Overall, the present findings support the view, that especially regarding cortisol responses, the changes applied to the protocol (i.e., implementation of a prolonged relaxing phase, glucose administration, more abrupt stress onset) have been effective. Stress exposure led to significant increases in cortisol levels, heart rate, and negative affect ratings as well as activations and deactivations in (pre)limbic regions. When individual cortisol increases were used as covariate, stronger responses in the hippocampus, amygdala, mPFC, and cingulate cortex were observed. Moreover, responses within the same regions predicted negative affect ratings throughout the protocol. Remarkably, an increasing deactivation over the two runs of Scan*STRESS* (exposure-time effect) was found, again, in the same structures and was further confirmed in an independent sample (Streit et al., 2014).

Regarding gender differences, the present data confirmed the well-known gender-specific cortisol response pattern with men exhibiting higher adrenocortical reactions than women (using OCs). Still, mean increases in cortisol were significant in the female as well as the male subsample, confirming a successful stress induction in both genders. Responses of the hippocampus, amygdala, cingulate cortex, thalamus, and striatal structures were found to be differentially associated with cortisol increases in women and men. For men, higher cortisol increases resulted in more activation of these striato-limbic structures whereas in women, higher cortisol increases were associated with more deactivation. However, no significant gender differences regarding exposure-time effects, affect nor heart rate measures were detected.

6.2 Evaluation of the improved Scan*STRESS* protocol

6.2.1 Cortisol responses

The first aim of the present thesis was the improvement of the Scan*STRESS* protocol and subsequently the evaluation of the changes made. Overall and particularly with regard to cortisol responses, these changes can be regarded as effective. Referring to the implementation of a prolonged relaxing phase prior to stress onset, a decrease in cortisol levels from time point -75 to -1 emerged (see Figure 2). Additionally, a slightly more pronounced anticipation response was detected in men (time point -75) but after relaxation, both genders exhibited similar mean cortisol levels (time point -1). Subsequently, cortisol values significantly increased whereby this was also valid for the female subsample taking OCs. As stated above, women tested in luteal phase might have exhibited even higher cortisol responses (Kirschbaum et al., 1999; Rohleder, Wolf, Piel, & Kirschbaum, 2003; Rohleder, Schommer, Hellhammer, Engel, & Kirschbaum, 2001; Uhart, Chong, Oswald, Lin, & Wand, 2006; Wolf, Schommer, Hellhammer, McEwen, & Kirschbaum, 2001).

According to the administration of a sugary drink 45 minutes prior to stress onset, the present results confirm previous findings suggesting that glucose facilitated more pronounced cortisol responses (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997; Zänkert et al., 2020). However, to verify this, the comparison to a placebo group (i.e., participants confronted with Scan*STRESS* receiving a placebo drink instead of glucose) would have been necessary. Although it was not an aim of the present thesis to evaluate the influence of sugar administration on responses to Scan*STRESS*; it can be speculated, that a placebo group would have achieved significantly lower cortisol stress responses compared to participants in a glucose-treated group.

Regarding the increased abruptness of stress onset implemented in the improved protocol it can be noted that this change yielded a more economical Scan*STRESS* protocol than earlier applications of the paradigm (Akdeniz et al., 2014; Lederbogen et al., 2011; Streit et al., 2017; Streit et al., 2014). In particular, the time required for the transfer and correct positioning of the participants into the scanner was minimized and furthermore, the subjects were confronted with the observation panel via live video stream without being presented to it beforehand as previously implemented (Streit et al., 2014). Comparing the improved protocol and the original one by Streit et al. (2014), the fact that the subjects were not introduced to the panel by the experimenter before entering the scanner room accelerated the timing of the protocol used for the present thesis. In this context, it has to be mentioned that this improved protocol requires sufficient trained technicians and a well-prepared scanner room to further reduce the transfer- and positioning-time needed. Moreover, it can be assumed that a personal introduction to the

panel might have led to an anticipation response which interferes with a stressor-specific cortisol response as described in Chapter 3. In addition to the enhancement of the overall timing of the experimental procedure of Scan*STRESS*, the improvement aimed at achieving more continuous measurement times of cortisol and affect levels (i.e. repeated collection of samples with similar time intervals). As previous studies collected varying and sometimes insufficient numbers of saliva samples or affect ratings (Noack et al., 2019), the present protocol can be regarded as a well-structured experimental protocol for biopsychological stress research. Again, trained experimenters and technicians are required for a correct implementation.

Moreover, it has to be acknowledged that the block design of Scan*STRESS* with frequent interruptions of the stress induction by control blocks might has hampered even more pronounced stress responses. This is supported by previous findings (Wang et al., 2005) and very recently by a study of Sandner et al. (2020) implementing a compact version of Scan*STRESS* – Scan*STRESS*-C – with shorter block duration (40 seconds instead of 60 seconds) and a rearrangement of the sequence of control and stress blocks to form two separate, non-randomized phases (i.e., first phase control blocks, second phase stress blocks). Here, the highest responder rate reported to date was achieved (73.7 %).

6.2.2 Affective and heart rate responses

Regarding the affective response to Scan*STRESS* the increase in negative affect ratings further proves the successful stress induction. Moreover, we detected a decrease in positive affect ratings but the lowest point was found after the implemented RS measurement and not – as expected – immediately after Scan*STRESS* completion. In this context, the composition of the PANAS scale has to be considered, as it contains adjectives like *interested*, *excited*, *alert,* and *active*. Thus, it may not be too surprising that the lowest ratings were found after RS measurement when participants had to lay still, with eyes open, looking at a fixation cross. Moreover, the present thesis intended to measure subjective stress levels whereby using a VAS asking, “How stressed do you feel?” might have been a more appropriate choice than the PANAS scale. However, asking this question would have interfered with the cover story (i.e., investigation of brain activation during maximal mental performance). In addition, the alternating presentation of stressful blocks and the respective control blocks (block design) might have also hampered the mood response to Scan*STRESS*. Nevertheless, at least referring to heart rate responses, the block design of Scan*STRESS* appears to be beneficial as it enables the comparison of stress and control blocks. Hence, the stress-eliciting impact of Scan*STRESS* was further documented by pronounced heart rate responses in stress compared to control blocks.

The expected gender differences were only found regarding cortisol measures. However, no response differences in women compared to men were detected when analyzing affective or heart rate responses. Given the literature on gender differences in response to laboratory stressors like the TSST, gender-specific affective responses in particular would have been expected (Buske-Kirschbaum et al., 2003; Helbig & Backhaus, 2017; Kelly et al., 2006; Kelly et al., 2008; Kirschbaum et al., 1999; Kirschbaum et al., 1996; Merz & Wolf, 2015). To date, only one study reported on gender-specific differences in subjective stress ratings after psychosocial stress exposure in the fMRI (Brugnera et al., 2018) using a self-report stress rating questionnaire which measures subjective stress in a similar way as a VAS does. Thus, based on the above, using a VAS might have produced detectable gender differences in mood states. Similarly, findings reporting on autonomic responses in general and gender-specific cardiac reactions in particular remain scarce and yielded heterogeneous results (see Chapter 2 for further information). Therefore, future studies might focus on affective and heart rate responses to Scan*STRESS* with particular emphasis on the impact of gender. Regarding subjective stress responses, the present results suggest that a paradigm presented in block design might not be the best choice.

6.2.3 Mean neural responses to Scan*STRESS*

The whole-brain analysis of mean responses to Scan*STRESS* without additional covariate or group variable supported what would be expected from the current literature. It revealed a distributed network of activations and deactivations including (pre)limbic regions such as the cingulate cortex (ACC and PCC), thalamus, insula, and mPFC. However, and in contrast to previous findings (Noack et al., 2019; Pruessner et al., 2008), neither activations nor deactivations were found for core-limbic regions such as the hippocampus or amygdala in the whole-brain analysis contrasting stress and control blocks (stress > control, control > stress). Instead, these core-limbic structures showed significant associations with cortisol and affective responses. Hence, it can be speculated that especially the more sophisticated analysis strategy applied with different analysis levels including various sources of variance and different predictors (e.g., cortisol increases), contributed to disentangle (pre)limbic reactions to psychosocial stress. Of course, this has to be verified in future studies, but the conclusion might be that (pre)limbic regions (i.e., a diffuse network as found in the present study) react to psychosocial stress in general while core-limbic regions might have a more specific effect and therefore influence other systems that respond to stress such as the HPA axis. Still, some (pre)limbic regions – as stated in Chapter 4 – might have rather coordinating effects, like the cingulate cortex and/or the mPFC, as they reached significance in most of the analyses.

Moreover, the present results confirm the hypothesis of Van Oort et al. (2017) and Menon (2011). Stress exposure induced activations and deactivation in SN- and DMN-related structures, proving their involvement in the processing of social-evaluative threat, forced failure, and negative feedback as implemented in Scan*STRESS*. Moreover, the results of the present thesis further support the view that CEN-related structures are not addressed when participants are confronted with negative evaluation (Van Oort et al., 2017).

6.2.4 Evaluation of the analysis strategy for Scan*STRESS* data

It can be summed up, that the more sophisticated analysis strategy applied has been effective and made it possible to find various effects. In particular, the fact that the reported findings – with the exception of the association between (pre)limbic and affective responses – are mainly based on whole-brain analyses (with *post-hoc* ROI-analyses) underlines the usability of this analysis strategy. As stated above, it can be further speculated that this analysis approach contributed to unravel psychosocial stress processing of specific brain regions and therefore, future studies may apply this strategy also in the context of other research questions going beyond the scope of this thesis.

Regarding the analysis of the interplay between (pre)limbic and affective responses to acute stress it has to be noted that revealing a whole-brain effect would have required to aggregate the ten negative affect values that were collected throughout the procedure artificially to only one value. Hence, a whole-brain analysis with additional covariate (grand mean centered) could have been applied as it was done for the calculated cortisol increase. However, such an aggregated measurement has not yet been established for the PANAS scale and simply applying the formula for calculating cortisol increases might be inappropriate. It is questionable what significance this value would have, since it is initially composed as the mean value from ten evaluations of 20 adjectives (summed value regarding ten positive and ten negative adjectives at each measurement time) and is then further summarized as a rate of increase.

6.3 The interplay of (pre)limbic and cortisol responses as well as (pre)limbic and affective reactions to psychosocial stress

According to the association between neural and HPA axis responses as well as between neural and affective reactions, the present findings once more underline the importance and impact of (pre)limbic structures in human stress processing (Herman et al., 2003; Herman et al., 2005; Jankord & Herman, 2008). The present study is the first to confirm associations between (pre)limbic responses and cortisol values in a whole-brain analysis. So far, findings regarding this interplay were based on ROI-analyses and were not always consistent (Noack et al., 2019).

The fact that the responses of identical (pre)limbic structures were found to predict negative affect ratings throughout the procedure further emphasizes the assumption that in healthy subjects, (pre)limbic circuits interact with HPA axis responses and are associated with affective reactions to acute psychosocial stress. Hence, the implementation of Scan*STRESS* might also be promising to investigate maladaptive stress processing in (pre)clinical samples and to elucidate vulnerability and resilience as central question of psychobiological stress research.

As already stated in Chapter 4, when integrating the present findings on the interplay between (pre)limbic and HPA axis responses into animal literature, some of the presented results are in line with animal findings and others are not (Noack et al., 2019). According to the classification of the results for each (pre)limbic structure given in Chapter 4, the present data corroborate to a more integrative perspective. First, this is the first work explicitly aiming at investigating the interplay between the brain's, HPA axis, and affective responses to psychosocial stress. Therefore, results relating to this investigation that have been reported so far were more or less secondary findings as the original study purpose(es) were different; with the exception of the study by Pruessner et al. (2008) that intended to introduce the MIST and typical cortisol, heart rate, skin conductance, and neural responses to this paradigm. Second, all of the results presented in the present thesis rely on whole-brain analyses which were subsequently confirmed by ROI-analyses. As stated above, most of the findings reported to date were ROI-based, minimizing their explanatory power. Third, however, it has to be acknowledged that some of the present results contradict animal findings which have been sufficiently verified (Herman et al., 2003; Herman et al., 2005; Jankord & Herman, 2008). Nevertheless, it cannot be concluded that this documents true species differences and as described in detail in Chapter 2, variability in stress-related response patterns were not only found in humans when comparing MIST- or Scan*STRESS*-findings but also within animal literature. Moreover, in this Chapter, the different stress intensity that can be applied to animals versus humans was mentioned. Hence, to simply assume that animal findings in stress research are in general transferable to humans and vice versa is contraindicated.

6.4 Exposure-time effect of (pre)limbic structures

The exposure-time effect is certainly a very unexpected finding of the present thesis. As mentioned above, one can speculate that this is a correlate of sensitization processes after repeated experience of uncontrollability, forced failure, and social-evaluative threat. If so, future research might focus on exposure-time effects in (sub)clinical samples suffering from chronic stress. It is of particular interest in this context that the exposure-time effect was found for the identical structures that were identified to be associated with HPA axis and negative

affect responses to stress. Moreover, the replication of this exposure-time effect in an independent sample (Streit et al., 2014) strengthens the assumption that further investigations in this context might help to elucidate mechanisms of interindividual differences related to vulnerability and resilience. This effect corroborates previous repeated measures rsFC analyses (Quaedflieg et al., 2015; Vaisvaser et al., 2013; Veer et al., 2012) and may help to integrate these findings more specifically as they are based on a different methodological approach (i.e., tasked-based brain responses).

However, we did not detect gender differences in exposure-time effects. Following the hypothesis that this exposure-time effect may help to uncover interindividual differences in the processing of chronic stress, gender differences might only be visible in brains of chronically stressed subjects. Considering the assumption of Grabowska (2017) that gender-specific differences in the brain could result from compensation mechanisms aimed at maintaining comparable intellectual abilities across genders or preventing maladaptive differences, it could be speculated that gender-specific exposure-time effects were not found in the present study as only healthy subjects participated. As stated above, the available results suggest that Scan*STRESS* can also be used in (pre)clinical samples and therefore it might be of particular interest to analyze gender-specific exposure-time effects in mental disorders showing distinct gender-differences (e.g., prevalence rate), like depression (Bangasser & Valentino, 2014; Kudielka & Kirschbaum, 2005).

6.5 Gender differences in associations between neural and cortisol responses to psychosocial stress

The present data do not support the view that neural stress-related responses of women and men involve different structures (Kogler et al., 2017; Seo et al., 2017; Seo et al., 2011; Wang et al., 2007). Instead, the current results document different associations for women and men between cortisol measures and responses of the identical striato-limbic areas. Moreover, focusing on either subsample, this differential interaction was confirmed, as men showed associations with activations (stress > control) and women with deactivations (control > stress). These gender-specific response patterns found in the present thesis are again the first to be based on the combination of whole-brain analyses and *post-hoc* ROI-analyses. Furthermore, these results acknowledge previous findings, indicating a positive association in men (i.e., more activation – more cortisol) and a negative association in women (i.e., more activation – less cortisol) (Kogler et al., 2016; Veer et al., 2011; Vogel et al., 2015).

In this context, striatal stress responses might explain gender-specific associations between neural and cortisol responses. Speculatively, pronounced striatal responses were evoked as they might be the basis of gender-specific processing of the key stress components of Scan*STRESS*, namely uncontrollability, repeated experience of forced failure, and social-evaluative threat. This is emphasized by results of a study implementing the MIST reporting gender differences in striatal responses (Kogler et al., 2015) and the fact that another study – not implementing psychosocial stress – did not report gender-specific striatal reactions during stress perception (i.e., serial subtraction as stressor) (Wang et al., 2007). Furthermore, this view is further supported by rsFC data (Kogler et al., 2016) and the analyses presented in Chapter 4, where the whole-brain results for the total sample did not reveal striatal reactions.

As stated in Chapter 5, the present data may only contribute to a better understanding of differences in the association of neural stress responses and cortisol increases of women taking OCs versus men. Hence, it might be of particular interest for future studies, to compare the association of neural and cortisol responses in men with those in women in different menstrual cycle phases. At first glance, it may seem surprising that so far, no fMRI study investigated psychosocial stress response differences in a sample comprising men as well as women in different menstrual cycle phases and those taking OCs. It is even more surprising given the abundance of literature on these differences applying laboratory stressors like the TSST (Kirschbaum et al., 1999; Kudielka et al., 2004b; Kudielka et al., 2009; Kudielka & Kirschbaum, 2005; Zänkert et al., 2019). However, as simple group-level analyses contrasting female and male neural responses to psychosocial stress paradigms failed to reveal consistent differences [in the present as well as in previous studies: Chung et al. (2016a); Chung et al. (2016b); Dahm et al. (2017); Kogler et al. (2015); Kogler et al. (2017)] it seems to be unlikely that corresponding differences between OC-taking and naturally cycling women would be overly large. Hence, the biggest limitation of the present thesis might be that only women taking OCs participated. The presented results therefore may be limited for conclusions regarding men and OC-taking women. Up to this point it can only be speculated whether the presented gender differences persist when the reactions in men are compared with those in women in different menstrual cycle phases. Nevertheless, given previous studies reporting mean cortisol stress responses of OC-taking women as being diminished with less variance, it is striking that the present thesis found associations between cortisol increases and neural responses even in the subsample of OC-taking women.

6.6 Final conclusion

The results of the present thesis show that associations of HPA axis and neural responses can be detected, when psychosocial stress is induced properly, and an analysis strategy is applied taking different sources of variance and analysis levels into account.

To conclude, the present thesis introduced an improved Scan*STRESS* protocol and analysis strategy for Scan*STRESS* data. Based on the effects found, the usability of this psychosocial stress paradigm suited for fMRI environments was confirmed and therefore we suggest to implement Scan*STRESS* and analyze Scan*STRESS* data as described in this thesis. Moreover, consistent associations between neural and HPA axis responses as well as neural and affective reactions were found acknowledging the interactions between different stress response systems. Furthermore, gender-specific associations between cortisol and striato-limbic stress responses were detected contributing to a better understanding of stress processing in healthy participants. In particular, the exposure-time effect may be a special perspective for research on the development of mental illnesses.

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8 APPENDIX

8.1 Supplemental Methods

Salivary cortisol analysis

In order to determine the cortisol concentration in the saliva sample we used a time-resolved fluorescence immunoassay. The saliva samples were stored at -20°C until analysis. After thawing, saliva samples were centrifuged at 2000 g for six minutes, which resulted in a clear supernatant of low viscosity. 100 µl of saliva were used for duplicate analysis (50 µl per well). Cortisol levels were determined employing a competitive solid phase time-resolved fluorescence immunoassay with flourometric end-point detection (DELFIA). 96-well-Maxisorb microtiterplates (Nunc) were coated with swine-anti-rabbit immunoglobulin. After an inkubation period of 48 hours at 4°C plates, were washed three times with washbuffer (pH = 7,4; contains sodiumphosphat and the Tween-40). In the next step, the plates were coated with a rabbit anti-cortisol antibody and incubated for 48 hours at 4°C. Synthetic saliva mixed with cortisol in a range from 0 - 100 nmol/L served as standards. Standards, controls (saliva pools) and samples were given in duplicate wells. 50 µl of biotin-conjugated cortisol was added and after 30 minutes of incubation the non-binding cortisol/biotin-conjugated cortisol was removed by washing (three times). 200 µl europium-streptavidin (Perkin Elmer, Rodgau, Germany) was added to each well and after 30 minutes and six times of washing 200 µl enhancement solution was added (Pharmacia, Freiburg, Germany). Within 15 minutes on a shaker the enhancement solution induced the fluorescence which can be detected with a VICTOR™ X4 Multilabel Plate Reader (Perkin Elmer, Massachusetts, USA). With a computer-controlled program, a standard curve was generated and the cortisol concentration of the samples were calculated. The intra-assay coefficient of variation was between 4.0 % and 6.7 %, and the corresponding inter-assay coefficients of variation were between 7.1 % and 9.0 %. The detection limit of the assays is 0.179 nmol/L.

8.2 Supplemental Results

Table 6.
Activated (stress > control) and deactivated (control > stress) structures during psychosocial stress (two tailed combined FWE-corrected for whole-brain, threshold $p < .05$) including *z*- and *p*-values as well as the localization of peak voxels.

Structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
lateral occipital cortex, inferior division	**left**	**70040**	**< .001**	**6.01**	**-56**	**-70**	**- 8**
brainstem	right			5.74	4	-40	-34
occipital fusiform gyrus				5.71	26	-66	-12
frontal pole	**right**	**6640**	**< .001**	**-5.50**	**4**	**60**	**-10**
frontal medial cortex	left			-5.04	- 8	52	-10
frontal orbital cortex				-4.67	-16	24	-22
frontal medial cortex	right			-4.62	8	34	-26
				-4.60	4	32	-26
postcentral gyrus	**right**	**4248**	**< .001**	**-5.27**	**62**	**-6**	**34**
				-5.18	38	-28	70
central opercular cortex				-5.26	44	-12	16
				-5.21	60	-4	8
planum polare				-5.21	58	-4	0
superior temporal gyrus, posterior division				-5.15	64	-10	- 4
precuneus cortex	**left**	**3371**	**< .001**	**-5.21**	**- 6**	**-66**	**24**
				-5.07	- 6	-58	16
cingulate cortex, posterior division				-5.10	- 6	-46	24
				-5.03	- 2	-42	36

Structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
central opercular cortex, insula	**left**	**2111**	**< .001**	**-4.89**	**-40**	**-14**	**14**
				-4.48	-58	-14	10
				-4.36	-56	- 4	6
postcentral gyrus				-4.47	-54	-18	46
middle temporal gyrus, anterior division				-4.37	-60	- 6	-14
planum polare				-4.35	-56	- 2	2
lateral occipital cortex, superior division	**left**	**1168**	**< .001**	**-4.76**	**-48**	**-62**	**40**
				-4.67	-52	-66	32
				-4.66	-52	-62	32
				-4.60	-46	-64	26

Global cluster maxima are in boldface.

Table 7.
Activated structures during psychosocial stress (stress > control) with *cortisol increase*s as covariate (FWE-corrected for whole-brain, threshold $p < .05$) including *z*- and *p*-values as well as the localization of peak voxels.

structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
frontal orbital cortex	**left**	**6134**	**< .001**	**4.04**	**-28**	**12**	**-26**
middle temporal gyrus,				4.03	-60	-22	-20
posterior division							
parahippocampal gyrus,				3.96	-32	-26	-20
posterior division							
middle temporal gyrus,				3.94	-56	-20	-20
posterior division							
cingulate cortex,	**right**	**1854**	**< .01**	**3.48**	**4**	**-44**	**22**
posterior division							
	left			3.18	- 4	-38	30
precuneus cortex	left			3.46	- 6	-62	12
	right			3.23	6	-70	26
	left			2.79	-10	-52	8

Global cluster maxima are in boldface.

Table 8.
p-values and Benjamini-Hochberg corrected significance levels for each ROI.

	***p*-value**	**Benjamini-Hochberg corrected significance level**
hippocampus, bilateral	**.007****	≤ .02
left	**.013***	≤ .025
right	.042*	≤ .04
parahippocampal gyrus	**.003****	≤ .015
amygdala, bilateral	**.016***	≤ .035
left	**.002****	≤ .01
right	.126	≤ .045
mPFC	**.001****	≤ .005
cingulate cortex,		
ACC	.305	≤ .05
PCC	**.015***	≤ .03

** $p \leq .01$ and * $p \leq .05$ depict *a-priori* significance level; values in boldface indicate ROIs that survived correction.

Table 9.
Activated (stress > control) and deactivated (control > stress) structures during psychosocial stress with *run* as regressor (two-tailed combined FWE-corrected for whole-brain, threshold $p < .05$) including *z*- and *p*-values as well as the localization of peak voxels.

structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
temporal pole	**right**	**24304**	**< .001**	**7.25**	**48**	**18**	**-26**
				6.60	54	12	-30
	left			6.27	-42	16	-32
lateral occipital cortex, inferior division	right			6.38	46	-68	-10
lateral occipital cortex, superior division	right			6.60	32	-86	12
frontal pole	**left**	**6438**	**< .001**	**6.00**	**- 8**	**46**	**44**
				5.75	- 2	58	4
	right			5.17	6	62	20
superior frontal gyrus	left			5.71	- 2	56	20
paracingulate cortex	left			5.38	- 6	42	34
cingulate cortex, posterior division	**left**	**320**	**< .05**	**4.00**	**- 4**	**-50**	**24**
precentral gyrus	**right**	**2481**	**< .001**	**-5.05**	**38**	**- 8**	**62**
				-4.38	22	-10	68
				-4.34	34	-26	68
	left			-4.72	-38	-16	62
superior frontal gyrus	left			-4.34	-16	-12	66

Global cluster maxima are in boldface.

Table 10.
Activated structures during psychosocial stress (stress > control, control > stress) with *run* as regressor (two-tailed combined FWE-corrected for whole-brain, threshold $p < .05$) including *z*- and *p*-values as well as the localization of peak voxels for the sample of Streit et al. (2014).

structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
paracingulate cortex	**left**	**40060**	**< .001**	**5.49**	**- 6**	**42**	**- 4**
middle temporal gyrus, anterior division	right			4.90	60	4	-20
lateral occipital cortex, posterior division				4.80	44	-60	24
inferior frontal gyrus, pars triangularis	**right**	**1916**	**< .01**	**4.26**	**52**	**30**	**10**
inferior frontal gyrus, pars opercularis				3.25	48	20	20
				3.18	40	14	24

Global cluster maxima are in boldface.

Table 11.
Comparison (mean ± *SD*) between women and men regarding cortisol levels [nmol/L] at each time point and cortisol increases [nmol/L].

	women (***n*** **= 31)**	**men** (***n*** **= 36)**	**df**	***t***	***p*-value**	***d***
-75 minutes	4.41 (± 1.94)	6.40 (± 4.61)	48.49	-2.36	**.022**	.563
-15 minutes	3.64 (± 1.64)	3.18 (± 1.58)	62.68	1.16	.251	-.286
- 1 minute	3.50 (± 1.38)	3.43 (± 1.51)	64.77	.21	.836	-.048
15 minutes	4.13 (± 1.77)	4.51 (± 1.97)	64.88	- .82	.413	.203
30 minutes	4.56 (± 2.49)	6.23 (± 3.41)	63.39	-2.31	**.024**	.559
50 minutes	4.75 (± 2.99)	7.19 (± 4.40)	61.83	-2.69	**.009**	.649
65 minutes	4.33 (± 2.18)	6.67 (± 4.42)	52.68	-2.79	**.007**	.671
80 minutes	4.20 (± 1.75)	6.33 (± 5.26)	43.74	-2.28	**.028**	.543
95 minutes	3.83 (± 1.46)	5.35 (± 4.31)	44.03	-1.99	.053	.472
110 minutes	3.50 (± 1.19)	4.39 (± 3.21)	45.74	-1.53	.132	.368
increase	1.61 (± 2.29)	4.90 (± 5.21)	56.13	-3.25	**.002**	.818

Significant *t*-tests are in boldface.

Table 12.
Structures of a gender-specific (men > women) cluster in response to stress > control with *cortisol increases* as covariate (two-tailed combined FWE-corrected for whole-brain, threshold $p < .05$) including *z*- and *p*-values as well as the localization of peak voxels.

structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
cingulate cortex, posterior division	**right**	**9413**	**< .001**	**4.34**	**32**	**-56**	**0**
				4.28	26	-46	- 2
				4.27	- 6	- 4	22
				3.87	14	-52	44
thalamus				4.01	16	-36	8
				4.00	4	-14	18

Global cluster maxima are in boldface.

Table 13.
Significant cluster in the female subsample in response to control > stress associated with *cortisol increases* as covariate (two-tailed combined FWE-corrected for whole-brain, threshold $p < .05$) including *z*- and *p*-values as well as the localization of peak voxels.

structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
lingual gyrus	**right**	**4081**	**< .001**	**3.92**	**28**	**-48**	**- 4**
				3.88	32	-56	- 2
				3.47	14	-70	2
precuneus cortex				3.85	14	-52	46
				3.24	10	-64	48
cingulate cortex,	**right**	**2404**	**< .001**	**3.64**	**14**	**- 8**	**48**
posterior division							
thalamus				3.61	6	-14	18
ncl. caudatus				3.30	6	8	10
cingulate cortex,				3.28	6	- 2	28
anterior division							

Global cluster maxima are in boldface.

Table 14.
Significant cluster in the male subsample in response to stress > control associated with *cortisol increases* as covariate (two-tailed combined FWE-corrected for whole-brain, threshold $p < .05$) including *z*- and *p*-values as well as the localization of peak voxels.

structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
frontal orbital cortex	**left**	**14564**	**< .001**	**4.75**	**-28**	**12**	**-24**
				4.35	-18	12	-28
				4.14	-20	18	-14
amygdala	right			4.43	12	- 6	-18
hippocampus	left			4.08	-22	-18	-16

Global cluster maxima are in boldface.

Table 15.
Activated structures in women during psychosocial stress (stress > control, control > stress) with *run* as regressor (two-tailed combined FWE-corrected for whole-brain, threshold $p < .05$) including *z*- and *p*-values as well as the localization of peak voxels.

structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
lateral occipital cortex, superior division	**right**	**4402**	**< .001**	**5.40**	**28**	**-84**	**28**
				5.35	34	-86	26
				4.75	34	-82	14
occipital fusiform gyrus				4.95	26	-72	-10
				4.82	26	-64	-12
temporal occipital fusiform cortex				4.73	32	-56	-12
frontal pole	**left**	**1714**	**< .001**	**4.55**	**- 8**	**48**	**44**
				4.28	-10	54	40
				4.27	- 4	56	38
	right			4.34	12	60	26
				4.26	6	64	18
				4.05	0	60	10
lateral occipital cortex, inferior division	**left**	**1439**	**< .001**	**4.67**	**-38**	**-80**	**6**
				3.85	-50	-78	-12
				3.83	-44	-78	-16
lateral occipital cortex, superior division				4.64	-32	-86	12
occipital pole				3.85	-24	-90	24
temporal pole	**right**	**782**	**< .001**	**4.86**	**48**	**18**	**-26**
				4.66	54	10	-32
frontal orbital cortex				3.95	32	18	-20
				3.87	28	20	-24
inferior temporal gyrus, anterior division				3.38	50	0	-38

structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
middle temporal gyrus, posterior division	**left**	**719**	**< .001**	**5.17**	**-52**	**-42**	**- 2**
				4.27	-66	-28	-10
				3.69	-56	-18	-18
				3.67	-68	-26	-18
superior temporal gyrus, posterior division				3.74	-66	-18	- 6
temporal occipital fusiform cortex	**left**	**376**	**< .05**	**3.85**	**-28**	**-60**	**-14**
				3.57	-26	-54	-18
parahippocampal gyrus, superior division				3.77	-24	-34	-20
occipital fusiform gyrus				3.62	-22	-72	-12
				3.51	-22	-76	-14
angular gyrus	**left**	**331**	**< .05**	**4.43**	**-54**	**-56**	**26**
lateral occipital cortex, superior division				3.25	-44	-64	16

Global cluster maxima are in boldface.

Table 16.
Activated structures in men during psychosocial stress (stress > control, control > stress) with *run* as regressor (two-tailed combined FWE-corrected for whole-brain, threshold $p < .05$) including *z*- and *p*-values as well as the localization of peak voxels.

structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
temporal pole	**right**	**3226**	**< .001**	**5.28**	**48**	**18**	**-26**
				5.35	54	8	-24
				5.06	54	14	-28
				5.00	56	10	-32
middle temporal gyrus,				5.37	52	4	-22
anterior division							
middle temporal gyrus,	**left**	**2668**	**< .001**	**5.64**	**-56**	**-16**	**-14**
posterior division							
temporal pole				5.14	-52	6	-26
				4.96	-44	14	-32
lateral occipital cortex,	**right**	**2176**	**< .001**	**4.69**	**48**	**-80**	**4**
inferior division							
				4.66	46	-68	-10
				4.37	34	-86	10
occipital pole				4.42	24	-94	26
				4.38	28	-92	28
paracingulate cortex	**left**	**1619**	**< .001**	**4.47**	**- 4**	**42**	**34**
				4.41	0	56	8
				4.35	0	54	14
superior frontal gyrus				4.45	- 2	44	50
				4.42	- 2	54	22
frontal pole				4.44	- 8	46	44

structure		**statistics**			**MNI coordinates**		
		K	***p***	***z***	**X**	**Y**	**Z**
lateral occipital cortex, superior division	**left**	**1258**	**< .001**	**4.41**	**-36**	**-88**	**8**
lateral occipital cortex, inferior division				4.29	-46	-80	- 2
				4.26	-48	-78	- 8
				3.94	-34	-82	0
occipital pole				4.08	-26	-94	8
				3.90	-30	-98	10

Global cluster maxima are in boldface.