Decoding chronic stress: Neural markers and their influence on chronic stress perception and mental health

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Foreword

In the present dissertation, three studies investigating chronic stress responses in daily life and their potential neural predictors are presented.

The research paper in Chapter 3 was written in close cooperation and joint first authorship with my project partner, Hannah L. Peter. Consequently, it was also included in her dissertation. Chapter 4 and 5 were exclusively written for the present thesis. The original research papers in the following chapters are either already published (Chapter 3 and 4) or currently under review (Chapter 5). Permissions for including these articles in my dissertation were granted by *Elsevier* (*Study I*: 'use of one's own article in a dissertation for non-commercial purposes') and *Nature* (*Study II*: 'Material in a paper submitted to Scientific Reports may also have been published as part of a PhD or other academic thesis'). *Study III* is currently under review in the Journal of Neuroscience Research (Wiley). The studies are listed below in order of their appearance. Co-author contributions are disclosed.

To improve readability, contents, tables, and figures of the manuscripts were numbered continuously and journal-specific reference styles were standardized and combined in a single reference section. Furthermore, all research paper appendices were integrated and are presented at the end of the dissertation. Original figures and original supplementary material can be found with the respective research paper or at the repository of the University of Regensburg at https://epub.uni-regensburg.de/view/projects/LawSTRESS_project.html. In this repository, also additional information and graphs, including questionnaire trajectories over measurement timepoints, can be found. All publications were written in the framework of the project *'Psychological, endocrine, central nervous and genetic predictors of chronic stress responses in daily life – a prospective-longitudinal quasi-experiment*' funded by the German Research Foundation (DFG; grant number WU392/9-1). Marina Giglberger received additional funding by the DGPs section 'Biological Psychology and Neuropsychology' (research fellowship during corona pandemic) and the 'Financial Incentive System to Promote Gender Equality' (FAS) of the University of Regensburg.

Study I

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List of abbreviations

AA	Ambulatory assessment
ACC	Anterior cingulate cortex
ACTH	Adrenocorticotropic hormone
AIC	Akaike information criteria
ANOVA	Analysis of variance
AVP	Aversive viewing paradigm
BET	Brain extraction tool
BOLD	Blood-oxygenation-level-dependent
BWAS	Brain-wide association studies
CAR	Cortisol awakening response
CEN	Central executive network
CFA	Confirmatory factor analysis
CFI	Comparative fit index
CG	Control group
CG.model	Models only containing the control group
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CTQ	Childhood Trauma Questionnaire
DELFIA	Dissociation-enhanced lanthanide fluorescence immune-assay
DMN	Default mode network
DNA	Deoxyribonucleic acid
EPI	Echo planar imaging
FEAT	FMRI expert analysis tool
FILM	FMRIB's improved linear model
FLIRT	FMRIB's linear image registration tool
fMRI	Functional magnetic resonance imaging
FNIRT	FMRIB's non-linear image registration tool
FoV	Field of view
FSL	FMRIB's software library
FWE	Familywise error
FWHM	Full width at half maximum
GC	Glucocorticoid
GLM	General linear model
GR	Glucocorticoid receptor
HADS	Hospital Anxiety and Depression Scale
HADS-A	Anxiety score of the Hospital Anxiety and Depression Scale
HADS-D	Depression score of the Hospital Anxiety and Depression Scale
HADS-G	General factor of the Hospital Anxiety and Depression Scale
HC	Hormonal contraception
HPA axis	Hypothalamic-pituitary-adrenocortical axis
LC	Locus coeruleus

MDD	Major depressive disorder
MEMS	Medication event monitoring systems
min	Minutes
MIST	Montreal Imaging Stress Task
ML	Maximum Likelihood
MNI	Montreal Neurological Institute
mPFC	Medial prefrontal cortex
MP-RAGE	Magnetization-prepared rapid gradient-echo
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
NA	Negative affect
PAF	Prüfungsangstfragebogen (test anxiety questionnaire)
PANAS	Positive and negative affect schedule
PFC	Prefrontal cortex
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
rANOVA	Repeated measures analyses of variance
RDoC	Research Domain Criteria
RIS	Regensburg Insomnia Scale
RMSEA	Root mean square error of approximation
ROI	Region of interest
SAM	Sympathetic adrenomedullary system
SCI	Stress and Coping Inventory
SCL-90-R	Symptom-Check-List
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SEM	Standard error of the mean
SET	Social-evaluative threat paradigm
SG	Stress group
SG.model	Models only containing the stress group
SMS	Short message service
SN	Salience network
SRMR	Standardized root mean square residual
Std.Error	Standard error
T	Timepoint
TE	Echo time
TICS	Trier Inventory of Chronic Stress
TLI	Tucker-Lewis index
TR	Repetition time
TSST	Trier Social Stress Test
TV	Television
vmPFC	Ventromedial prefrontal cortex

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Summary

Although it is widely acknowledged that chronic stress significantly increases the risk of various disorders, there is still limited understanding of the biopsychological mechanisms underlying this stress-disease relationship. As the brain is mainly responsible for the interpretation of a stimuli as stressful and orchestrates subsequent psychobiological stress responses, it appears crucial to investigate the neural processes elicited by acute stress as well as interindividual differences in central nervous stress regulation.

To pursue this objective, studies with a prospective-longitudinal design and a predefined long-lasting stress period, along with the application of neuroimaging methods combined with ecologically valid methods of field research for stress measurement, seem promising.

Thus, we conducted the LawSTRESS project, which investigated long-term stress responses of 452 law students from different Bavarian universities. The sample was divided into two cohorts: cohort A, consisting of 204 participants mainly from the University of Regensburg, and cohort B, comprising 248 students from other Bavarian universities who followed a less detailed study protocol without visiting the laboratory in Regensburg. Each cohort was further subdivided into a stress group (SG; cohort A: n = 97 and cohort B: n = 129) and a control group (CG; cohort A: n = 107 and cohort B: n = 119). While the SG experienced a long-lasting and substantial stress period due to their preparation for the first state examination, the CG was in the mid-phase of their study program with normal study-related workload.

Students were studied over a 13-months period, with assessments conducted at six different sampling points: twelve months prior exam (t1), three months (t2), and one week before the exam (t3), during the exam (t4), one week (t5), and one month after the exam (t6). The LawSTRESS project employed a multimodal and multidimensional approach to assess chronic stress responses. In cohort A, perceived stress measured through repeated ambulatory assessments (AA) at each sampling point, was evaluated alongside the cortisol awakening response (CAR), an established marker of cortisol regulation. Additional stress-related variables such as anxiety or depression symptoms were assessed through online questionnaires at all sampling points except t4. Furthermore, a subsample of cohort A (n = 124) underwent a magnetic resonance imaging (MRI) scanning session at baseline (t1), which included the acute stress paradigm Scan*STRESS*.

Study I (Chapter 3) examined the impact of chronic exam stress on different facets of students' psychological well-being. Moreover, the relationship between perceived daily life stress and

the CAR during this prolonged stress period was investigated. Significant differences in the trajectories of the SG and the CG could be found. The SG experienced heightened levels of perceived stress, anxiety, depression, sleep disturbances, and different dimensions of chronic stress compared to the CG. Additionally, a blunted CAR was observable in the SG during the exam (t4). Remarkably, neither perceived stress levels nor other psychometric predictors – namely anxiety, depression, and test anxiety – had an impact on the alteration of the CAR.

Investigating the potential predictive value of neural markers on chronic stress outcomes in daily life, *Study II* (Chapter 4) focused on the acute stress responses of the amygdala, hippocampus, and medial prefrontal cortex (mPFC) elicited by Scan*STRESS*. Changes in activation levels in these brain regions were found to be significantly linked to the trajectory of perceived stress, but not with the CAR. These neural reactions could be indicators of an individual stress response pattern showing a certain stability over time and contexts and thus predicting chronic stress perception in real life.

Study III (Chapter 5) examined the interplay between anxiety, depression, and neural stress regulation mechanisms. Stress-related activation changes in a striato-limbic cluster were related with anxiety but not with depression under baseline conditions. Moreover, a sex-specific effect could be found with stronger associations for men compared to women in striatal and temporal regions. Using this neural marker to predict the increase of anxiety levels due to the exam in the SG revealed no significant results. However, our findings indicate that these regions play a role in determining an individual's susceptibility to stress-related disorders.

In conclusion, the findings of the present thesis can be broken down into two strands. First, it provides evidence of the substantial burden faced by law students. Second, these results can be seen in a broader sense of chronic stress, as the preparation period for the first state examination is used as model for chronic stress exposure. Thus, it could be shown that long-term stress is associated with negative impacts on psychological well-being and a reduction in the CAR. Furthermore, stress-induced activation changes in the (pre)limbic regions amygdala, hippocampus, and mPFC could predict the increase in perceived stress levels due to a long-lasting and significant stress period. Additionally, striato-limbic structures showed associations with the extent of anxiety symptoms at a baseline level. Thus, these neural activation patterns might be linked to an individual's vulnerability or resilience regarding chronic stress exposure.

Despite some methodological limitations, our findings further elucidated temporal trajectories of chronic stress and identified neural predictors. Integrating imaging data with a detailed examination of the individual's everyday life employing AA is a powerful tool to enhance our understanding of human behavior. This synergy allows us to explore more deeply the neural mechanisms that play a crucial role in the transition from acute to chronic stress. By gaining these insights, we can better understand the stress-disease relationship, identify at-risk individuals, and develop tailored prevention and intervention strategies.

Chapter 1

1 Introduction and outline of the thesis

In 2023, 24% of the adult population in the United States reported high levels of stress, reflecting a 5% increase over the last four years (American Psychological Association, 2023). In Germany, prevalence rates are similar (26% in 2021), and the perception of situations as stressful appears to be continuously rising (Techniker Krankenkasse, 2021). Social demands, stress at work or university, high expectations – psychological stress is an inevitable phenomenon of our everyday life (Almeida et al., 2002). For most individuals, occasional and moderate stress experiences are assumed to have no negative health consequences; successful coping may even strengthen resilience (Jin et al., 2014). However, chronic or excessive stress is a risk factor for several disorders, including major depressive disorder (MDD), anxiety, sleep disorders, cardiovascular diseases, and diseases resulting from dysregulated immune functions (Chrousos, 2009; Cohen et al., 2007; Hammen, 2005). Although the biological mechanisms mediating this stress-disease relationship are not fully understood to date, it is considered as certain that dysregulations in stress-relevant systems, such as the sympathetic adrenomedullary system (SAM) or the hypothalamus-pituitary-adrenal (HPA) axis along with concomitant alterations in cortisol activity, are a key factor (O'Connor et al., 2021; Tsigos and Chrousos, 1994).

Since the brain is responsible for the interpretation of a situation as stressful and also initiates and orchestrates the neuroendocrine stress response, it appears crucial to investigate neural stress processes (De Kloet et al., 2005; McEwen, 2007). Interindividual differences in neural stress regulation might reveal potential vulnerability or resilience factors. First studies regarding stress regulation and mechanisms were conducted in animal samples (Herman et al., 2005, e.g., 2003; Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009). With the advent of imaging techniques like MRI or positron emission tomography the investigation of neural processes in humans became possible. Nonetheless, for the examination of the human neural stress response, suitable stress eliciting paradigms had to be created. In the last decades, several psychosocial stress paradigms for the MRI environment have been developed, e.g., the Montreal Imaging Stress Task (MIST; Dedovic et al., 2005) or Scan*STRESS* (Streit et al., 2014). In healthy individuals, acute stress responses in (pre)limbic and frontal regions have been repeatedly reported (Berretz et al., 2021; Henze et al., 2021, 2020; Noack et al., 2019; Qiu et al., 2022). Remarkably, these regions are also known to react differently in clinical samples like MDD or anxiety disorder patients (Boehme et al., 2014; Dong et al., 2022b;

Ming et al., 2017). For example, patients with MDD or remitted depression exhibited decreased stress-induced activation changes in the ventromedial prefrontal cortex (vmPFC) and increased activation in the middle cingulate cortex, precuneus, and paracentral lobule , in comparison to healthy controls (Ming et al., 2017). Although these cross-sectional results are important, longitudinal designs would greatly enhance our understanding of the processes leading from acute to chronic stress and the development of stress-related diseases. Showing that variations in the brain's reaction to stress can be detected prior to the emergence of chronic stress symptoms is a crucial step in establishing a possible causal relationship.

Thus, the *brain-as-predictor* approach, introduced by Berkman et al. (2013), seems to be a promising new tool. Its general aim is to connect neural findings from laboratory contexts with longer-term, ecologically valid outcomes. First results are encouraging. For instance, in consumer psychology, sales forecasting significantly improved after incorporating neuroscientific data (e.g., Varga et al., 2021). Using an example from clinical research, the onset of depression three to four years later could be predicted by resting state connectivity patterns (Shapero et al., 2019). With the spread of smartphone usage and other electronic devices for data storage, ambulatory assessments, i.e., data collection in the individual's natural environment, has become more feasible. This assessment method is convincing due to its increased ecological and external validity, as well as its high reliability resulting from real-time and real-life measurements (Trull and Ebner-Priemer, 2014). Thus, combining MRI data with ambulatory assessments could be a powerful tool for the investigation of mechanisms involved in the transition from acute to chronic stress and the development of stress-related diseases (Gadassi Polack et al., 2021).

Nevertheless, a suitable study design has to meet certain requirements: First, the recruitment of a cohort that will experience a prolonged period of stress in the future, along with a control group, is necessary. Second, state-of-the-art biopsychological laboratory techniques and AA measures should be applicable in the chosen cohort. The preparation for the first state examination in law satisfies these conditions. It is known as one of the most demanding academic periods in the German university system. In Bavaria, students must complete six written exams of several hours each within eight days. They dedicate approximately a year to preparing for this examination due to the high failure rates (up to 30%), limited opportunities for retaking the exam, and the significant impact of the final result on future career prospects. Previous research has revealed that academic stress, especially during exam periods, leads to

substantial increases in perceived stress levels and changes in stress-relevant biological systems, such as the HPA axis (Bunevicius et al., 2008; Burger et al., 2014; Duan et al., 2013; Koudela-Hamila et al., 2020). However, these results are mostly cross-sectional, and the investigated stress periods were considerably shorter than in the present project – called LawSTRESS.

Studying law students over 13 months, we used a prospective-longitudinal design, which allows us not only the assessment of variables of interest under basal conditions but also their trajectories across longer-term stress exposure. While students preparing for the state examination in law constituted the stress group, students in the mid-phase of their study program with the usual university-related stress load were assigned to the control group. Furthermore, a multimethod and multidimensional approach that integrates data from controlled laboratory settings and ambulatory assessments was employed. The AA comprises estimations of perceived stress levels in the participants' daily routine as well as the collection of saliva samples in the morning to measure the CAR.

The present thesis aims to investigate the consequences of chronic exam stress and their neural predictors. In Chapter 2, we first provide a brief overview of the research topics stress and its neural regulation processes before presenting studies that have combined MRI and AA. Chapter 3 introduces the first study (*Study I*), focusing on possible alterations in perceived stress levels, the CAR, their association, and other stress-related psychometric variables due to long-term academic stress exposure (Giglberger et al., 2022). Building on this, *Study II* presented in Chapter 4 addresses whether neural acute stress processing can act as predictor for chronic stress responses in daily life (Giglberger et al., 2023). Chapter 5 comprises *Study III*, which examines the association between neural acute stress responses and anxiety as well as depression (Giglberger et al., 2024, submitted). Finally, the findings of the presented results are integrated into a general discussion in Chapter 6.

Chapter 2

2 Theoretical background

Stress is an unavoidable element of everyday life. However, its definition remains challenging. As a first and very generic attempt, Hans Selye defined stress as the non-specific response of the body to any demand (Selye, 1970, 1936). In an effort to introduce a more specific description beyond stress as sole threat to homeostasis, Levine and Ursin (1991) interpreted stress as a process that includes the stimulus, its perceptual processing, and the subsequent behavioral and physiological output. Similarly, according to transactional stress theory, stress arises when a situational demand is perceived as threatening and exceeds the individual's own coping strategies (Lazarus and Folkman, 1984). In a recent endeavor, Koolhaas et al. (2011) sought to further clarify the stress concept by emphasizing a clear distinction between stressors and everyday challenges. For the authors, acute stress is defined as a process triggered by an environmental demand that exceeds an individual's natural regulatory capacity, especially in unpredictable and incontrollable situations.

2.1 Neurobiology of stress regulation

2.1.1 The SAM system and the HPA axis

Two key systems are involved in the regulation of stress responses: the SAM system and the HPA axis (Agorastos and Chrousos, 2022; Chrousos, 2009; Herman et al., 2020). The SAM system, also known as locus-coeruleus-noradrenalin-system, reacts immediately (within seconds) to a stressor. Once activated by the hypothalamus, the locus coeruleus (LC) in the brain stem is stimulated, leading to higher levels of noradrenaline in the brain and the activation of adrenergic receptors on preganglionic sympathetic neurons in the spinal cord (Gunnar and Quevedo, 2007; Morilak et al., 2005; Ulrich-Lai and Herman, 2009). These, in turn, triggers the production of the catecholamines adrenaline and noradrenaline by the adrenal medulla which causes the so-called *fight-or-flight response* proposed by Cannon (1915). It is characterized by an elevated arousal, vigilance, and focused attention (Chrousos and Gold, 1992), the rapid mobilization of metabolic resources as well as the acceleration of breathing, blood pressure, and heart rate (Gunnar and Quevedo, 2007; Osborne et al., 2020). The slower acting HPA axis is activated when corticotropin-releasing hormone (CRH) and arginine vasopressin are released in the paraventricular nucleus (PVN) of the hypothalamus. These hormones trigger the secretion of adrenocorticotropic hormone (ACTH) in the anterior

pituitary gland, which then targets the adrenal cortex to produce and release glucocorticoids (GCs) and adrenal androgens. The primary GC in humans is the steroid hormone cortisol, which impacts the individual's immune system regulation, brain function, behavior as well as the modulation of metabolic and cardiovascular processes (De Kloet et al., 2019; Gunnar and Quevedo, 2007; McEwen, 1998; Ulrich-Lai and Herman, 2009). The effects of elevated GCs take minutes to hours to evolve since they act on their target tissues also via changes in gene transcription (Sapolsky et al., 2000). GCs can bind to two types of receptors: the high affinity mineralocorticoid receptor (MR) and the low affinity glucocorticoid receptor (GR). Regarding the acute stress response, it is supposed that MRs play a role in determining the HPA axis' sensitivity or threshold of the stress response due to its high occupancy rate of 80 – 90% under basal conditions. In contrast, GRs are only occupied during the peak of the circadian rhythm of the HPA axis or when GC concentration is elevated. They activate brain pathways which result in downregulation of CRH production (negative feedback) and thus in a termination of the HPA axis stress response (see 2.2.2 "Cortico-limbic regulation of the HPA axis"). Maintaining a balance between MR- and GR-mediated actions appears to be crucial for restoring homeostasis by the properly initiation and termination of the stress response (De Kloet et al., 2019, 2018; Gunnar and Quevedo, 2007; Mourtzi et al., 2021).

HPA axis activity and its final product cortisol can be measured in various body fluids or tissues including saliva, urine, blood, or hair. Each procedure has its advantages and disadvantages, captures a different time interval of cortisol release, and a different state of biological availability of cortisol. For instance, while hair cortisol levels reflect the cortisol production of the last months, blood and saliva samples represent current concentrations (Gatti et al., 2009; Hellhammer et al., 2009; Kirschbaum et al., 2009). Moreover, it has to be considered that cortisol secretion occurs in a pulsatile as well as a circadian manner, which seemed to be independently regulated (Levine et al., 2007). Salivary cortisol is frequently used as a biomarker for psychological stress due to its ease of use in both controlled laboratory settings and field studies that have emerged to examine cortisol regulation in daily life.

2.1.2 Cortico-limbic regulation of the HPA axis

In the last decades, (pre)limbic brain structures, which can evaluate potential external threats and have indirect connections to the PVN, got more and more attention regarding stress regulation (Herman et al., 2003). The literature thus emphasized the importance of the

stressor type. Reactive stressors involve actual sensory stimuli that intensify demands on the system (e.g., pain, responses of the immune system, or a physical injury), while anticipatory stressors focus on an individual's perception and evaluation of a stimulus as a threat, such as in social challenges or unfamiliar situations (Boonstra, 2013; Dedovic et al., 2009; Herman et al., 2016, 2003). In animal studies, especially the modulating impact of hippocampus, amygdala, and mPFC on the endocrine stress response was examined (Herman et al., 2005; Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009).

Besides its role in the circadian glucocorticoid rhythm, the hippocampus is involved in the inhibition of the HPA axis stress response, although this inhibition is strongly stressor-dependent (Herman et al., 2005, 2003). For instance, lesion studies using stressors like restraint or open field exposure revealed a corticosterone hypersecretion following hippocampal damage and thus support the inhibitory role of the hippocampus whereas studies using physical challenge (like ether inhalation) found no change in the secretion of corticosterone (for a summary see Herman et al., 2003). Frontal regions are also linked to the modulation of HPA axis activation, even though in a more complex way. The more ventral components of the mPFC appear to have an excitatory effect on the HPA axis. The dorsal part is involved in its inhibition, albeit also in a stressor-dependent manner with an inhibitory effect only for anticipatory stressors like restraint (for a summary see Jones et al., 2011; Myers et al., 2012; Radley et al., 2006). The amygdala shows a similar dependency on stressor type and anatomical subdivision. Medial as well as central nuclei of the amygdala excite HPA axis responses, but they react on different stressor modalities. While central nuclei are involved in the processing of reactive but not anticipatory stressors, the medial amygdala reveals its excitatory influence solely on generating anticipatory responses (Dayas et al., 2001; Ma and Morilak, 2005). Another subnucleus of the amygdala, the basolateral amygdala, is activated by both stressor types and seems to have an excitatory influence as well (for a summary see Jankord and Herman, 2008; Myers et al., 2012).

Taken together, strong evidence for the involvement of limbic regions in HPA axis stress processing is undoubtedly given, but their interplay is complex and depends on stressor characteristics and anatomical subdivisions. Moreover, the literature mentioned so far deals solely with animal studies. These findings cannot necessarily be transferred to humans as stressor type and duration are often hardly comparable making stress research in humans essential (see also 2.2 "Stress research in humans"; Henze et al., 2020; Van Oort et al., 2017).

2.1.3 Chronic stress

Stress is a double-edged sword: It triggers adaptive processes, enabling coping with threats, but prolonged exposure can lead to maladaptive responses, such as hormonal imbalances and changes in behavior (McEwen, 2004). The adaption to stress involves complex interactions between several physiological systems, leading to short-term benefits, such as energy mobilization, but can result in long-term negative health consequences, including depression, anxiety, sleep disorders, cardiovascular diseases as well as diseases resulting from dysregulated immune functions (Chrousos, 2009; Cohen et al., 2007; Hammen, 2005). Despite the abundance of evidence supporting the stress-disease relationship, the specific biopsychological processes leading from acute to chronic stress and potentially to disease are not yet fully understood. However, it appears that a dysregulation of the HPA axis plays a critical role in mediating the association between stress and malady (O'Connor et al., 2021; Tsigos and Chrousos, 1994).

Key concepts include the role of habituation and sensitization (Herman, 2013). Dynamic habituation, where physiological responses to repeated stressors diminish over time, is involved in the successful adaptation to chronic stress. It is evident in a reduced activation of the HPA axis and is mediated by neural mechanisms, particularly within prelimbic regions, including the PFC, the amygdala, and the paraventricular thalamus. Studies have shown that the inactivation of these areas disrupts habituation, while their activation seems necessary for it to occur (Bhatnagar et al., 2002; Grissom and Bhatnagar, 2011; Weinberg et al., 2010). Although this process is important for adaptation, it does not necessarily return the system to a normal physiological state. Instead, long-term changes can occur in CNS circuits, e.g., increased expression of CRH (Herman, 2013). These changes can lead to sensitization, where chronic stress increases reactivity to new stressors. However, non-habituating to stress is not an option, as it maintains or heightens stress responses, leads also in sensitization, and can result in maladaptive outcomes. In contrast to non-habituation, the glucocorticoid exposure associated with habituation processes may not be sufficient to induce physiological or behavioral outcomes or the onset of the cumulative damage might require substantially longer. The specific mechanisms behind the transition from adaptive to maladaptive stress responses remain poorly understood. Thus, more research of neural circuits and HPA axis alterations involved in this shift is needed.

2.2 Stress research in humans

As previously noted, first investigations into acute and chronic stress responses relied on animal samples. Undoubtedly, these findings hold tremendous relevance for stress research. However, direct transferability to humans is not always feasible (Henze et al., 2020; Van Oort et al., 2017). Stress paradigms commonly employed in animals, such as restraint stress or immobilization, significantly differ from human stress experiences in laboratories and everyday life. These differences pertain to stress intensity, duration, and the psychological components that induce stress. While stress studies in humans face methodological and ethical limitations compared to animal studies, they also offer unique opportunities, such as asking for subjective experiences and perceptions. Furthermore, over the past few decades, several methods that enhance our understanding of stress regulation in humans have been developed. In this context, I will highlight specific findings and methods relevant for the present thesis.

2.2.1 The cortisol awakening response

The CAR represents a distinct increase of cortisol levels in the first 30 to 45 minutes after morning awakening (Pruessner et al., 1997; Stalder et al., 2016). It can be observed from early infancy to older adulthood (Almeida et al., 2009; Fries et al., 2009). The methodological advantages of the CAR, including its assessment in natural settings as well as its combined features of a reactivity index and aspects tied to circadian regulation, make it a widely used measure of HPA axis (re)activity (Stalder et al., 2016). The regulatory mechanisms of the CAR are not yet fully understood. However, they seem to differ from the basal diurnal secretion pattern, since the CAR is evoked by the morning awakening and superimposed upon the circadian rhythm (Clow et al., 2010; Wilhelm et al., 2007). Clow et al. (2010) emphasized the role of the suprachiasmatic nucleus (SCN) in the regulation of the CAR through different pathways, which modulate the pre-awakening ACTH signaling and sensitivity, and the post-awakening adrenal ACTH sensitivity.

In healthy adults, its magnitude ranges between a 50 to 156% rise in salivary cortisol levels (Clow et al., 2004). This magnitude is associated with different physiological and psychological factors, at both an inter- and intraindividual level. Sex and age have been identified as physiological factors associated with the CAR magnitude (Wright and Steptoe, 2005). Moreover, twin studies revealed a moderate heritability of the CAR (Wüst et al., 2000).

Regarding psychosocial influences, associations with negative prior-day experiences as well as with upcoming demands of the day have been found (Adam et al., 2006; Clow et al., 2010; Dahlgren et al., 2009; Doane and Adam, 2010; Stalder et al., 2010). However, studies have also shown an impact of situational factors on the magnitude of the CAR, for which investigators should control, e.g., time of awakening or ambient light level (Hellhammer et al., 2007; Law et al., 2013; Stalder et al., 2022, 2016).

Since its identification, the CAR has been researched extensively and linked to a wide range of psychosocial, physical, and mental health parameters. While in patients with post-traumatic stress disorder (PTSD) a blunted CAR could be observed (De Kloet et al., 2007; Wessa et al., 2006), MDD and its symptom severity were associated with an increased CAR albeit results have been mixed (Dedovic and Ngiam, 2015; Dienes et al., 2013; M. Pruessner et al., 2003; Vreeburg et al., 2009). Regarding physical health problems, alterations of the CAR can be linked among others to adiposity (Steptoe et al., 2004), systemic hypertension (Wirtz et al., 2007), and coronary artery disease (Bhattacharyya et al., 2008). Hence, deviations from a typical CAR pattern indicate maladaptive neuroendocrine processes (Chida and Steptoe, 2009; Clow et al., 2004; Fries et al., 2009; Kudielka and Wüst, 2010). In the present thesis, which employed data from the LawSTRESS project, alterations of the CAR as indicator for HPA axis activity in response to chronic stress were investigated (Chapter 3).

2.2.2 MRI in human stress research

An important model in stress research is the biphasic-reciprocal model proposed by Hermans et al. (2014). It describes the dynamic shifts of neural resources triggered by neuromodulators. According to the authors, acute stress leads to an increased activity in regions of the salience network (SN) at the cost of the central executive network (CEN). During the recovery period, this process is reversed to return to homeostasis. The SN is a neurocognitive system linked to attention and vigilance, and comprises the hypothalamus, anterior cingulate cortex (ACC), amygdala, thalamus, insula, striatum, and the brainstem (Hermans et al., 2011; Seeley et al., 2007). The CEN on the other hand is related to executive control functions and is localized in parietal regions and regions of the prefrontal cortex (PFC; Vincent et al., 2008).

The model is primarily based on animal data, but with the advent of imaging techniques like MRI the investigation of neural processes in humans became feasible. First studies often showed increased stress-related activity in SN but not in CEN regions. Additionally, in human

stress processing, structures of the default mode network (DMN) were involved (Seo et al., 2011; Van Oort et al., 2017). Core regions of this neurocognitive network are the vmPFC, posterior cingulate cortex, dorsal medial prefrontal cortex, inferior parietal lobule, lateral temporal cortex, and the hippocampus (Buckner et al., 2008). Thus, acute stress exposure seems to have an impact on structures of the SN and the DMN but not the CEN.

In a meta-analysis by Kogler et al. (2015b), activation patterns of different stress paradigms were integrated. Convergent activation could only be found for the right inferior frontal gyrus and the anterior insula. In separate analyses for physiological and psychological stressors, additional clusters emerged. Thus, physiological stress led to heightened activity in regions associated with pain processing, e.g., in the insula or striatum; psychosocial stressors, however, were related with activation in the superior temporal gyrus and consistent deactivation in parts of the striatum. As we are interested in psychosocial stress and it appears that the activation patterns of the two stressor types just partially overlap, the following section focuses solely on research of psychosocial stress processing (Kogler et al., 2015b; Qiu et al., 2022).

2.2.2.1 Psychosocial stress induction

Psychosocial stress arises in situations inducing social threat like social evaluation, social exclusion, or performance situations and is characterized by uncontrollability, novelty, and unpredictability (Dickerson and Kemeny, 2004; Kogler et al., 2015a). Tasks incorporating these key components exhibit the strongest cortisol responses and the longest recovery periods (Dickerson and Kemeny, 2004). The most widely used psychosocial stress paradigm in the laboratory is the Trier Social Stress Test (TSST; Kirschbaum et al., 1993). It consists of a public speaking (job interview) and a mental arithmetic task in front of an unresponsive investigator panel. With cortisol increases in 70% – 80% of the participants, it is a robust laboratory stress induction paradigm (Dickerson and Kemeny, 2004; Kirschbaum et al., 1993; Zänkert et al., 2019). Thus, MRI suitable paradigms similar to the TSST might have the strongest probability to elicit a stress response of comparable strength.

In a recent review and meta-analysis (Berretz et al., 2021), stress-responsive activation patterns from five different psychosocial stress paradigms were integrated, namely from the MIST (Dedovic et al., 2005), the Scan*STRESS* (Streit et al., 2014), the social-evaluative threat paradigm (SET; Eisenbarth et al., 2016), the aversive viewing paradigm (AVP; Henckens et al.,

2009), and the Cyberball paradigm (Williams et al., 2000). Two clusters of convergent activation including the insula, claustrum, and the inferior frontal gyrus and one cluster of deactivation with its peak in the parahippocampal gyrus extending to the amygdala were found. Since the Cyberball paradigm entails no social judgment component, a second analysis across all other stress tasks was conducted. Additional clusters of enhanced activation, comprising striatal and temporal regions, and clusters of deactivation, including the cingulate gyrus and the superior temporal gyrus, were revealed (Berretz et al., 2021).

However, the two MRI suitable stress paradigms exhibiting the greatest similarities with the TSST are the MIST and the Scan*STRESS*. As Scan*STRESS* is of importance for the present thesis and as findings from studies using the same stress induction procedures are more comparable than findings from different stress paradigms (Van Oort et al., 2017), the following section focuses on Scan*STRESS*.

2.2.2.2 Scan*STRESS*

Scan*STRESS* is a stress induction paradigm for functional MRI (fMRI) environments developed by our group (Streit et al., 2014). It is based on the TSST (Kirschbaum et al., 1993) and aims at inducing psychosocial stress using components like time pressure, forced failure, pressure to perform, social-evaluative threat, uncontrollability, and unpredictability (Streit et al., 2014). We recently modified the original protocol without changing the paradigm itself (Henze, 2021). The Scan*STRESS* protocol used for the present thesis is presented in Figure 1. A prolonged relaxation phase of about 45 minutes was implemented and the participants were given a sugary drink (75 g glucose in 200 ml herbal tea) about 45 minutes before stress onset facilitating cortisol reactivity (Kirschbaum et al., 1997; Zänkert et al., 2020). Approximately 25 minutes prior stress, a brief training session consisting solely of the Scan*STRESS* control blocks had to be completed. After moving into the scanner tube and the recording of pre-measurements (e.g., localizer), Scan*STRESS* started immediately. The paradigm consists of a block design. During the stress blocks, participants are confronted with challenging mental rotation and serial subtraction tasks while being observed by an investigator panel in professional attire that is visible via life video stream. The two investigators (one female and one male) give standardized, negative feedback (“Work harder” and “Error”) using a red buzzer. Moreover, time pressure is induced, and task difficulty and speed are adapted to the participant’s performance. The control blocks consist of simple number and figure matching

tasks without feedback, observation, or time pressure. The 60-s blocks are presented in alternating order in two runs of 11:20 minutes each. Between the two runs, the participants receive additional negative feedback on their individual performance and a subsequent request to improve by the investigator panel.

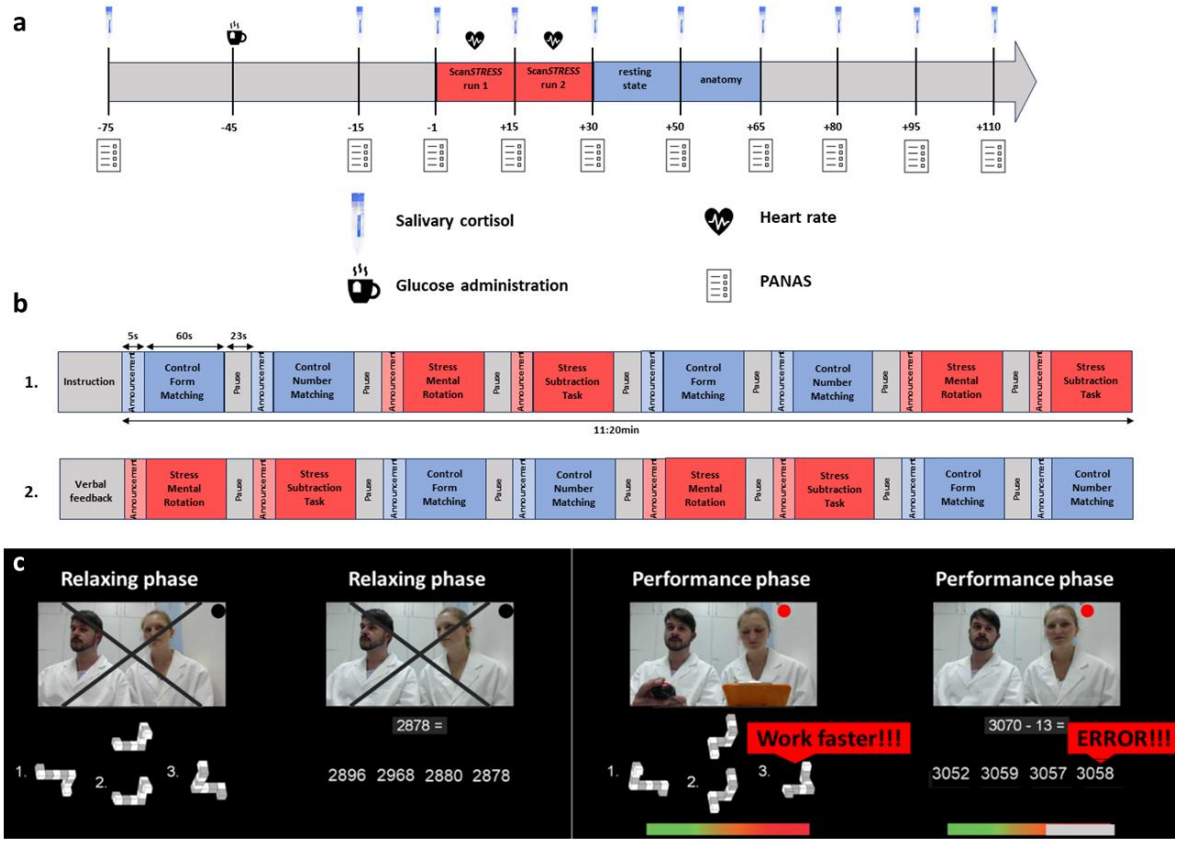

Figure 1. Scan*STRESS*. (1a) Scanning protocol including affective, heart rate, and cortisol measures, (1b) Visualization of the block design of Scan*STRESS* with two runs, (1c) Example screens of the two different conditions (control vs. stress) and the two different tasks within each condition.
Note. PANAS: Positive and Negative Affect Schedule (Watson et al., 1988); Figure is modified after Henze et al. (2020).

Meanwhile, Scan*STRESS* and a recently developed short version of Scan*STRESS* (Scan*STRESS*-C; Sandner et al., 2020) have been implemented in several studies. In the present section, only studies with healthy samples which reported psychological, cortisol, and/or neural measures in response to Scan*STRESS* are included. Studies that implemented Scan*STRESS* just as acute stressor but did not report any results (e.g., Sikka et al., 2020) or reanalyzed already described data (e.g., Henze et al., 2021) will not be presented.

Subjective feelings of stress, nervousness, and negative affect, as well as the heart rate were elevated in response to stress in all studies publishing this data (Akdeniz et al., 2014; Bärtl et

al., 2024; Chand et al., 2020; Dimitrov et al., 2018; Dimitrov-Discher et al., 2022; Henze et al., 2020; Herrmann et al., 2020; Konzok et al., 2021; Kuhn et al., 2023, 2021; Lederbogen et al., 2018; Liu et al., 2023; Nowak et al., 2020; Sandner et al., 2021, 2020; Schwarze, 2021; Speicher et al., 2023). Additionally, in 14 studies, a significant cortisol stress response could be observed in the total sample with varying rates of cortisol responders (Akdeniz et al., 2014; Bärtl et al., 2024; Dahm et al., 2017; Dimitrov et al., 2018; Henze et al., 2020; Hu et al., 2022; Konzok et al., 2021; Kuhn et al., 2021; Lederbogen et al., 2018; Liu et al., 2023; Nowak et al., 2020; Ren et al., 2023; Sandner et al., 2021, 2020; Speicher et al., 2023). The criterion for a responder is a cortisol increase of at least 1.5 nmol/l (Miller et al., 2013). In two studies (Dimitrov-Discher et al., 2022; Streit et al., 2014), no significant change in cortisol levels could be detected, and one study found a decrease over all sampling points (Kuhn et al., 2023). Table 1 provides an overview of psychological, heart rate, and cortisol responses in studies using either Scan*STRESS* or Scan*STRESS*-C.

Table 1. Overview of studies measuring subjective stress ratings, heart rate, and cortisol responses to Scan*STRESS* and Scan*STRESS*-C.

Authors	Version	Sample size	Subjective stress rating	Heart rate	Cortisol
Akdeniz et al. (2014)	Scan*STRESS*	80 (39 men; 41 women)	⇧ negative affect	⇧	⇧
Bärtl et al. (2024)	Scan*STRESS*	61 (healthy) (30 men; 31 women)	⇧ negative affect	⇧	⇧
Chand et al. (2020)	Scan*STRESS*-C	38 (only men)	⇧ nervousness	⇧	
Dahm et al. (2017)	Scan*STRESS*	86 (36 men; 50 women)			⇧ responder: *n* = 55
Dimitrov et al. (2018)	Scan*STRESS*	86 (36 men; 50 women)	⇧ negative affect	⇧	⇧ responders: *n* = 47
Dimitrov-Discher et al. (2022)	Scan*STRESS*	42 (only men)	⇧ stress rating	⇧	⇔
Henze et al. (2020)	Scan*STRESS*	67 (36 men; 31 women)	⇧ negative affect	⇧	⇧ responders: *n* = 38

Authors	Version	Sample size	Subjective stress rating	Heart rate	Cortisol
Herrmann et al. (2020)	Scan*STRESS*	39 (only men)	⇧ nervousness		
Hu et al. (2022)	Scan*STRESS*	70 (39 men; 31 women)			⇧
Konzok et al. (2021)	Scan*STRESS*	61 (31 men; 30 women)	⇧ negative affect	⇧	⇧ responders: *n* = 31
Kuhn et al. (2021)	Scan*STRESS*	39 (only men)	⇧ stress rating		⇧ responders: *n* = 10
Kuhn et al. (2023)	Scan*STRESS*	140 (73 men; 67 women)	⇧ stress rating	⇧	⇩ responders: *n* = 43
Lederbogen et al. (2018)	Scan*STRESS*	57 (32 men; 25 women)	⇧ stress rating	⇧	⇧
Liu et al. (2023)	Scan*STRESS*	77 (40 men; 37 women)	⇧ stress rating		⇧
Nowak et al. (2020)	Scan*STRESS* (only run2)	45 (only men)	⇧ stress rating	⇧	⇧
Ren et al. (2023)	Scan*STRESS*	77 (42 men; 35 women)			⇧
Sandner et al. (2020)	Scan*STRESS*-C	40 (28 men; 12 women)	⇧ negative affect	⇧	⇧ responders: *n* = 28
Sandner et al. (2021)	Scan*STRESS*-C	81 (41 men; 40 women)	⇧ negative affect	⇧	⇧
Schwarze (2021)	Scan*STRESS*-C	47 (22 men; 25 women)	⇧ stress rating		
Speicher et al. (2023)	Scan*STRESS*	20 men	⇧ neg. affect	⇧	⇧ *n* = 12
	Control task	20 men	⇧ neg. affect	⇧	⇧ *n* = 14
Streit et al. (2014)	Scan*STRESS*	42 (22 men; 20 women)			⇔

Note. ⇧ = significant increase; ⇩ = significant decrease; ⇔ = no significant change. Table is modified and extended after Henze (2021).

Regarding neural findings, activations describe higher responses to stress than control conditions (stress > control) and deactivations higher activity during control than during stress blocks (control > stress). Findings concerning activation changes due to acute stress exposure with Scan*STRESS* in (pre)limbic structures have been mixed. For the hippocampus, activations as well as deactivations have been reported. Amygdala and mPFC are more often found to be deactivated than activated. Concerning the ACC, the response direction seems to depend on the subregion with activations in the dorsal ACC and deactivations in the subgenual and perigenual ACC. In contrast, consistent activations could be observed in striatal structures. Table 2 provides an overview of the neural findings for stress-sensitive structures of studies using either Scan*STRESS* or Scan*STRESS*-C.

Table 2. Overview of the neural findings of studies using Scan*STRESS* or Scan*STRESS*-C.

Region	Activations	Deactivations
Hippocampus	Akdeniz et al. (2014), Dahm et al. (2017), Dimitrov-Discher et al. (2022), Lederbogen et al. (2018), Liu et al. (2023), Streit et al. (2014)	Bärtl et al. (2024), Dahm et al. (2017), Henze et al. (2020), Herrmann et al. (2022), Kuhn et al. (2023)
Amygdala	Akdeniz et al. (2014), Dimitrov-Discher et al. (2022), Streit et al. (2014)	Dahm et al. (2017), Henze et al. (2020), Hu et al. (2022), Kuhn et al. (2023), Liu et al. (2023), Sandner et al. (2020)
mPFC	Kuhn et al. (2023)	Akdeniz et al. (2014), Bärtl et al. (2024), Dimitrov-Discher et al. (2022), Henze et al. (2020), Herrmann et al. (2022), Konzok et al. (2021), Liu et al. (2023), Sandner et al. (2020), Streit et al. (2014)

Region		Activations	Deactivations
ACC	ACC total	Hu et al. (2022), Lederbogen et al. (2018), Liu et al. (2023)	Dimitrov-Discher et al. (2022), Henze et al. (2020), Hu et al. (2022), Streit et al. (2014)
	dorsal	Akdeniz et al. (2014), Dahm et al. (2017), Sandner et al. (2020)	
	subgenual perigenual		Akdeniz et al. (2014), Dahm et al. (2017)
Striatum		Akdeniz et al. (2014), Dahm et al. (2017), Lederbogen et al. (2018), Liu et al. (2023), Streit et al. (2014)	

Summing up, Scan*STRESS* recruits (pre)limbic and striatal structures, but the response direction (i.e., activation or deactivation) in certain brain (sub)regions, such as hippocampus or ACC, differs between studies. These differences may arise due to changes in its protocol (e.g., Scan*STRESS* vs Scan*STRESS*-C, duration of the relaxation period), the implementation of Scan*STRESS* in a greater study protocol (e.g., emotion regulation or cognitive tasks before Scan*STRESS*), divergent analyses techniques or the sample selection. Nevertheless, it elicits robust psychological, heart rate, and neuroendocrine stress responses and thus seems to serve as robust stress induction paradigm for the fMRI environment.

2.2.3 Ambulatory assessment

By using ambulatory assessment, data from individuals in their natural environment can be collected (Trull and Ebner-Priemer, 2014). It, thereby, often comprises a multimodal approach including for instance self-report, questionnaire, as well as physiological and biological data (Ebner-Priemer and Kubiak, 2010; Trull and Ebner-Priemer, 2014). The popularity and feasibility of AA has highly benefited from technological advances, increasing digitalization, and the widespread use of smartphones and other wearable data storage devices (Conner and Mehl, 2015; Trull and Ebner-Priemer, 2020). Compared to more traditional assessment methods (e.g., laboratory tasks, questionnaires, or interviews), the AA promises several

advantages (Trull and Ebner-Priemer, 2020): First, as already mentioned, the AA is conducted during the individual's daily life and, thus, increases ecological and external validity (Conner and Mehl, 2015; Trull and Ebner-Priemer, 2014). Additionally, higher reliability can be achieved by intensive measurement repetition. These also enables the examination of within-processes and dynamic changes of mental states over time (Shiffman et al., 2008). Lastly, the AA captures momentary experiences by real-time measurements and can thus reduce the retrospective bias. It therefore offers the opportunity to examine potential influencing factors in addition to dynamic mood processes (Reichert et al., 2021). Although significant efforts have been made to correlate alterations in biological stress systems with changes in the subjective emotional perception of stress, the results have been inconsistent at best (Cohen et al., 2000; Knight et al., 2021; Murphy et al., 2010; Oswald et al., 2004; Pulopulos and Kozusznik, 2018).

Despite those advantages and the inevitable usefulness of AA designs for behavioral stress research (Schlotz, 2019), it is not meant to substitute questionnaires assessing retrospective experiences and behavior. The two methodologies can complement each other by measuring different facets of the same construct (Conner and Barrett, 2012; Shiffman et al., 2008).

Referring to the above-mentioned covariation problem of self-report and biological data (Campbell and Ehlert, 2012; Fahrenberg, 1979), momentary assessments could be more strongly associated with stress-related biological processes than retrospective and trait self-report measures (Conner and Barrett, 2012). For instance, up to now, several AA studies have found an association of salivary cortisol levels collected throughout the day with momentary affect (Jacobs et al., 2007; Joseph et al., 2021; Schlotz, 2019). Thus, different stress indicators might be reliable associated when appropriate methods are applied. Taking this into account, Chapter 3 reports on our findings regarding a potential association between self-reported momentary stress assessed via AA, and the CAR during a long-lasting stress period.

2.2.4 Combining neural findings with ambulatory assessment data

We want to understand and – in the best case – predict human behavior and its underlying mental processes. Self-report and questionnaire data have often been the methods of choice. However, such data have drawbacks, including retrospective biases, response biases (e.g., social desirability, no extreme responding), or problems with introspective abilities, such as mental processes leading to the response/behavior are not fully conscious aware (Berkman

and Falk, 2013; Gadassi Polack et al., 2021). For instance, in consumer psychology, feedback gathered from test-viewers about potential new TV shows frequently diverges from subsequent audience ratings. This discrepancy suggests that viewers' decisions regarding a particular TV show are influenced by unconscious processes (Berkman and Falk, 2013). However, the brain activity of viewers watching a series of commercials was able to predict the success of these commercials in media markets, and outperformed the subjective reports of the commercials' effectiveness (Bell et al., 2018; Falk et al., 2012). Thus, neural measures might be a good source to explain and predict human behavior. However, one major criticism MRI has to face is its ecological validity. Data is collected in a highly-controlled environment and participants lie immobile in a scanner tube, which is problematic to compare with the individual's real world (Shamay-Tsoory and Mendelsohn, 2019). This is where the AA steps into. Due to its real-time and real-life measurements a higher reliability and ecological validity can be reached (Trull and Ebner-Priemer, 2014). Combining these two methods – neuroimaging and AA – may enhance the generalizability of laboratory findings, and thus constitute a powerful approach for a better understanding of human behavior (Gadassi Polack et al., 2021).

In the last years, the advantages of this coalescence have led to an increased number of studies using both approaches. Between 2017 and 2020, 40 studies with the combined approach were published, more than in the previous decade (Gadassi Polack et al., 2021). The main constructs examined by AA were daily affect/stress, social interactions, self-regulation/craving, and psychopathology symptoms. Regarding task-based neural measures, social and emotional paradigms were most commonly used followed by inhibition/self-regulation tasks, reward processing, and risk taking (Gadassi Polack et al., 2021). In their review, Gadassi Polack et al. (2021) emphasize three important considerations that researchers should keep in mind before conducting a multi-method study: the order of the measurements (MRI first, AA first, MRI during AA period, or varying order for subgroups), the similarity of the assessed constructs, and the analysis strategy to integrate data from different sources. The studies included in this review most often conducted the MRI scan first, followed by the AA period. Regarding construct similarity, especially studies with a focus on either emotion processing/regulation, inhibition/craving, or social constructs paid attention on the usage of equivalent constructs in both methods. The analytical techniques applied varied

strongly ranging from simple correlations to mediator or moderator analyses (Gadassi Polack et al., 2021).

Although MRI and AA are both rather recent research methods, and their combination is even newer, the growing number of studies using this multi-method approach and their initial results, are encouraging. Regarding especially emotional and social results, activity patterns in limbic and frontal regions could be associated with daily-life affective states and perceived social threat ratings (Bastiaansen et al., 2018; Berhe et al., 2023; Forbes et al., 2011; Hua et al., 2021; James et al., 2023; Provenzano et al., 2018; Sequeira et al., 2021). For instance, stronger activation of the left amygdala during an emotion regulation task was linked to higher reactivity in negative affect in daily life, whereas increased recruitment of frontal regions was related with lower negative affect reactivity (Bastiaansen et al., 2018). A study that employed the MIST to induce stress in the scanner reported negative associations between stress-induced deactivation in the hippocampus and amygdala, and perceived activity-related stress scores in daily life. Additionally, higher task-induced activation changes in the anterior insula were related to increased daily-life negative affect ratings (Vaessen et al., 2023).

Another step further, is the attempt to predict human behavior using neural markers. Berkman et al. (2013), therefore, introduced the *brain-as-predictor* approach. Its general aim is the uncovering of connections between neural activity in laboratory contexts and longer-term, ecologically valid outcomes. While the traditional neuroscience approach tries to map psychological processes on certain neural mechanisms, the *brain-as-predictor* approach seeks to predict real-world outcomes by using the previous found neural processes (Berkman and Falk, 2013). Up to now, this approach has been proven as valuable in different contexts and for several research questions ranging from consumer behavior/decisions to social interactions and health outcomes (Bertocci et al., 2016; Kühn et al., 2016; Powers et al., 2016; Tompson et al., 2019; Varga et al., 2021). For instance, activity changes in the lateral PFC during emotion induction were associated with later decreases in negative as well as positive emotional inertia in daily life. Additionally, lateral occipital cortex and vmPFC activation were able to predict negative/stress inertia and positive emotional inertia, respectively (Waugh et al., 2017). Mentioning an example from clinical psychology, connectivity alterations in the DMN and the cognitive control network allowed the prediction of the onset of depression three to four years later (Shapero et al., 2019). Despite its growing popularity and use, no

study has yet applied the *brain-as-predictor* approach to examine the link between neural acute stress responses and subsequent chronic stress measures or health consequences.

Taken together, the combination of MRI and AA is a powerful method to study human behavior. MRI measures can elucidate internal psychological processes and AA can compensate for their limited ecological validity. With the *brain-as-predictor* approach, researchers aiming at the investigation of longitudinal mechanisms and the prediction of behavior, emotions, cognitions, and health outcomes. First results using this approach are encouraging. Thus, it appears to be a promising tool to investigate the relation between neural processes in the context of stress and its predictive value on future stress-related consequences and disorders.

2.3 Contributing to human stress research – the LawSTRESS project

In the last decades, substantial research on the processing of stress within different systems and their interactions have been conducted, as detailed in preceding chapters. Despite these efforts, there remains a lack of comprehensive understanding regarding the transition from acute to chronic stress in humans, the psychoneuroendocrine changes that accompany it, and the interindividual differences that contribute to this transition.

Studies incorporating the following features might provide valuable insights into human psychobiological stress research: First, a prospective-longitudinal design, along with an assessment of real baseline levels of stress related variables, should be employed. Second, a study cohort comprising both a healthy control group and a group of healthy individuals who will experience a long-lasting and significant stress period in a well-defined future timeframe should be examined. Third, within this cohort, it should be feasible to apply state-of-the-art and innovative biopsychological laboratory methods as well as ecologically valid assessments to study participants' experiences and behaviors in their daily lives.

The (preparation for the) first state examination for German law students largely fulfills the mentioned requirements. It involves six written exams (in Bavaria) lasting several hours each, spread across eight days, along with an oral exam at a later date. The failure rate ranges from 24% to 30%, and students have only one chance to retake the exam. The final mark significantly impacts their future careers, leading to an intense preparation over approximately one year. All in all, it is regarded as one of the most stressful academic periods.

2.3.1 Chronic stress in students as model for chronic stress exposure

In general, students are considered as a resilient and healthy part of the population, but evidence is accumulating that suffering from high mental load reflected by high levels of psychological stress, depression, and anxiety are widespread among students (Bunevicius et al., 2008; Dastan et al., 2023; Gallea et al., 2021; Grützmacher et al., 2018; Heilmann et al., 2015; Herbst et al., 2016; Sheldon et al., 2021; Weber et al., 2020). Compared with samples of the same age and the general population, the prevalence rates of mental health problems in students are heightened indicating an increased vulnerability (Burger et al., 2014; Gallea et al., 2021; Ibrahim et al., 2013; Rabkow et al., 2020; Rotenstein et al., 2016). The challenges triggering stress are diverse ranging from relationship problems to high expectations or lack of resources (Hamaideh, 2011; Hurst et al., 2013). Across all disciplines, academic stress in general and exams in particular represent a significant source of stress (Hurst et al., 2013). For instance, in studies with medical students, elevated levels of depression and anxiety (Burger et al., 2014; Scholz et al., 2014), and a high number (50%) of students with burnout symptoms developed during their university studies could be observed (Ishak et al., 2013). In a recent report from the University of Berlin, 2145 students from various disciplines reported significantly higher levels of impairment due to physical and psychological symptoms, with law students at the upper end of the reported subjective stress experience (Dastan et al., 2023). Moreover, in a sample of 306 German law students from different semesters, depression symptoms were more frequently reported than in other student populations (Rabkow et al., 2020).

Academic stress influences also biological processes. Exam stress could be associated with alterations in cardiovascular and immunological parameters (Kamezaki et al., 2012; Koudela-Hamila et al., 2020; Loft et al., 2007). For instance, a study including 20 students reported a reduction in the absolute number of Natural Killer cells during an examination period compared to the baseline measurement one month earlier. Furthermore, during the same period, the production of proinflammatory cytokines interleukin-6 and tumor necrosis factor-α in monocytes stimulated by Lipopolysaccharide was increased (Maydych et al., 2017). Regarding neuroendocrine markers, associations between academic stress and salivary as well as hair cortisol were reported (Duan et al., 2013; Koudela-Hamila et al., 2020; Loft et al., 2007; Stetler and Guinn, 2020). The results for stress triggered by academic exams and the cortisol awakening response have been mixed. In a study including men only, students in preparation

for an important examination exhibited a blunted CAR compared to an age-matched comparison group. Moreover, perceived stress levels were negatively related with the CAR (Duan et al., 2013). Koudela-Hamila et al. (2020) used a within-subject design and examined the CAR of students at the beginning of the semester and during the exam period at the end of the semester. During the examination days, a heightened cortisol level at the awakening and a lower increase of cortisol in the first 30 minutes could be observed (Koudela-Hamila et al., 2020). However, studies with opposite results (Hewig et al., 2008; Weik and Deinzer, 2010) or even no impact of examination stress on the CAR (González-Cabrera et al., 2014; O'Flynn et al., 2018) can be found. The methodological heterogeneity, e.g., exam periods of different durations and intensities, differences in the composition of samples, baseline measurements, and the general university systems of different countries, makes a comparison difficult. Nonetheless, the findings indicate a considerable stress load among university students, especially during exam periods, which is associated with changes in biological processes. As noted above, the LawSTRESS project investigated an exceptionally long and continuous exam period – specifically, the (preparation for the) first state examination in law – which is thus supposed to serve as a good model to examine chronic stress.

2.3.2 LawSTRESS: project outline

In the LawSTRESS project, law students were recruited in two different cohorts. Cohort A mainly consisted of students from the University of Regensburg. Cohort B comprised students from other Bavarian universities (Passau, Munich, Erlangen-Nürnberg, Augsburg) who had a less detailed study protocol without laboratory visits in Regensburg. Participants preparing for the first state examination in law were assigned to the SG, while those who did not experience this particular stress period and were in the mid-phase of their study program constituted the CG. The same study protocol was applied to both groups, except for the administration of the exam in the SG. Students were assessed at six sampling points over a period of 13 months (t1: one year, t2: three months, t3: one week prior exam, t4: exam, t5: one week, t6: one month thereafter).

At the first sampling point, questionnaire data (demographics, psychometrics, physical health, health behavior, and university studies related variables), along with genetic and hair samples were collected. Participants in cohort A completed an AA that involved ten pseudo-randomized queries per sampling day, assessing students' stress perception and the collection

of saliva samples in the morning to measure the CAR. Cohort B ran through a less detailed AA consisting only of a morning and an evening query. A subsample of cohort A was additionally examined using fMRI. The scanning session included the stress paradigm Scan*STRESS*, two resting-state sequences and an anatomical scan. At the sampling points t2 (-3 months), t3 (-1 week), t5 (+1 week), and t6 (+1 month), trajectory questionnaires measuring psychometrics, physical health, health behavior, and university studies related variables were administered. The AA was completed at all sampling points (t2 – t6). At t6, an additional hair sample was collected.

In summary, the LawSTRESS project encompasses all the aforementioned components designed to yield valuable insights into stress trajectories and their predictors. It employs a prospective-longitudinal design, which includes a real baseline measurement, thereby facilitating the analysis of temporal transitions, ranging from a healthy state with acute stressors to a state of chronic stress. Its quasi-experimental framework features a healthy control group and a cohort of participants anticipated to experience a prolonged and substantial stress period within a clearly defined future timeframe. The design enhances the capability to identify true associations between vulnerability factors and the environment. Increasingly requested in genetics, quasi-experiments are supposed to offer increased statistical power compared to correlational studies, as experimental methodologies diminish uncontrolled correlations between genetic and environmental factors (Bakermans-Kranenburg and Van IJzendoorn, 2015; Van IJzendoorn et al., 2011; for information about gene-environment effects in the LawSTRESS project see Peter et al. (2022) and Peter et al. (2023)). This methodological advantage also extends to studies examining alternative predictors, such as neural markers, which are a primary focus of the present thesis. Finally, the targeted cohort allows the application of both biopsychological laboratory techniques and detailed AA for the investigation of the participants' experiences and behaviors in their daily life.

Chapter 3

3 Daily life stress and the cortisol awakening response over a 13-months stress period - findings from the LawSTRESS project

3.1 Abstract

The LawSTRESS project is a controlled prospective-longitudinal study on psychological, endocrine, central nervous and genetic predictors of responses to long-lasting academic stress in a homogenous cohort. In this first project report, we focused on the association between daily life stress and the CAR. The CAR, a distinct cortisol rise in the first 30 to 45 minutes after morning awakening, is a well-established marker of cortisol regulation in psychoneuroendocrinology.

Law students from Bavarian universities (total *n* = 452) have been studied over a 13-months period at six sampling points starting 12 months prior exam. The stress group consisted of students experiencing a long-lasting and significant stress period, namely the preparation for the first state examination for law students. Law students assigned to the control group were studied over an equally long period without particular and sustained stress exposure.

To investigate stress-related alterations in the CAR, we examined a subsample of the LawSTRESS project consisting of 204 students with 97 participants from the SG (69.1% female, mean age = 22.84 ±1.82) and 107 from the CG (78.5% female, mean age = 20.95 ±1.93). At each sampling point, saliva samples for cortisol assessment were collected immediately upon awakening and 30 as well as 45 minutes later. Perceived stress in daily life was measured by repeated ambulatory assessments (about 100 queries over six sampling points).

The time course of perceived stress levels in the two groups differed significantly, with the SG showing an increase in perceived stress until the exam and a decrease thereafter. Stress levels in the CG were relatively stable. The CAR was not significantly different between groups at baseline. However, a blunted CAR in the SG compared to the baseline measure and to the CG developed over the measurement timepoints and reached significance during the exam. Remarkably, this effect was neither associated with the increase in perceived stress nor with anxiety and depression symptoms, test anxiety and chronic stress at baseline.

The present study successfully assessed multidimensional stress trajectories over 13 months and it documented the significant burden, law students preparing for the first state examination are exposed to. This period was related to a blunted CAR with presumed physiological consequences (e.g., on energy metabolism and immune function). Mean

psychological stress levels as well as the CAR returned to baseline levels after the exam, suggesting a fast recovery in the majority of the participants.

3.2 Introduction

While the occasional experience of moderate stress is assumed to constitute an inevitable element of everyday life with no negative health consequences in most individuals, chronic stress is a significant risk factor for several disorders, including depression, anxiety, sleep disorders, cardiovascular diseases as well as diseases resulting from dysregulated immune functions (Chrousos, 2009; Cohen et al., 2007). A dysregulation in the HPA axis seems to be a key factor in mediating the stress-disease relationship (O'Connor et al., 2021; e.g., Tsigos and Chrousos, 1994). However, while there is ample evidence for this association between stress and malady, the biopsychological mechanisms mediating this link are not fully understood. In our view, studies could possibly make a useful contribution to human psychobiological stress research, if they combine a prospective-longitudinal design - including an appropriate assessment of baseline levels of stress-related variables - with a research cohort of healthy participants that will be exposed to a long-lasting and significant stress period in a clearly predictable future period and an appropriate control group. Additionally, the application of state-of-the-art biopsychological laboratory methods and ecologically valid assessments of the participants' experience and behavior in everyday life should be feasible in such a cohort. These requirements are met to a high degree by (the preparing for) the first state examination for German law students. This state examination is commonly considered one of the most stressful academic exam periods in the German university system. It consists of six (in Bavaria) written exams of several hours each within eight days (and an oral exam at a later date). The failure rate is about 24% to 30%, the exam can be repeated only once, and the final mark is of major importance for the future career. Usually, the students prepare intensively for this exam for about one year. Although in general, it can surely be assumed that university students constitute a relatively healthy part of the population, academic stress was shown to be a severe burden for many of them. Increased depression and anxiety scores were found in medical students (Burger et al., 2014) and, in a review paper, it was reported that about 50% of the students develop significant burnout symptoms in the course of their university studies (Ishak et al., 2013). Moreover, academic stress was found to be related to salivary and hair cortisol levels (Koudela-Hamila et al., 2020; Stetler and Guinn, 2020) as well as to changes in immune functions (Maydych et al., 2017). It should be noted that the stress periods in previous studies have been significantly shorter and / or less continuous than in the present project. In the LawSTRESS project, we studied law students over a 13-months period. Students preparing

for the state examination have been assigned to the SG, while law students, who did not experience this specific stress period, constituted the CG (see https://doi.org/10.5283/epub.51240 for additional information).

In the present manuscript, we focus on the cortisol awakening response and perceived stress in daily life. The CAR represents a distinct increase of cortisol levels in the first 30 to 45 minutes after morning awakening (Pruessner et al., 1997; Stalder et al., 2016). The regulatory mechanisms of the CAR are not yet fully understood but they differ from the basal diurnal secretion pattern, since it is evoked by the morning awakening and superimposed upon the circadian rhythm (Wilhelm et al., 2007). Amongst others, the CAR was found to be related to various stress-related disorders, including the risk of developing a major depression (Adam et al., 2010), posttraumatic stress disorder (Wessa et al., 2006) or systemic hypertension (Wirtz et al., 2007). Studies examining the association between the CAR and perceived chronic stress and related concepts reported mixed results (Chida and Steptoe, 2009). An increased CAR was linked to chronic work overload and worrying (Schlotz et al., 2004), whereas a blunted CAR was found in subjects reporting burnout symptoms (Oosterholt et al., 2015) and in parents taking care of mentally ill children (Barker et al., 2012). However, in a recent review it has been concluded that studies with more reliable methodologies predominantly found chronic stress to be related to an attenuated CAR (for review see Law and Clow, 2020). These methods include, e.g., a sampling time verification, elaborate statistical analyses including relevant confounding variables (Stalder et al., 2016) and a longitudinal design (O'Connor et al., 2021). Results regarding the association between academic stress phases and the CAR are, as well, not fully consistent (Duan et al., 2013; Weik and Deinzer, 2010).

As saliva samples for the later assessment of the CAR can easily be collected and temporarily stored outside a laboratory, this measure is well suitable for ambulatory settings. Daily life research methods, known as ecological momentary assessment, experience sampling method or ambulatory assessment, cover a wide range of methods, from momentary self-report up to physiological methods, aiming at capturing experience and behavior over the course of an individual's everyday life. The potential advantages of AA are higher reliability due to real-time measurements, higher ecological validity due to real-life measurements and an increased precision due to repeated measurements within individuals (Trull and Ebner-Priemer, 2014). A combination of self-administered salivary cortisol assessments with an AA design offers the opportunity to investigate variance in circulating cortisol and covariance with self-reported

stress in daily life. For example, salivary cortisol levels collected throughout the day were shown to be associated with momentary negative affect in several AA studies (Jacobs et al., 2007; Schlotz, 2019). These and other encouraging findings support the view that reliable associations between indicators of different stress response levels (here: momentary stress ratings and cortisol) can be found when appropriate methods are applied, although a lack of significant covariation of stress indicators is a well-known phenomenon (Campbell and Ehlert, 2012; Fahrenberg, 1979).

For the present study, we hypothesized that long-term stress exposure, defined as preparation for the first state examination for German law students, results, on average, in an increase of perceived stress and other stress-related psychometric variables during the preparation phase and a decrease thereafter, while non-exam students would stay relatively stable in these variables. Moreover, we expected a blunted mean CAR in this period in the stress group compared to the control group. Across all measurements over the observation period, we assumed a significant negative association between the CAR and the perceived stress levels. As interindividual differences can certainly influence the CAR, the predictive value of psychometric variables recorded at the first sampling point, namely anxiety and depression symptoms, test anxiety and chronic stress, on the time course of the CAR over the observation period was tested.

3.3 Methods

3.3.1 Sample

In cooperation with Bavarian faculties of law, 470 students were recruited via social media, flyers and presentations in university as well as commercial law school courses and lectures. In total, 452 law students from the universities of Regensburg (*n* = 154), Passau (*n* = 115), München (Munich; *n* = 85), Erlangen-Nürnberg (Nuremberg; *n* = 49), Würzburg (*n* = 28) and Augsburg (*n* = 21) completed at least the first sampling point. The whole study protocol was completed by 415 participants. Reasons for dropping out were the postponement of the exam to a timepoint after study ending (*n* = 19), no reactions to contact requests (*n* = 15), quitting without reasons (*n* = 16) or other reasons (*n* = 4).

Participants were recruited in two different cohorts. Cohort A comprised of 204 students mainly from the University of Regensburg. Cohort B consisted of 248 law students from the other Bavarian universities who underwent a modified examination protocol that did not

include laboratory visits in Regensburg. Each cohort consisted of a stress group (cohort A: *n* = 97 and cohort B: *n* = 129), experiencing a long-lasting and significant stress period, namely the preparation for the first state examination for law students, and a control group (cohort A: *n* = 107 and cohort B: *n* = 119). It is important to note that CG participants had a typical workload for law students in the mid-phase of their study program.

Individuals who met any of the following (self-reported) criteria were excluded: current psychiatric, neurological, or endocrine disorders, treatment with psychotropic medications or any other medication affecting central nervous system or endocrine functions, regular night-shift work. The study was approved by the local ethics committee. All participants provided written informed consent and received monetary compensation as well as a feedback report on their individual study results.

3.3.2 General procedure

The study protocol provided six sampling points (t1 – t6) over 13 months. T1 for the SG was scheduled one year before the exam; the remaining appointments were three months (t2) and one week (t3) prior exam, at the weekend during the eight-days exam period (t4), as well as one week (t5) and one month (t6) thereafter. The same procedure, except the exam at t4, applied to the CG. Data collection lasted three years from March 2018 until April 2021. Adjusted to the dates of the state examination, the SG started each March or September, with the last group initiated in March 2020. The CG participants started interleaved to the SG each May or November. An additional CG was assessed in July 2019 (see supplementary Figure 11 for a description of the nested data collection in cohorts A and B). In the SG, 36.9% of the students postponed their examination date after t1. Consequently, t2 to t6 were adjusted accordingly in these participants to fit the new exam dates, only the baseline measure at t1 could not be repeated.

At t1, written informed consent was obtained and exclusion criteria were checked. An online questionnaire battery was submitted via SoSci Survey (https://www.soscisurvey.de/; Leiner, 2021) to assess baseline data, psychometrics, physical health, health behavior, and university studies related variables. Moreover, a buccal swab for later DNA analysis as well as a hair sample were collected. The material for the first AA was handed out along with a detailed instruction. Furthermore, 124 participants of cohort A were examined using fMRI (results not presented here). At t2 (-3 months), t3 (-1 week), t4 (exam), t5 (+1 week) and t6 (+1 month)

only the AA and a trajectory questionnaire were administered except for t4, where only the AA was conducted. At t6 a second hair sample was collected. Cohort B had the same study design as cohort A but they did not take part in the fMRI examination and they ran through a slightly less detailed AA (see section 2.3 Ambulatory assessment). In the present manuscript, only AA data (including the CAR) and questionnaire data are presented (Figure 2).

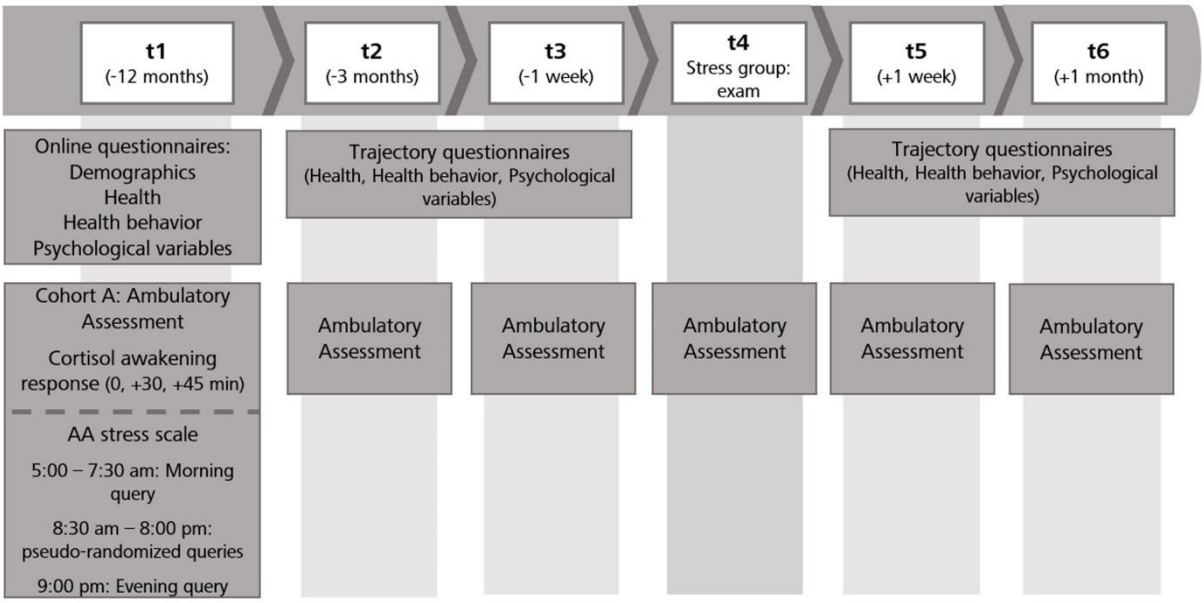

Figure 2. Timing of the data collection for the ambulatory assessment and questionnaires.
Note. Trajectory questionnaires comprised health, health behavior and psychological variables. For an overview of the entire study procedure of the LawSTRESS project see https://doi.org/10.5283/epub.51920.

3.3.3 Ambulatory assessment

The AA for cohort A consisted of an assessment of current perceived stress via the AA stress scale, a short morning and evening questionnaire and the collection of saliva samples after awakening for later assessment of the CAR.

The AA was carried out via the combined smartphone app and web platform movisensXS (Version 1.3.2 to 1.5.13; movisens, Karlsruhe, Germany). At measurement timepoints t1, t2, t5, and t6, ten queries appeared on two consecutive working days. Queries were announced by an acoustic and vibration alarm. To limit the study related burden at the timepoints close to the exam (t3 and t4), queries were presented on one day only. T4 in the SG (not in the CG) was scheduled at the weekend in the middle of the eight-days exam period. The first daily query took place immediately at the individually chosen awakening time between 5:00 and 7:30 a.m. and the last one at 9:00 p.m. The remaining queries were presented at pseudo-randomized times between 8:30 a.m. and 8:00 p.m. with a minimum interval of 60 minutes

between two queries. Across all measurement points, we collected 100 queries per participant. Those who did not have a compatible smartphone were equipped with a device provided by the institute (Motorola G4, Motorola Play G4, Motorola Play G6).

The CAR assessment was based on three saliva samples, obtained on the first day of each AA phase. Only at t1, we assessed the CAR on both sampling days. Saliva samples were collected using cortisol Salivettes (Sarstedt, Nümbrecht, Germany) immediately after awakening as well as 30 and 45 minutes later. Participants were instructed not to eat, drink (except from water), smoke or brush their teeth during this period. To increase compliance and sampling accuracy, functional and non-functional ('fake') electronic monitoring devices to verify times of sample collection (MEMS caps, AARDEX Ltd., Zug, Switzerland) were used in 57.6% – 75.4% (varying over sampling points) of the measurements (Broderick et al., 2004; Kudielka et al., 2003). Moreover, at each saliva sampling the participants were instructed to transfer a random three-digit code to the sampling tube that was briefly presented via smartphone.

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). 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%.

In cohort B, the participants received a hyperlink including the AA stress scale and either the morning or evening questionnaire via SoSci Survey (SMS and e-mail) in the morning at 7:30 a.m. and in the evening at 9:00 p.m. which had to be answered within 90 minutes. This resulted in 12 queries per respondent across all measurement timepoints.

3.3.4 Questionnaires

3.3.4.1 Questionnaires at t1

At t1, a survey was administered online via SoSci Survey. This battery included demographic variables (age, gender etc.), university studies related questions (e.g., academic study time, leisure time, career aspirations), health (behavior) related variables (including height, weight, smoking, alcohol and drug consumption, acute and chronic somatic complaints, disease history and medication use). Furthermore, sleep disturbances were measured with the Regensburg Insomnia Scale (RIS, Crönlein et al., 2013), psychosomatic symptoms with the somatization items from the Symptom-Check-List (SCL-90-R, Franke and Stäcker, 1995), test

anxiety with the test anxiety questionnaire (Prüfungsangstfragebogen (PAF), Hodapp et al., 2011), anxiety and depression symptoms with the Hospital Anxiety and Depression Scale (HADS, Herrmann-Lingen et al., 2011), chronic stress with the Trier Inventory of Chronic Stress (TICS, Schulz et al., 2004) and coping behavior with the Stress and Coping Inventory (SCI, Satow, 2012). To explore child maltreatment retrospectively, the Childhood Trauma Questionnaire (CTQ, Bernstein et al., 2003) was administered.

3.3.4.2 Trajectory questionnaire

To examine the participants' experience and behavior across the study, some of the questionnaires used at t1 were also applied at subsequent timepoints. Besides the university studies and health related questions, the RIS and the HADS were used at t2, t3, t5 and t6. To reduce the burden of the study protocol there was no assessment at t4. The TICS was administered at t2 and t5.

3.3.4.3 AA questionnaire

To measure momentary perceived stress, a five-items AA stress scale was used, consisting of the following items: 'I am under time pressure', 'I am relaxed', 'I am tense', 'I am overstrained' and 'I am disappointed with my performance'. The factor analyses applied to construct this scale based on an original 18-item version are described in the supplements (see supplementary Methods section AA stress scale and Table 9). Additionally, in the first query after awakening, four items related to sleep (e.g., 'The quality of sleep last night was ... ') and stress anticipation (e.g., 'I am confident that I can cope well with today's tasks') were added (Powell and Schlotz, 2012). In the last query, six extra items were asked regarding events of the day (e.g., 'I had an argument with someone today').

3.3.5 Statistical analyses

3.3.5.1 University studies and health related variables

To assess the impact of exam preparation on the participants´ health and behavior, several university studies and health related variables measured over the 13-months period have been used. Analyses of Variances (ANOVAs) for repeated measures with the relevant variables as within-subject factors were calculated using IBM SPSS Statistics (Version 25, IBM, Corp., Armonk, New York, USA). Group (SG vs. CG) was added as between-subject factor,

Greenhouse-Geisser corrections were applied where appropriate and only adjusted results are reported. The entire study sample (*n* = 452) was included in this analysis.

3.3.5.2 AA stress scale and the cortisol awakening response

As the CAR was not assessed in cohort B, the association between the CAR and the AA stress scale was examined in *n* = 204 participants (cohort A). We computed hierarchical models using R (version 4.0.3; R Core Team, 2020). The models were estimated with Maximum Likelihood (ML) and the significance level was set at α = .05. The explained variance of the final models was calculated via conditional R squared for the overall explained variance and via marginal R squared for the variance explained by the fixed effects (Nakagawa and Schielzeth, 2013).

The time course of the AA stress scale was calculated using generalized linear mixed models computed with the package glmmTMB (Brooks et al., 2017). In this two-level model, the variable *group* (0 = CG, 1 = SG), the variable *timepoint* as linear, quadratic and cubic trend and the interactions between these time trends and *group* were included. AA values were clustered in participants, hence random intercepts and slopes for timepoint by participants were estimated to account for dependencies in the data.

To test for alterations in the CAR, we computed three level linear mixed models with cortisol measurements (level 1) nested within timepoints (level 2), nested within participants (level 3). The packages nlme (Pinheiro, Bates, DebRoy, Sarkar and R Core Team, 2021) and MuMIn (Barton, 2013) were used for the analysis. We added random intercepts for both participants and timepoints as well as random slopes for minutes. The variable *timepoint* was entered as categorical variable and recoded, thus, model intercept parameters represented cortisol at the first timepoint. The CAR at the first level was modelled with the categorical variable *minutes* consisting of 0, 30 and 45 minutes after awakening. The final model contained the following fixed effects: *timepoint*, *group*, and the interactions *minutes x group*, *minutes x timepoint*, *group x timepoint*, and *minutes x timepoint x group* to test for differences between the two groups at the six timepoints (model 1). As covariates, we added the *hormonal status* (0 = women not using hormonal contraceptives, 1 = women using hormonal contraceptives and 2 = men), its interaction with *minutes* and the person-mean centered variable *awakening time* and *awakening time x minutes* because these two variables were shown to have an impact on the CAR in our data (model 2).

To further investigate alterations in the SG, similar three level models only containing the SG were computed (SG.model). The predictors were added separately as main effects, in interaction with *minutes* and *timepoint* and as three-way interaction (*minutes x timepoint x predictor*) to test if the predictor had an influence on the alterations of the CAR. In total, we tested seven models, one for each of the predictors (AA stress scale over the time course, anxiety and depression symptoms, test anxiety, work overload, excessive demands from work and chronic worrying at t1). For the AA stress scale, we computed a mean value of the ten queries of the AA stress scale for the day of the CAR assessment, which was centered on the person mean. The other predictors were grand-mean centered. To test for a possible influence on our findings, we also added *post-hoc* the self-report items 'sleep duration' and 'sleep quality', that were assessed on saliva sampling days as part of the AA morning questionnaire, to the SG.model.

Cortisol data was log-transformed to base 10. Seventeen cortisol values were excluded because of participants' nonadherence to the study protocol and physiologically implausible values (e.g., only one extremely high value within one CAR measurement). The residuals of the final models displayed satisfactory approximation to normal distribution.

3.4 Results

3.4.1 Demographic, university studies related and psychological variables

Demographic information of the sample is presented in Table 3. No differences between cohort A and B could be observed in the examined variables. Therefore, only results from the total sample are presented. None of the demographic variables differed significantly between the SG and the CG, except for age ($t(450) = -11.96$, $p < .001$), and none of the reported study related and psychological variables (anxiety, depression, etc., see below) differed significantly at baseline (ps > .113) except the subscale social tensions of the TICS ($t(450) = 1.92$, $p = .056$). Regarding the self-report of academic study time in hours per week, significant differences between the SG and the CG over time were observed with a significant main effect for *timepoint* ($F[3.14, 1184.72] = 185.69$, $p < .001$, $\eta^2 = .33$), as well as a significant interaction *timepoint x group* ($F[3.14, 1184.72] = 164.33$, $p < .001$, $\eta^2 = .30$). For students in the SG, a rise in academic study time until t3 and a distinct decrease thereafter was found. The CG, in contrast, stayed relatively stable. In the last months prior exam, students in the SG indicated

spending 49.12 ±14.90 hours per week with study related issues, while students in the CG indicated spending 34.98 ±14.19 hours per week.

Table 3. Demographic characteristics of the total sample, cohort A and cohort B.

	Total sample		Cohort A		Cohort B	
	Stress group	Control group	Stress group	Control group	Stress group	Control group
n	226	226	97	107	129	119
Age (Mean ± *SD*)	22.98* (±1.71)	21.04* (±1.75)	22.84* (±1.82)	20.95* (±1.93)	23.09* (±1.62)	21.11* (±1.59)
Women	*n* = 165 (73.0%)	*n* = 175 (77.4%)	*n* = 67 (69.1%)	*n* = 84 (78.5%)	*n* = 98 (76.0%)	*n* = 91 (76.5%)
Women using hormonal contraceptives	*n* = 105	*n* = 106	*n* = 49	*n* = 50	*n* = 56	*n* = 56
BMI (Mean ± *SD*)	22.22 (±3.10)	21.90 (±2.82)	22.37 (±2.67)	22.02 (±3.18)	22.10 (±3.39)	21.79 (±2.47)

Note. *SD* = standard deviation. Cohort A (*n* = 204) consisted mainly of law students from Regensburg; Cohort B (*n* = 248) consisted of law students from other Bavarian universities who completed a less elaborate study protocol. * marks significant differences between stress and control group.

We found a significant main effect for *timepoint* and a significant interaction *timepoint x group* for the variables anxiety and depression symptoms and sleep disturbances. In contrast to the CG, we observed a distinct and statistically significant increase in anxiety and depression symptoms as well as in sleep disturbances until t3 in the SG. All variables decreased after the exam to similar levels measured at t1 and in the CG (test statistics can be found in Table 3).

At baseline, 17.0% of the SG and 19.0% of the CG participants already exceeded the clinically relevant score of 11 for anxiety symptoms, which is consistent with previous findings in student cohorts (Bunevicius, Katkute, & Bunevicius, 2008; Moreira de Sousa, Moreira, & Telles-Correia, 2018). At t3, this proportion reached 47.7% in the SG and decreased thereafter to the initial level. The same pattern was found for depression symptoms (cut-off ≥ 11) and sleep disturbances (cut-off ≥ 13). At t3, 19.2% exceeded the cut-off for depression symptoms (t1 = 3.0%) and 5.2% for sleep disturbances (t1 = 0.4%).

Regarding the different facets of chronic stress measured with the TICS, the scales work overload, work discontent, excessive demands from work, lack of social recognition, social

tensions, social isolation, and chronic worrying showed an increase in the SG compared to the CG (test statistics can be found in Table 4; figures can be found on https://doi.org/10.5283/epub.51240).

Table 4. Test statistics for repeated measures ANOVAs for stress-related questionnaire variables.

		F	*p*	η^2
HADS				
Anxiety symptoms	timepoint	44.37	<.001	.10
	timepoint x group	33.06	<.001	.08
Depression symptoms	timepoint	44.67	<.001	.10
	timepoint x group	27.53	<.001	.07
RIS				
Sleeping problems	timepoint	20.23	<.001	.05
	timepoint x group	18.72	<.001	.05
TICS				
Work overload	timepoint	0.13	.875	.00
	timepoint x group	18.35	<.001	.04
Social overload	timepoint	6.93	.001	.02
	timepoint x group	0.82	.436	.00
Pressure to perform	timepoint	8.18	<.001	.02
	timepoint x group	2.50	.085	.01
Work discontent	timepoint	0.46	.620	.00
	timepoint x group	6.93	.001	.02
Excessive demands from work	timepoint	4.36	.014	.01
	timepoint x group	19.58	<.001	.05
Lack of social recognition	timepoint	0.44	.631	.00
	timepoint x group	3.37	.038	.01
Social tensions	timepoint	0.04	.947	.00
	timepoint x group	4.68	.011	.01
Social isolation	timepoint	5.48	.005	.01
	timepoint x group	4.22	.016	.01
Chronic worrying	timepoint	0.46	.624	.00
	timepoint x group	8.24	<.001	.02

3.4.2 Stress-induced alterations in the AA stress scale and the cortisol awakening response

The most important self-report instrument of the present study was the AA stress scale. In cohort A, it was assessed in an extensive AA design with 100 queries per participant, while in cohort B we applied a less extensive design comprising only 12 queries for each participant. In the following, only the results for cohort A (n = 204) are presented.

On average, participants who completed at least the first timepoint, responded to 91.35 (±11.19) out of 100 queries. The model containing a cubic trajectory represented the best fit for the data (compared to the preceding model: linear model ΔAIC = 3751.34; quadratic model ΔAIC = 1209.52; cubic model ΔAIC = 268.22). The trajectory of the perceived stress levels differed significantly between the CG and the SG (*timepoint x SG b* = .39, $p < .001$; *timepoint*^{2} *x SG b* = -.19, $p < .001$; *timepoint*^{3} *x SG b* = .02, $p < .001$). In the SG, mean perceived stress increased until the exam and showed a decline thereafter. The stress levels in the CG stayed relatively stable with just a slight linear increase (*timepoint b* = .05, $p < .001$) (see Figure 3 & supplementary Table 10). There was no significant difference between the two groups at t1 (*SG b* = .09, $p = .059$). Since the covariate *sex* showed no significant effect on perceived stress, the parameter was excluded from the final model. The overall explained variance of the final model was 65.1% and the variance explained by the fixed effects was 8.9%. It should be noted that perceived stress levels in cohort B were higher in both the stress and the control group over the entire study period, but the overall trajectories in SG and CG were very similar to those shown in Figure 3 (see supplementary Table 11 and 12).

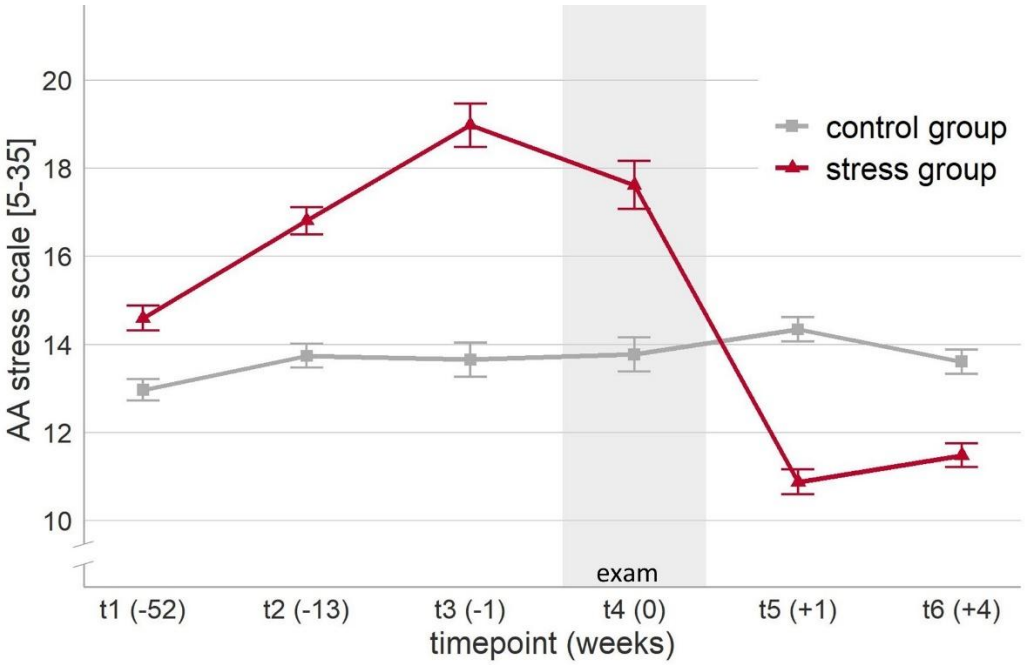

Figure 3. Time course of mean perceived stress levels (± standard error of the mean; SEM) in the stress group (SG) and the control group (CG) over the study period (cohort A).

The compliance rate regarding saliva sampling was rather high. Among participants who completed all timepoints, an average of 20.78 (±0.88) out of 21 saliva samples have been successfully collected resulting in 4009 observations.

A key hypothesis of the present study was the assumption of a decreased mean CAR over the 13-months period in the SG compared to the CG due to exam preparation. Our findings are consistent with this hypothesis. Due to a significant model improvement, the covariates *awakening time*, *hormonal status*, and their interaction with *minutes* (model 1 - model2: ΔAIC = 158.67) were included in the final model. We found a significant increase of cortisol after awakening (*0 min b* = .80, $p < .001$; *30 min b* = .35, $p < .001$; *45 min b* = .36, $p < .001$) with no difference between SG and CG at the first timepoint (*SG x min ps* ≥ .292). Compared to the CG, the SG showed significantly lower mean cortisol values 30 and 45 minutes after awakening during the exam (*SG x t4 x 30 min b* = -.07, $p = .041$; *SG x t4 x 45 min b* = -.10, $p = .004$) (see Figure 4 & Table 5). The overall explained variance of the model was 86.0%; 25.2% thereof could be explained by the fixed effects.

The models for further analysis within the SG comprised 97 students, 86 of whom completed the whole study protocol. Compared to the baseline measure at t1, lower cortisol concentrations during the exam at t4 (*t4 x 30 min b* = -.12, $p < .001$; *t4 x 45 min b* = -.16, $p < .001$) could be observed. At t2 at awakening and at t3, a trend for lower cortisol concentrations

became visible (*t2 x 0 min b* = -.05, *p* = .075; *t3 x 30 min b* = -.05, *p* = .073; *t3 x 45 min b* = -.06, *p* = .075). The full output of the model can be found in Table 13 in the supplements. The overall explained variance for the full model was 84.0% and the variance explained by the fixed effects was 24.6%. The *post-hoc* tested variables *sleep duration* and *sleep quality* and their interaction with *minutes* and *timepoint* did not lead to an improvement of the model, so the significant CAR effect was not explained by concomitant changes in reported sleep behavior (*duration*: ΔAIC = -12.72; *quality*: ΔAIC = -19.68).

Table 5. Parameter estimates for overall effects for the final group model (model 2).

Fixed Effects	Estimate	Std. Error	*p*
Intercept	.80	0.03	**<.001**
30 min	.35	0.03	**<.001**
45 min	.36	0.03	**<.001**
SG	.02	0.04	.517
SG x 30 min	-.02	0.03	.415
SG x 45 min	-.04	0.04	.292
T2	-.01	0.03	.753
T3	-.01	0.03	.801
T4	-.01	0.03	.648
T5	-.07	0.03	**.023**
T6	-.10	0.03	**.001**
T2 x 30 min	-.01	0.03	.616
T2 x 45 min	-.04	0.03	.157
T3 x 30 min	.00	0.03	.918
T3 x 45 min	-.02	0.03	.480
T4 x 30 min	-.03	0.03	.250
T4 x 45 min	-.04	0.03	.188
T5 x 30 min	-.01	0.03	.638
T5 x 45 min	-.02	0.03	.594
T6 x 30 min	.02	0.03	.538
T6 x 45 min	.03	0.03	.432
SG x t2 x 0 min	-.04	0.04	.392
SG x t2 x 30 min	-.02	0.03	.625
SG x t2 x 45 min	.03	0.03	.450
SG x t3 x 0 min	.02	0.04	.703
SG x t3 x 30 min	-.04	0.03	.252
SG x t3 x 45 min	-.03	0.03	.385
SG x t4 x 0 min	.00	0.04	.960
SG x t4 x 30 min	-.07	0.03	**.041**
SG x t4 x 45 min	-.10	0.03	**.004**
SG x t5 x 0 min	.03	0.04	.498
SG x t5 x 30 min	.06	0.03	.085
SG x t5 x 45 min	.05	0.03	.106
SG x t6 x 0 min	.07	0.04	.109

SG x t6 x 30 min	.06	0.03	.060
SG x t6 x 45 min	.06	0.03	.092
Covariates			
Women using HC	.02	0.03	.541
Women using HC x 30 min	-.11	0.02	**<.001**
Women using HC x 45 min	-.10	0.03	**<.001**
Men	-.03	0.04	.465
Men x 30 min	-.07	0.03	**.007**
Men x 45 min	-.10	0.03	**.001**
Awakening time	.15	0.01	**<.001**
Awakening time x 30 min	-.12	0.02	**<.001**
Awakening time x 45 min	-.16	0.02	**<.001**

Random Effects	*SD*	Correlation	
		(Intercept)	30 min
Subject (Intercept)	0.17		
30 min	0.10	-.71	
45 min	0.13	-.76	1
Timepoint (Intercept)	0.20		
30 min	0.15	-.70	
45 min	0.19	-.74	1
Residual	0.10		

Note. Std.Error: Standard error; *SD*: Standard deviation; Min: Minutes after awakening; SG: Stress group; T: Timepoint; HC: Hormonal contraception.

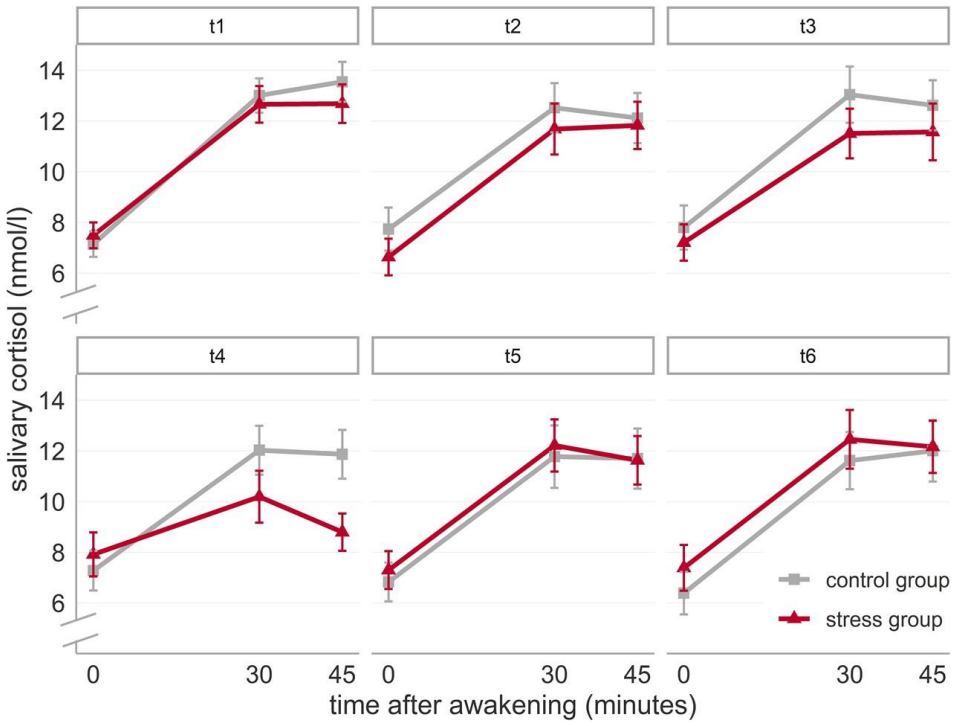

Figure 4. Mean cortisol values (± SEM) for the stress group (SG) and control group (CG) over the study period.

Note. Timepoints: t1 (1 year before the exam), t2 (-3 months), t3 (-1 week), t4 (during exam in the stress group), t5 (+1 week), t6 (+1month).

3.4.3 Predictors of the alterations in the cortisol awakening response

Adding the stress scale to the SG.model on level 2 did not lead to a significant improvement (ΔAIC = -12.14). Thus, we failed to find an association between the cortisol awakening response and perceived stress. Furthermore, we could not detect significant associations between any of the predictors measured at t1, namely anxiety and depression symptoms, test anxiety as well as chronic stress (work overload, excessive demands at work and chronic worrying) and the alterations of the CAR (all ΔAICs < -9.65; for fit indices of the models see supplementary Table 14).

3.5 Discussion

In this report, we present the first results from the controlled prospective-longitudinal LawSTRESS project. Here, we examined the effects of the long-term preparation for and the exposure to the first state examination for German law students on perceived stress and the cortisol awakening response. The combination of a longitudinal design with a baseline measurement about one year prior exam offered the opportunity for a detailed analysis of the trajectories of different stress-related variables including the CAR and their interrelations.

In the stress group, we found significant increases in self-reported anxiety and depression symptoms, sleep disturbances as well as regarding several facets of perceived chronic stress until the exam. Furthermore, perceived stress in everyday life – measured at high frequency with the AA stress scale – increased significantly until the examination period, whereas non-exam students stayed relatively stable. At closer inspection, a considerable number of participants could be identified who temporally clearly exceeded the cut-off levels for anxiety and depression. We also found clear evidence for a fast recovery. Mean anxiety, depression and stress levels as well as reported sleep disturbances returned to initial levels four weeks after the exam. These results confirm and expand previous findings and they highlight the impact of academic stress on students' health and well-being (González-Cabrera et al., 2014; Koudela-Hamila et al., 2020).

Regarding cortisol regulation, a blunted CAR during the examination days (t4) in the SG compared to the baseline measure and to the CG could be observed. This effect is driven by lower cortisol concentrations 30 and 45 minutes after awakening and not by a higher awakening value as observed in other studies (Koudela-Hamila et al., 2020; Weik and Deinzer, 2010). It has to be noted that t4 data were assessed at the weekend between exam days and

that a lower CAR on regular weekend days compared to regular weekdays has been previously reported (Schlotz et al., 2004). However, based on a pilot study (self-report in *n* = 197 law students from Regensburg), we concluded that in the final phase of the exam preparation and during the actual exam block a typical weekend-weekday rhythm does not exist. Our finding that momentary stress levels at t4 were slightly lower than at t3 but still very high, supports this view (see Figure 2). Furthermore, our statistical model controlled for awakening time. Moreover, the significant effect at t4 was preceded by a trend for a reduced CAR at t2 and t3, suggesting a plausible development over time, peaking during the examination days. In our view, this apparent temporal trajectory provides further support for the assumption that the observed blunted CAR at t4 indeed is a valid finding. To date, studies investigating the influence of stress due to academic examinations on the CAR have not yielded consistent results. In the context of examination stress, enhanced morning cortisol responses (e.g., Hewig et al., 2008; Weik and Deinzer, 2010) as well as dampened cortisol levels after awakening (e.g., Duan et al., 2013; Koudela-Hamila et al., 2020) or even no change in the CAR (e.g., O'Flynn et al., 2018) have been reported. However, this partly contradictory results pattern can probably be explained by methodological differences, e.g., heterogeneous samples or varying durations and intensities of the exam period. Law and Clow (2020) recently concluded that studies with convincing designs and reliable methods relatively consistently reported a decreased CAR to be linked to chronic stress. In their cross-sectional study in male students, Duan et al. (2013) observed a blunted CAR in timely proximity to an examination period compared to a control group. This effect was more pronounced in students with higher perceived stress levels. The CAR was assessed twice in a longitudinal study by Koudela-Hamila et al. (2020), once at the beginning of the semester and once at the end, one week prior the examination period. Heightened cortisol levels at awakening as well as reduced subsequent increases were found. Based on a longitudinal design, a real baseline measurement, a control group and a long stress period, our study could confirm these findings. Moreover, we also had the opportunity to collect saliva samples at two timepoints after the exam and, on average, we found a distinct and quick recovery of the CAR already one week after the exam. On the one hand, this trajectory is perfectly in line with those of our measurements of anxiety, depression and perceived stress. On the other hand, the velocity of this change that could be interpreted as indicator of a fast regeneration of cortisol regulation back to normal, is somewhat unexpected.

The finding of a blunted CAR in males and females shows indications for a down-regulation of the HPA axis and hypocortisolism due to chronic examination stress. Interestingly, we could not find a preceding hyperactivity of the HPA axis, as often proposed in the context of a developing hypocortisolism due to ongoing stress (Fries et al., 2005; Miller et al., 2007). However, evidence for this plausible model is scarce. Miller et al. (2007) conducted a meta-analysis mainly based on cross-sectional data and they showed an inverse association between cortisol and the time since stressor onset. Nevertheless, they highlighted the need for longitudinal studies and that the impact of chronic stress on the HPA axis activity seems to depend not only on the timing of the stressor but also on several different features of the stressor and characteristics of the person experiencing it (Boggero et al., 2017; Miller et al., 2007). We assume that, at least in our cohort, long-term examination stress triggered a temporary hypocortisolism. Apparently, HPA axis activity on average seemed to quickly return to baseline levels after the exam. Nevertheless, we suggest that a temporal hypocortisolism in a critical period might be of great psychobiological relevance, considering the numerous effects of cortisol on energy metabolism, mood, and immune function (Sapolsky et al., 2000). First results of a longitudinal study by McGregor, Murphy, Albano, and Ceballos (2016) implicated an association between a flattened CAR due to university studies and a decrease in CD+19 lymphocytes. Further research is needed to examine possible effects of this short-term reduction in morning cortisol. In summary, we found that chronic examination stress in young and healthy students was related to a temporary reduction of the CAR, followed, on average, by a rapid recovery. Interestingly, this mean course of the CAR appears consistent with the mean trajectories of the measured psychometric variables.

While both, perceived stress assessed in everyday life as well as the CAR showed the *a priori* postulated changes over the measurement timepoints, they were not significantly associated. In general, a lack of consistent correlations between subjective stress experience and markers of cortisol regulation is a well-known phenomenon. Moreover, previous studies on the association between CAR measurements and self-reported perceived stress on the same day yielded inconsistent results (M. Pruessner et al., 2003; Weekes et al., 2008). Nevertheless, we hypothesized that a significant association between perceived stress and the CAR, theoretically representing indicators of the same construct 'stress', might become visible in the present study as several features of our design presumably enhanced the validity of our measurements (extensive AA) and facilitated the emergence of within- as well as between-

subject variability. The fact that we still failed to confirm this hypothesis is in line with a recent review by Schlotz (2019) concluding that the probability for the detection of a significant association between momentary stress and cortisol measures collected over the day increases when both variables are measured simultaneously or with only a short time delay to the stressor or daily hassle. Unfortunately, such an approach was not feasible in the present study. Consistent with the absence of a significant association between the AA stress scale and the CAR, stress-related psychological dimensions assessed at baseline, namely anxiety and depression symptoms, test anxiety and perceived chronic stress, did not significantly predict the ascertained CAR effect.

In our view, our study has several strengths, but it surely also has some limitations that need to be considered. First, our participants were young, healthy students with presumably above-average intelligence and socioeconomic status compared to the general population. Therefore, while our cohort was suitable to specifically study academic stress, a generalization of our findings to the general population may be less valid. Secondly, we cannot rule out a certain selection bias as we found that compared to the Bavarian average, our sample achieved better grades in the state examination. Also, the failure rate was higher in Bavaria (24% - 30% in 2019 and 2020) than in our sample (13.1%). It thus appears possible, that particularly less excellent students tended to expect a very stressful exam preparation period and consequently did not accept the extra burden related to participation in our study. Therefore, our findings may underestimate the general stress load related to the first state examination for German law students to a certain extent. However, 32% of the participants did not disclose the exam grade they had received a few months after the last measurement timepoint. Therefore, we certainly cannot rule out that this subgroup on average got lower grades, which, in part, could also explain the difference between the Bavarian average and our sample. Thirdly, our control group was very conservatively chosen. Although the participants were not preparing for the first state examination, they did experience 'usual' university studies related strain, including minor exams. Finally, to increase the CAR assessment quality, we applied several methods (electronic monitoring devices, random codes, encouragement to report non-compliance). Unfortunately, a reliable technique to verify the exact awakening time was not available in the present study and we cannot rule out that this limitation had a confounding effect to a certain extent. However, at least a group-specific effect of this potential confounder appears unlikely as a delay between awakening

and collecting the first sample should result in erroneously high cortisol levels at awakening. This was not observed in our study (see Figure 3).

In conclusion, we were able to assess psychological stress trajectories over 13 months in law students preparing for a major exam and in a control group. A significant increase of perceived stress, anxiety and depression symptoms could be documented and the number of participants showing temporally anxiety and depression scores well-above the clinically relevant cut-off scores appears alarming. These stress-related psychological changes were paralleled by the stepwise development of a blunted CAR, although within participants psychological stress and the CAR were not significantly associated. Fortunately, mean psychological stress levels as well as mean cortisol awakening responses normalized briefly after the exam, suggesting a quick and distinct recovery. It appears conceivable that successfully undergoing this demanding period may improve the individual stress coping strategy and capacity. On the other hand, we certainly cannot rule out that the experience of this exceptionally long stress period may also have a sensitizing effect on psychobiological responses to future stress exposures in vulnerable individuals.

Chapter 4

4 Neural responses to acute stress predict chronic stress perception in daily life over 13 months

4.1 Abstract

The importance of amygdala, hippocampus, and mPFC for the integration of neural, endocrine, and affective stress processing was shown in healthy participants and patients with stress-related disorders. The present manuscript which reports on one study-arm of the LawSTRESS project, aimed at investigating the predictive value of acute stress responses in these regions for biopsychological consequences of chronic stress in daily life. The LawSTRESS project examined law students either in preparation for their first state examination or in the mid-phase of their study program over 13 months. Ambulatory assessments comprising perceived stress measurements and the CAR were administered on six sampling points (t1 = -1 year, t2 = -3 months, t3 = -1 week, t4 = exam, t5 = +1 week, t6 = +1 month). In a subsample of 124 participants (SG: 61; CG: 63), Scan*STRESS* was applied at baseline. In the SG but not in the CG, amygdala, hippocampus, and (*post-hoc* analyzed) right mPFC activation changes during Scan*STRESS* were significantly associated with the trajectory of perceived stress but not with the CAR. Consistent with our finding in the total LawSTRESS sample, a significant increase in perceived stress and a blunted CAR over time could be detected in the SG only. Our findings suggest that more pronounced activation decreases of amygdala, hippocampus, and mPFC in response to acute psychosocial stress at baseline were related to a more pronounced increase of stress in daily life over the following year.

4.2 Introduction

While our knowledge on mechanisms mediating the link between chronic stress exposure and disease risk is still fragmentary, it appears undisputed that individual differences in the brain's interpretation of and response to external and internal stressors constitute a significant determinant of vulnerability for or resilience to stress-related pathology (McEwen, 2007). Although animal models have substantially contributed to our understanding of stress processing in the central nervous system (CNS) and of the interplay with other systems including the HPA axis (Herman et al., 2016; Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009), they cannot necessarily be transferred to humans (Henze et al., 2020; Van Oort et al., 2017). Research on stress regulation in the human CNS has been facilitated by the advent of psychosocial stress paradigms, such as the MIST (Dedovic et al., 2005) or Scan*STRESS* (Henze et al., 2020; Streit et al., 2014), reliably inducing both, robust neural and HPA axis responses in an fMRI environment. While some differences between animal and human models became evident (Van Oort et al., 2017), an involvement of (pre)limbic regions, such as amygdala, hippocampus, and mPFC, well-established in animals, could be confirmed in humans as well (Henze et al., 2020; Herman et al., 2016; Jankord and Herman, 2008; Pruessner et al., 2008; Ulrich-Lai and Herman, 2009). The importance of these brain regions for the integration of CNS and neuroendocrine stress processing is further highlighted by their repeatedly found relation with individual cortisol responses to acute stressors (Henze et al., 2020; Khalili-Mahani et al., 2010; Lederbogen et al., 2011; Pruessner et al., 2008). Moreover, subjective stress ratings were associated with neural activation changes in response to stress in amygdala, hippocampus, and different subregions of the prefrontal cortex as well (Albert et al., 2015; Henze et al., 2020; Orem et al., 2019; Wheelock et al., 2016). In a conjunction analysis, the vmPFC was revealed as region of common activation for cortisol and subjective reactivity to acute stress, suggesting an integrative role of the vmPFC in endocrine and affect regulation (Wheelock et al., 2016). Acute and chronic stress regulation in general and the integration of CNS, endocrine, and affective stress processing in particular, depends on limbic (most notably but not exclusively on amygdala and hippocampus) and prefrontal brain regions (Albert et al., 2015; Henze et al., 2020; Jankord and Herman, 2008; Jovanovic et al., 2011; Khalili-Mahani et al., 2010; Lederbogen et al., 2011; Orem et al., 2019; Pruessner et al., 2008; Wheelock et al., 2016). In patients with stress-related disorders including major depressive disorder, anxiety, or PTSD, functional and structural alterations in the same brain regions have

been observed (Ball et al., 2013; Hariri, 2015; Helm et al., 2018; Henigsberg et al., 2019; Holzschneider and Mulert, 2011; Jaworska et al., 2015). In addition, neural acute stress responses in various patient groups differed from those in healthy subjects with changes predominantly found in limbic and striatal-prefrontal regions (Dong et al., 2022b; Hariri, 2015; Ming et al., 2017). For instance, in patients suffering from major depressive disorder, altered responses to acute psychosocial stressors like the MIST or Scan*STRESS* have been repeatedly observed in cortico-limbic regions including amygdala, hippocampus, vmPFC, and dorsolateral prefrontal cortex (Dong et al., 2022b; Ming et al., 2017). A history of childhood maltreatment was reported to augment the effects (Dong et al., 2022a; Zhong et al., 2020). Although these cross-sectional findings are important, longitudinal designs would allow to contribute substantially to the investigation of mechanisms mediating the shift from acute to chronic stress and, eventually, to the evolvement of stress-related diseases. Demonstrating that differences in the brain's neural response to stress can be observed before the manifestation of signs of chronic stress could serve as one important prerequisite for the assumption of a causal link.

Therefore, the so-called brain-as-predictor approach appears promising in biopsychological stress research. It aims at revealing connections between neural activity in laboratory contexts and longer-term, ecologically valid outcomes (Berkman and Falk, 2013). To date, no study has used this approach to investigate the link between acute stress responses and chronic stress outcomes or health consequences. However, for other research questions, it has been already proven as valuable. For instance, Shapero et al. (2019) used this approach and discovered that resting state connectivity measures significantly predicted the onset of depression three to four years later. Another study by Urry et al. (2006) found activation patterns in the amygdala and prefrontal regions during an emotion regulation task to be related to diurnal cortisol slopes assessed later at home.

The current study focused on the investigation of the predictive value of acute neural stress responses on chronic stress outcomes in real life. It is part of the LawSTRESS project, a prospective-longitudinal study on long-lasting academic stress in a homogenous cohort. It examined German law students either in preparation for their first state examination (SG) or without upcoming particular stress exposure (CG) over 13 months. Besides a suitable cohort, a valid multidimensional stress assessment with methods appropriate for usage in everyday life was essential for the implementation of such a longitudinal study. Thus, we used repeated

ambulatory assessments, allowing an ecologically valid recording of momentary stress-relevant experience and a higher reliability due to repeated real-time and real-life measurements (Trull and Ebner-Priemer, 2014). The AA consisted of perceived stress ratings combined with the collection of saliva samples after awakening to measure CAR. The CAR represents a distinct increase of cortisol levels in the first 30 to 45 minutes after morning awakening (J. C. Pruessner et al., 2003; Stalder et al., 2016) and differs from the basal diurnal secretion pattern (Wilhelm et al., 2007). It is modulated by the SCN via the HPA axis and the sympathetic nervous system (Clow et al., 2010). The CAR was found to be associated with a wide range of psychosocial and health related variables, making it a promising tool in psychobiological stress research (Stalder et al., 2016). So far, it is unknown if neural stress responses are a significant predictor of CAR trajectories over a longer stress period in healthy participants. However, the CAR was indeed shown to be related to indicators of chronic stress. While in early reports findings have been mixed, a current review stated that studies with more reliable methodologies predominantly found associations between chronic stress and an attenuated CAR (Law and Clow, 2020). Chronic stress, in turn, was shown to be related to alterations in limbic and prefrontal regions (Jankord and Herman, 2008; Jovanovic et al., 2011).

Based on this, the current study arm of the LawSTRESS project sought to test the hypothesis that responses to acute psychosocial stress in amygdala, hippocampus, and mPFC are significantly associated with changes in perceived stress and the CAR due to long-lasting exam stress. As the fMRI scan has been performed at baseline, a significant difference between the two groups (SG vs. CG) regarding neural and salivary cortisol responses have not been expected. Moreover, as suggested by the brain-as-predictor approach and as supplementary objective, we intended to contribute to the ecological validation of the used stress paradigm.

4.3 Methods and Materials

4.3.1 Sample

Law students in preparation for their first state examination were assigned to the chronic stress group as it is considered as one of the longest and most stressful exam periods in the German university system. Students with a typical workload in the mid-phase of their study program constituted the control group. The present manuscript reports on data from a LawSTRESS subsample including all 124 (SG: 61; CG: 63) participants who underwent MRI (for

a description of the entire study sample see Giglberger et al., 2022). Of these, six did not complete the entire study protocol. For all fMRI analyses, 13 participants were excluded due to pronounced motion artefacts after motion correction (i.e., absolute movement > 3 mm during at least one run; *n* = 12) or due to poor image acquisition (*n* = 1), resulting in 111 participants (fMRI sample). Demographic information is presented in Table 6.

Table 6. Demographic characteristics of the sample included in the analyses of the present report.

	Total sample		fMRI sample	
	Stress group	Control group	Stress group	Control group
n	61	63	56	55
Age (Mean ± standard deviation)	22.73 (±1.58)	21.10 (±2.05)	22.75 (±1.61)	21.16 (±2.05)
Women	*n* = 36 (59.0%)	*n* = 45 (71.4%)	*n* = 33 (58.9%)	*n* = 37 (67.3%)

Women not using hormonal contraceptives (19.8%; no statistically significant difference between groups, *p* = .533) were scheduled for the MRI session during the luteal phase of the menstrual cycle (Wolfram et al., 2011), confirmed by urinary ovulation test kits (gabmed GmbH, Köln, Germany).

Individuals who met any of the following (self-reported) criteria were not included: current psychiatric, neurological, or endocrine disorders, treatment with psychotropic medications or any other medication affecting CNS or endocrine functions, regular night-shift work or MRI-scanner contraindications. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee at the University of Regensburg, Germany ("Ethikkommission der Universität Regensburg"; https://www.uni-regensburg.de/ethikkommission/startseite/index.html). All participants provided written informed consent and received monetary compensation as well as individualized feedback on their study results.

4.3.2 General procedure

A detailed overall description of the LawSTRESS project can be found elsewhere (https://epub.uni-regensburg.de/51920/; Giglberger et al., 2022). For the present report, the following aspects are relevant: The protocol comprised six sampling points (t1 – t6) over 13

months. T1 for the SG was one year prior exam. The remaining appointments were three months (t2) and one week (t3) prior exam, at the middle of the eight-days exam period (t4), as well as one week (t5) and one month (t6) thereafter. The same procedure, except t4 (exam), applied to the CG. Data collection was carried out in different cohorts and lasted from March 2018 until April 2021.

At t1, an online questionnaire battery was submitted via SoSci Survey (https://www.soscisurvey.de/; Leiner, 2021) to assess baseline data, psychometrics, physical health, health behavior, and university studies related variables. Subsequently, an appointment at the laboratory was arranged to hand out the material for the first AA (including assessment of the CAR) along with a detailed instruction. During a second appointment at the laboratory, an MRI scanning session (including the stress paradigm) took place for the MRI subsample. Further AA were conducted at t2 to t6. In the present manuscript, only AA (including the CAR) and fMRI data are presented (Figure 5).

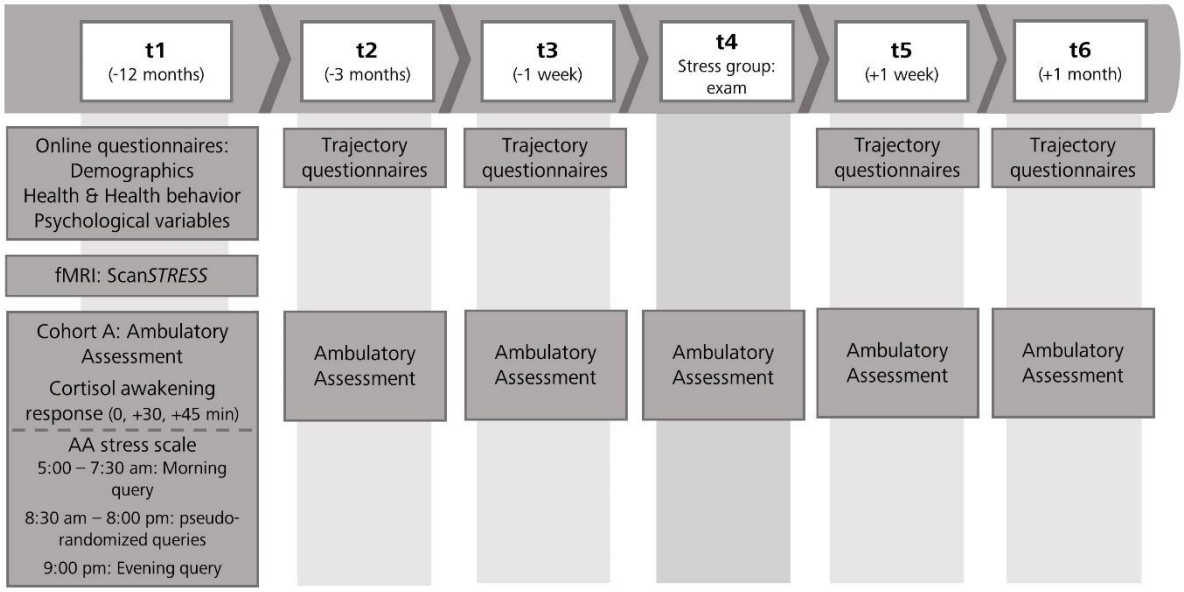

Figure 5. Timing of the data collection for the ambulatory assessment and the MRI measurement. For an overview of the entire study procedure of the LawSTRESS project see https://epub.uni-regensburg.de/51920/.

4.3.3 Ambulatory Assessment

A detailed description of the AA can be found in section 3.3.3. In brief, the AA consisted of an assessment of momentary perceived stress with the five-items AA stress scale (Giglberger et al., 2022) ten times a day (movisensXS; version 1.3.2 to 1.5.13; movisens, Karlsruhe, Germany) and the assessment of the CAR. At t1, t2, t5, and t6, the AA was conducted on two consecutive

working days, while at the sampling points close to or during the examination days (t3 and t4), it took place on single days only. On the first day of each AA, participants collected three saliva samples (Salivettes®, Sarstedt, Nuembrecht, Germany) for later CAR assessment immediately after awakening and 30 and 45 minutes later. Only at t1, we assessed the CAR on both sampling days.

4.3.4 Scan*STRESS*

Scan*STRESS* is a stress induction paradigm for fMRI conditions developed by our group, predominantly aiming at inducing social-evaluative threat and uncontrollability as stress-inducing psychological components (Henze et al., 2020; Streit et al., 2014). Briefly, this block design paradigm consists of two runs containing two conditions (stress vs. control) each. During stress blocks, participants are prompted to perform visually presented arithmetic and mental rotation tasks under time pressure. Task speed and difficulty are adapted to the participant's performance ensuring frequent failure and uncontrollability. After trials and between runs, a previously introduced observation panel gives standardized negative feedback regarding work speed and error frequency. During control blocks, simple figure and number matching tasks have to be performed in the absence of time pressure, observation and negative feedback. Saliva samples were collected at ten timepoints (t = -75, -15, -1, +15, +30, +50, +65, +80, +95, +110 minutes relative to stress onset). Test sessions were scheduled between 1:00 and 5:00 p.m. For a detailed overview of the used Scan*STRESS* protocol see Supplementary Methods section "Scan*STRESS* protocol" and Henze et al. (2020).

4.3.5 fMRI data acquisition and analysis

Imaging data was acquired on a Siemens MAGNETOM Prisma 3T MRI scanner (Siemens Healthcare, Erlangen, Germany) equipped with a 64-channel head coil. Functional images were acquired using a series of blood-oxygenation-level-dependent gradient (BOLD) echo planar imaging images (EPI) with the following parameters: TR = 2000 ms, TE = 30 ms, flip angle = 90°, matrix size = 64 x 64 mm^2, FoV = 192 mm, 37 slices, slice thickness = 3 mm, 1 mm gap, voxel size = 3 mm isotropic, interleaved. The following parameters were used for anatomical pictures: TR = 2400 ms, TE = 2.18 ms, flip angle = 9°, voxel size = 0.8 mm isotropic, distance factor = 50%. The complete MRI session included two resting state sequences after the stress paradigm which are beyond the scope of this manuscript.

Data analyses were carried out with FSL 6.0 (Jenkinson et al., 2012; Smith et al., 2004) using FEAT version 6.0 (Woolrich et al., 2004, 2001). The first five EPI volumes were discarded to account for inhomogeneities of the magnetic field. For a detailed description of the pre-statistics processing and the used analysis steps see Henze (2021). *Z* (Gaussianized *t/F*) statistic images were thresholded *a priori* non-parametrically using clusters determined by z > 2.3.

For each participant, general linear models (GLMs) were defined containing a total of 12 regressors – six conditions (stress arithmetic subtraction, stress figure rotation, control numbers, control figures, announcement of stress, announcement of control) and six motion regressors. In a first step, one GLM was computed for each participant and each run (first level analysis, z > 2.3) to account for scanner drifting. Next, we analyzed mean responses for each participant over both runs (second level, z > 2.3). On a third level, a group analysis (mixed effects, z > 2.3) was conducted to study the overall neural stress response (Henze et al., 2020). For the main task effects (stress > control; control > stress), corrections via the familywise error (FWE) for multiple comparison at a significance level of $p < .025$ (two-tailed combined test FWE $p < .05$) were applied.

To test the predictive value of certain brain regions on the trajectory of our stress measures, we extracted mean beta values of our *a priori* defined regions of interest (ROIs) amygdala, hippocampus, and mPFC from second level analysis (stress > control). For amygdala and hippocampus, we used masks from the Harvard-Oxford Atlas; for mPFC, we created a mask from the deactivation pattern found in Henze et al. (2020) associated with the anatomical mPFC region (Figure 6). ROI-analyses were performed using fslmaths and featquery.

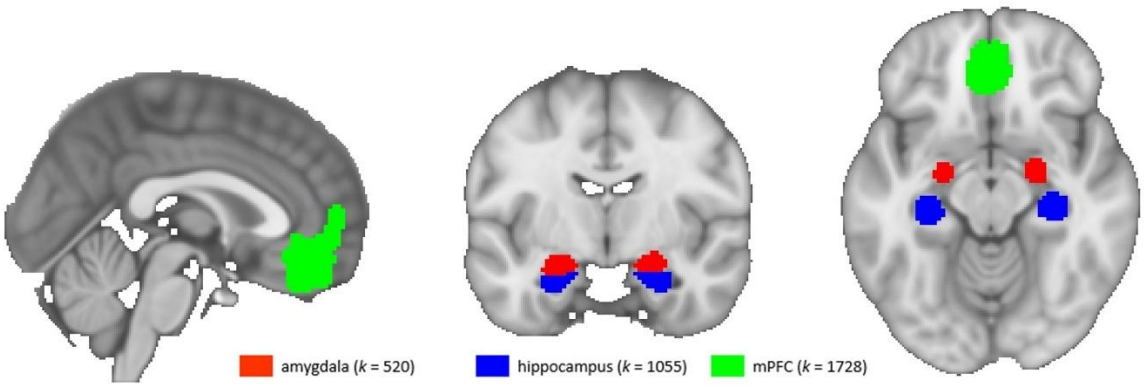

Figure 6. Masks of the used regions of interest amygdala, hippocampus, and medial prefrontal cortex (mPFC). k = number of voxels.

4.3.6 Statistical analyses

Data were analyzed using R (version 4.0.3, R Core Team, 2020), the rstatix (Kassambara and Alboukadel, 2021), glmmTMB (Brooks et al., 2017), nlme (Pinheiro, Bates, DebRoy, Sarkar and R Core Team, 2021) and MuMIn (Barton, 2013) packages. All models were estimated with Maximum Likelihood and the significance level was set at $\alpha = .05$.

4.3.6.1 Scan*STRESS* cortisol response

For the acute stress response, log-transformed cortisol values served as within-subject factors and sex as between-subject factor in a repeated measures ANOVA. Greenhouse-Geisser corrections were applied where appropriate and only adjusted results are reported.

4.3.6.2 AA stress scale and the CAR

The time course of the AA stress scale was calculated using generalized linear models. In this two-level model, the variable *group* (CG = 0; SG = 1), the variable *timepoint* as linear, quadratic, and cubic trend and the interactions between these time trends and *group* were included allowing a different curvilinear time course for each group. AA values were clustered in participants, hence random intercepts and slopes for timepoint by participant were estimated to account for dependencies in the data.

The model testing the predictive value of our ROIs on the rise of perceived stress until the exam comprised the timepoints t1 – t4. The continuous variable *ROI* (*amygdala*, *hippocampus,* and *mPFC*) was included in separate models (due to different time trends; see 3.2) for SG and CG as main effect and in interaction with the time trends (SG: *timepoint*, *timepoint*^{2}; CG: *timepoint*). For explorative analyses, models for the unilateral ROIs were calculated additionally.

To test for alterations of the CAR in the SG, we computed three level linear mixed models with cortisol measurements (level 1) nested within timepoints (level 2), nested within participants (level 3). We added random intercepts for both, participants and timepoints, as well as random slopes for minutes (0, 30, and 45 minutes after awakening). The final model contained the variables *timepoint* (t1 - t6) and *minutes* as well as the covariates *hormonal status* (dummy-coded; reference category: women not using hormonal contraceptives) and *awakening time* in minutes (person-mean centered). Cortisol data was log-transformed.

The analyses testing the predictive value of our ROIs on alterations of the CAR comprised the timepoints t1 – t4. The ROIs were added separately as main effects, in interaction with *minutes* and *timepoint* and as three-way interaction (*minutes x timepoint x ROI*) in separate models for SG and CG.

4.4 Results

4.4.1 Scan*STRESS*: cortisol and neural responses

On average, cortisol levels showed a significant rise in response to acute laboratory stress exposure ($F_{3.29,\ 388.19}$ = 32.69, p < .001, η^2 = .22). As expected, men showed higher mean responses than women ($F_{3.29,\ 388.19}$ = 9.22, p < .001, η^2 = .07). However, the female subsample also exhibited a significant cortisol rise ($F_{3.07,\ 239.52}$ = 7.46, p < .001, η^2 = .09; Figure 7a). During Scan*STRESS*, activations (stress > control) emerged in a cluster including the bilateral insula, frontal, and occipital regions, whereas deactivations (control > stress) arose in two clusters comprising, among others, medial regions of the PFC, hippocampus, and amygdala (all in cluster1) and angular gyrus (cluster2; two-tailed combined FWE-corrected p < .05; Figure 7b; for peak voxels see supplementary Table 15). Mean beta values of the *a priori* defined ROIs amygdala, hippocampus, and mPFC (all bi- and unilateral) are shown in Table 7. As expected, no significant differences were found between SG and CG, neither for the cortisol nor the fMRI analyses.

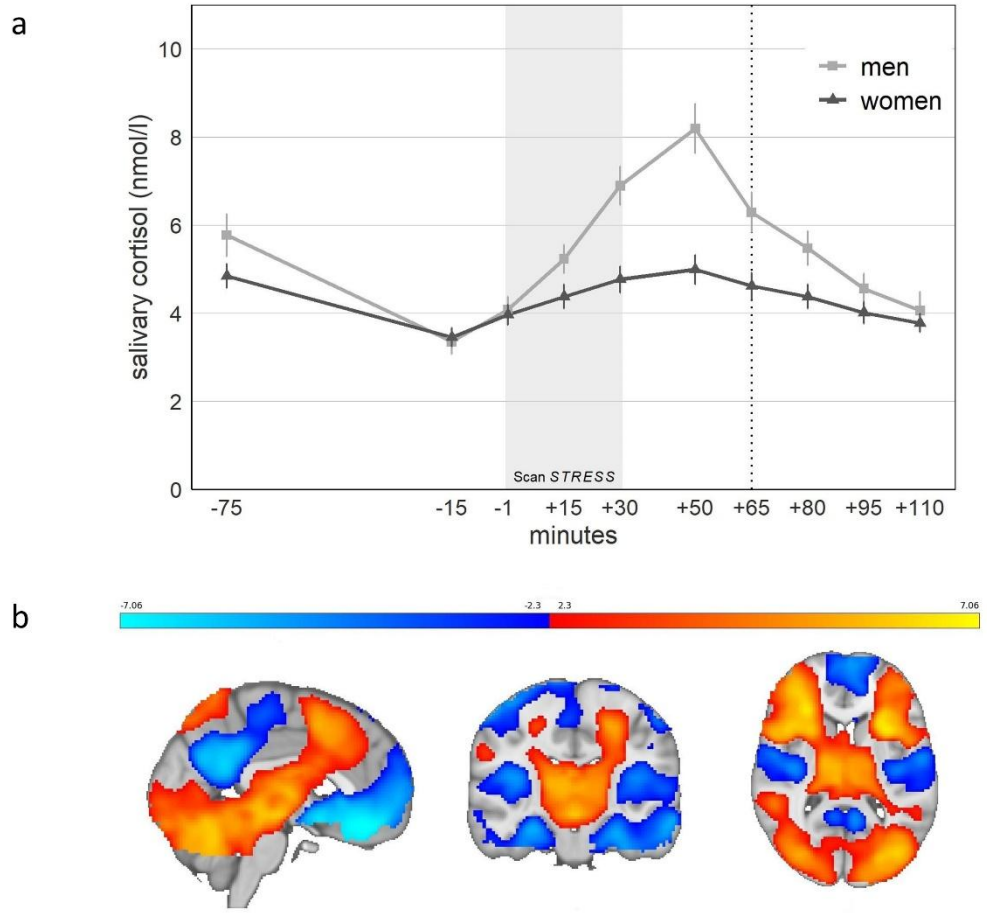

Figure 7. Responses to Scan*STRESS*. (7a) Salivary cortisol responses in women and men (± SEM) (dotted line = end of the scanner session). (7b) Activations (red to yellow) and deactivations (blue) in response to psychosocial stress induction (z > 2.3).

Table 7. Mean beta values ± *SD* of the main task effect stress > control for the *a priori* defined ROIs amygdala, hippocampus, and mPFC (bi- and unilateral).

		n	Mean	*SD*
amygdala	bilateral	109 (SG: 56)	-.09	.30
	left	108 (SG: 56)	-.09	.31
	right	109 (SG: 55)	-.07	.30
hippocampus	bilateral	111 (SG: 56)	-.11	.26
	left	111 (SG: 56)	-.12	.29
	right	111 (SG: 56)	-.10	.24
mPFC	bilateral	85 (SG: 42)	-.22	.48
	left	88 (SG: 44)	-.24	.49
	right	80 (SG: 40)	-.19	.48

4.4.2 Association between neural responses and perceived stress

On average, participants who completed at least the first timepoint responded to 89.01 out of 100 AA stress scale queries. A model containing a cubic trajectory represented the best model fit (compared to the preceding model: linear model ΔAIC = 2227.52; quadratic model ΔAIC = 697.02; cubic model ΔAIC = 142.53). The trajectory of perceived stress levels differed significantly between groups (*timepoint x SG b* = 0.39, $p < .001$; *timepoint*^{2} *x SG b* = -0.20, $p < .001$; *timepoint*^{3} *x SG b* = 0.02, $p < .001$). In the SG, mean perceived stress increased until the exam and showed a decline thereafter. Stress levels in the CG stayed relatively stable (supplementary Figure 12 & supplementary Table 16). There was no significant difference between groups at t1 (*SG b* = 0.10, $p = .074$).

Testing our hypotheses, only the timepoints until the exam (t1 – t4) especially in the SG (SG.model) were of interest. Nevertheless, also models for the CG (CG.model) were calculated to explore potential influences of the ROIs on perceived stress levels without particular stress exposure. A quadratic trajectory for the SG.model (compared to the preceding model: linear SG.model ΔAIC = 740.38; quadratic SG.model ΔAIC = 174.44) and a linear one for the CG.model (compared to the preceding model: ΔAIC = 421.01; *timepoint b* = 0.01, $p = .413$) represented the best fit.

4.4.2.1 Amygdala

Entering the beta values of the amygdala activation to the SG.model led to an improvement (ΔAIC = 4.53). They significantly predicted the increase of stress perception until the exam. In detail, a stronger decrease in amygdala activation to acute stress was related to a steeper curve of perceived stress levels and a peak closer to t4 (Figure 8a and Table 8). In an explorative analysis, this result was found in both hemispheres (timepoint x left amygdala *b* = -0.21, $p = .006$; timepoint^2 x left amygdala *b* = 0.06, $p < .001$; timepoint x right amygdala *b* = -0.21, $p = .017$; timepoint^2 x right amygdala *b* = 0.05, $p = .004$). Entering beta values of the amygdala response to the CG.model did not lead to an improvement (ΔAIC = -0.95).

4.4.2.2 Hippocampus

Adding the beta values of the hippocampus activation also improved the SG.model (ΔAIC = 2.52) with similar results as for the amygdala (Figure 8b and Table 8). Explorative analyses indicated a predictive value for the right hippocampus (timepoint x right hippocampus *b* = -

0.28, p = .008; timepoint^2 x right hippocampus b = 0.07, p = .002), but not for the left (no model improvement ΔAIC = -0.41). The CG.model showed no improvement after entering the hippocampus beta values (ΔAICs = -1.14).

4.4.2.3 mPFC

Since neither the SG.model nor CG.model parameters improved after adding the beta values of the mPFC (SG.model: ΔAIC = -3.23; CG.model: ΔAIC = -0.50), no significant influence of this ROI was assumed. However, in an explorative analysis, an effect of the right mPFC on the trajectory of the SG could be found (timepoint^2 x right mPFC b = 0.02, p = .008; supplementary Figure 13).

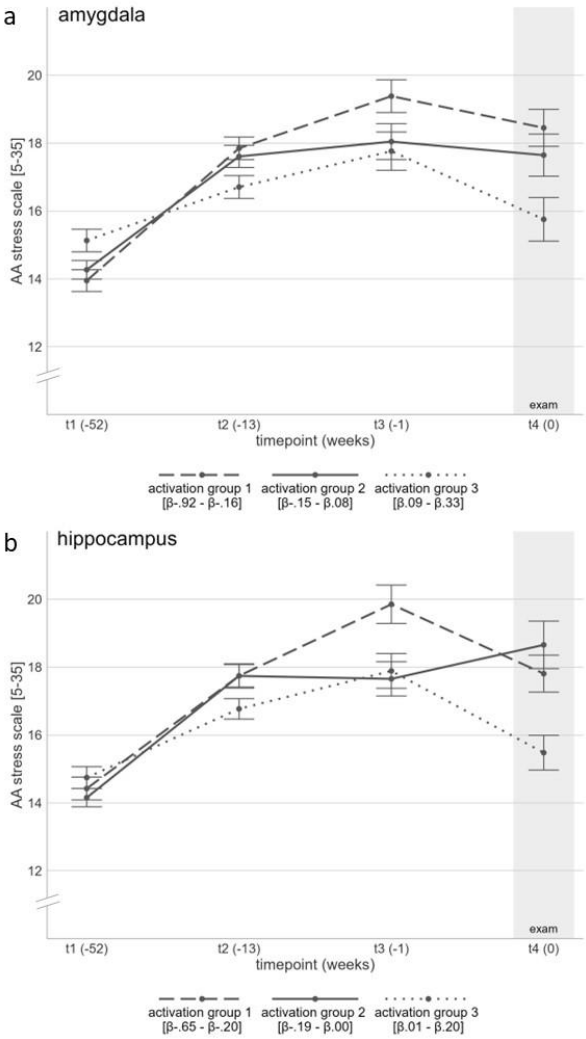

Figure 8. Time course of the *AA stress scale* (±SEM) in the stress group. For illustrative purposes, participants were divided into three groups of equal size according to their amygdala (8a) and hippocampus (8b) responses (indicated by β values), respectively.
Note. t = timepoint; t1 = one year before the exam, t2 = three months prior exam, t3 = one week prior exam and t4 = in the middle of the exam period.

Table 8. Parameter estimates for overall effects for the final SG.model with amygdala or hippocampus activation as predictor.

Fixed Effects	Estimate		*SE*		Significance	
	Amygdala	Hippocampus	Amygdala	Hippocampus	Amygdala	Hippocampus
Intercept	2.64	2.63	0.04	0.04	**<.001**	**<.001**
Timepoint	0.22	0.21	0.02	0.03	**<.001**	**<.001**
Timepoint^2	-0.06	-0.06	0.01	0.01	**<.001**	**<.001**
ROI	0.07	-0.01	0.13	0.16	.578	.956
Timepoint x ROI	-0.21	-0.22	0.08	0.10	**.009**	**.025**
Timepoint^2 x ROI	0.05	0.06	0.02	0.02	**.002**	**.006**
Random Effects	*SD*		Correlation Intercept			
	Amygdala	Hippocampus	Amygdala		Hippocampus	
Participant (Intercept)	0.28	0.28				
Timepoint	0.13	0.13	-.27		-.28	

4.4.3 Association between neural responses and the CAR

The models for the CAR analysis within the SG comprised 61 students of whom 55 completed the whole study protocol. Consistent with our finding in the complete SG sample (Giglberger et al., 2022), a blunted CAR was found at t4 (*t4 x 30 min b* = -0.12, *p* = .002; *t4 x 45 min b* = -0.18, *p* < .001; supplementary Figure 14 & supplementary Table 17). Adding beta values of the ROI activations as predictors led neither in the models for the SG (amygdala ΔAIC = -8.34; hippocampus ΔAIC = -10.68; mPFC ΔAIC = -22.08) nor in the ones for the CG (amygdala ΔAIC = -17.90; hippocampus ΔAIC = -19.44; mPFC ΔAIC = -17.16) to improvements. Thus, no significant associations between activation patterns in our ROIs and alterations of the CAR could be detected.

4.5 Discussion

For stress research, animal models are indisputably relevant, but their findings cannot necessarily be directly transferred to humans. Stress paradigms like restraint stress, commonly used in animals, show substantial differences compared to human stress experience in laboratories and everyday life regarding stress intensity, duration, and stress inducing psychological components. Nevertheless, the importance of amygdala, hippocampus, and mPFC for the integration of CNS, endocrine, and affective stress processing, first documented in rodents (Herman et al., 2016; Hermans et al., 2014; Jankord and Herman,

2008; Ulrich-Lai and Herman, 2009), was confirmed in studies with healthy participants (Henze et al., 2020; Lederbogen et al., 2011; Orem et al., 2019; Pruessner et al., 2008) and patients with major depression, anxiety, or PTSD (Ball et al., 2013; Helm et al., 2018; Henigsberg et al., 2019; Holzschneider and Mulert, 2011; Jaworska et al., 2015). The overarching aim of the present study was to explore if stress processing in these regions is related to later stress responses outside the laboratory. Therefore, we assessed the predictive value of neural responses to acute psychosocial stress in amygdala, hippocampus, and mPFC for biopsychological consequences of chronic stress exposure in daily life. Due to their relevance for a meaningful investigation of this research question, two features of our study design should be emphasized (Berkman and Falk, 2013). First, we used a protocol that reliably induces psychosocial stress in the MRI scanner (Henze et al., 2020; Streit et al., 2014). The observed cortisol responses support the assumption that neural activation changes were indeed responses to psychosocial stress and not mere indicators of task-related mental load. Our ROIs amygdala, hippocampus, and mPFC show on average decreased activation, hence activation was higher during control than stress conditions. This is in line with previous findings examining neural responses to acute stress and, in particular, their relation to cortisol responses (Dedovic et al., 2009; Henze et al., 2020; Pruessner et al., 2008). Limbic regions play an important role in the regulation of the HPA axis stress response. It is assumed that their active state during rest leads to a tonic inhibition of HPA axis activity, changing to a disinhibition facilitating stress hormone release after stress onset (Dedovic et al., 2009; Pruessner et al., 2008). Ongoing processing and contextualization of potentially threatening sensory information might be a default mode of limbic regions which might be disrupted as a response to a stimulus perceived as threatening, thus promoting the focus on the threatening task at hand (Dagher et al., 2009; Pruessner et al., 2008).

The second key feature is the longitudinal and detailed assessment of stress-related variables in everyday life allowing prospective registration of changes over time. In the stress group, we found significant increases in stress levels in everyday life until the exam, whereas non-exam students stayed relatively stable. The perceived stress trajectory of the SG could be predicted by individual activation changes in amygdala and hippocampus; the higher the decrease of activation in these ROIs during acute stress at baseline, the greater the perceived stress increase until the exam twelve months later. Consistent with our longitudinal data, lower hippocampus activation during the MIST was recently reported to be related to scores on a

daily life stress scale in a cross-sectional study (Ren et al., 2023). Associations with the mPFC could not be observed in our study which might partly be due to a decreased number of participants in this model. However, an explorative analysis suggested that the right mPFC was putatively related to the perceived stress changes in the SG. Remarkably, the unilateral analyses for the hippocampus also revealed only a significant association with the right hemisphere which was repeatedly found to be associated with negative emotions (Adolphs et al., 2001; Jansari et al., 2011; Rodway et al., 2003; Sato and Aoki, 2006). Additionally, stress seems to stimulate greater right hemispheric involvement (Ocklenburg et al., 2016). Amygdala activation and hippocampus volume were shown to predict depression and PTSD symptoms (Mattson et al., 2016; Rauch et al., 2000; Villarreal et al., 2002). Complementing these clinical findings, the present study suggests that also in the healthy brain, limbic responses to stress-related content may serve as predictor for the experience of chronic stress.

Moreover, with all due caution, we would like to offer the speculation that our study provided evidence for a certain individual stability of stress processing over time and contexts: Over time, as acute neural stress responses were associated with perceived stress reactions one year later; and over contexts, since the experience of acute stress in a scanner differs from the experience of daily hassles and ongoing stressors in real life. The idea that stress processes are to some extent stable, is supported by genetic studies reporting significant heritabilities for volumes of subcortical regions, like amygdala and hippocampus (e.g., Den Braber et al., 2013). Also, their reactivity to negative stimuli and functional coupling with other stress-relevant brain regions seem to be under genetic influence (Drabant et al., 2006; Jiang et al., 2021; Lonsdorf et al., 2011).

No associations between activation changes in our ROIs and stress levels at baseline or the trajectory of the perceived stress levels in the CG could be detected. This finding supports the view that interindividual differences in stress processing mainly become visible when stress response systems are challenged. Consistent with this notion, internalizing symptoms one to four years after an fMRI session could be predicted by threat-related amygdala reactivity, but only in participants experiencing relatively high life stress during this time period (Swartz et al., 2015).

Furthermore, we asked if our data contribute to research on the ecological validity of laboratory stress paradigms. From that perspective, the present findings are indeed encouraging. They provide novel evidence for a significant association between acute

responses to psychosocial challenge in brain regions involved in stress processing and a longer-term, ecologically valid outcome, namely the development of chronic stress symptoms in real life.

Regarding cortisol awakening responses, SG participants showed, compared to their baseline, a significant decrease during the examination days (t4). We assume that the blunted CAR at t4 can be interpreted as a temporary hypocortisolism in otherwise healthy, young students (Giglberger et al., 2022). An association between neural stress response patterns and alterations in the CAR could not be detected. Convincing evidence for an influence of amygdala, hippocampus, and mPFC on HPA axis stress responses has been found in animal as well as human studies (Herman et al., 2016; Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009). Moreover, while the regulation of the CAR was shown to be modulated by prelimbic regions (Clow et al., 2010; Contreras and Gutierrez-Garcia, 2018) and associations between the CAR and chronic stress have been found (Law and Clow, 2020), it was mainly reported to be unrelated to cortisol reactivity to experimentally-induced psychological stress (Bouma et al., 2009). This is in line with the assumption that the CAR is modulated by additional regulatory mechanisms, for instance a direct influence of the adrenal cortex by the suprachiasmatic nucleus (Clow et al., 2010). This and perhaps other mechanisms may partly explain why on the one hand academic stress in our study affected the CAR, and why on the other hand, this alteration was not associated with activation changes in our limbic ROIs.

Our study has some limitations that need to be considered. First, we cannot rule out a certain selection bias in our sample. Students who already felt stressed by their regular study program and who anticipated an exceedingly stressful exam phase did possibly not volunteer to participate in a study that was related to (modest) additional burden. Therefore, it might be possible that we underestimated the mean stress load in the SG to a certain extent. Second, although we applied several methods to increase the quality of our CAR assessment (electronic monitoring devices, random codes, encouragement to report non-compliance), a reliable technique to verify the exact awakening time was not available in the present study. Thus, a confounding effect to a certain extent cannot be ruled out. However, at least a group-specific effect of this potential confounder appears unlikely as a delay between awakening and collecting the first sample should result in erroneously high cortisol levels at awakening. This was not observed in our study (Giglberger et al., 2022). Third, missing data in our ROI analyses led to different sample sizes in the models including amygdala (n_{SG} = 56; n_{CG} = 53),

hippocampus (n_{SG}= 56; n_{CG} = 55), and mPFC (n_{SG} = 42; n_{CG} = 43). Finally, while the detailed protocol and information on previous publications of the LawSTRESS project have been made easily accessible (https://epub.uni-regensburg.de/51920/), the study was not preregistered. Taken together, the LawSTRESS project was designed as prospective-longitudinal study and used a multimethod and multidimensional approach to assess chronic academic stress. While the experience of this first state examination is specific for German law students, it can be assumed that the overall psychosocial profile of this stressful period is generally comparable to stress that individuals frequently experience in schools, universities, or at the workplace. We found that a more pronounced decrease of activation in amygdala and hippocampus in response to acute psychosocial stress was related to a more pronounced increase of stress over the following year. These neural responses could be indicators of an individual stress response pattern showing a certain stability over time and contexts and significantly predicting chronic stress perception in real life. In that sense, it appears promising to further pursue the idea in future studies that the described neural stress responses may serve as stress vulnerability or resilience marker. Our study supports the view that the brain-as-predictor approach can be a useful strategy in human psychobiological stress research.

Chapter 5

5 Associations between neural stress responses and symptoms of anxiety and depression

5.1 Abstract

Anxiety and depression disorders show high prevalence rates and stress is a significant risk factor for both. However, studies investigating the interplay between anxiety, depression, and stress regulation in the brain are scarce.

The present manuscript included 124 law students (61 preparing for the first state examination) of the LawSTRESS project. Anxiety and depression symptoms were assessed using the Hospital Anxiety and Depression Scale, psychosocial stress was induced with the imaging stress paradigm Scan*STRESS*.

Anxiety, but not depression scores, were significantly related to neural stress responses in a striato-limbic cluster. Moreover, relative to women, men showed stronger associations between anxiety scores and activity in striatal and temporal clusters. A bifactor model of the HADS suggested a general factor characterized by tension, nervousness, and cheerlessness, which was associated with activation changes in a similar but more circumscribed cluster than anxiety. In the LawSTRESS project, the HADS was assessed at five timepoints (one year, three months, one week prior exam, one week, one month thereafter) and thus an exploratory trajectory-analysis could be performed. It confirmed the relationship between anxiety scores and striatal stress responses at baseline but revealed no predictive value of the neural measure across timepoints.

Our results suggest that - in healthy young participants - neural acute psychosocial stress responses in striato-limbic structures are associated with anxiety, supporting the assumption that these regions are related to individual differences in vulnerability to stress-related disorders. A correlation with depression scores could not be found and possible explanations are discussed.

5.2 Introduction

In 2019, one in eight people worldwide was living with a mental disorder, with anxiety and depression disorders being the most common and women being more frequently affected than men (GBD 2019 Mental Disorders Collaborators, 2022; Otten et al., 2021; Seedat et al., 2009). During the COVID-19 pandemic, the number of affected people rose with an estimated increase of 25.6% and 27.6% for anxiety and MDD, respectively (Santomauro et al., 2021). Stressful life events and the exposure to chronic stress have been shown to be significant risk factors for both onset and maintenance of anxiety and depression disorders (Chrousos, 2009; Hammen, 2005; Monroe et al., 2019). Since the brain is the key organ orchestrating the interpretation of external and internal stimuli as well as complex psychobiological stress responses, it appears crucial to investigate individual differences in neural stress processing to further elucidate the stress-disease relationship (De Kloet et al., 2005; McEwen, 2007).

Using fMRI in healthy participants, striato-limbic and frontal activation changes in response to acute psychosocial stress exposure have been repeatedly reported (Berretz et al., 2021; Henze et al., 2021, 2020; Noack et al., 2019; Qiu et al., 2022). These regions are also known to be activated differently in individuals with anxiety disorders or depression. For instance, emotional stimulation, like the presentation of emotional faces or words, revealed altered activations in striato-limbic structures including the amygdala, insula, ncl. caudatus and ACC in patients with anxiety disorders such as posttraumatic stress disorder, social anxiety disorder, or specific phobia (Bruehl et al., 2014; Etkin and Wager, 2007). MDD patients showed, compared to healthy controls, mostly increased activity during rest in striatal and limbic regions and altered neural responses to emotional tasks (Gong et al., 2020; Hamilton et al., 2012; Palmer et al., 2015).

Only recently, first studies investigated neural responses to psychosocial stress in clinical samples. In MDD as well as in anxiety disorder patients, activation patterns differing from those in healthy control participants have been predominantly found in limbic and striato-prefrontal regions (Boehme et al., 2014; Dong et al., 2022b; Ming et al., 2017). For instance, in patients with social anxiety disorders relative to healthy controls, increased activation of the insula and decreased activation of the ventral striatum could be observed during a stress anticipation task (Boehme et al., 2014).

Studies in patient samples are undoubtedly very important. However, to identify risk factors and to trace potentially relevant temporal trajectories, they should be complimented by

studies in healthy individuals with varying levels of anxiety and depression. To date, only a few studies investigated associations between anxiety or depression and acute neural stress responses in healthy participants. Dedovic et al. (2014) used a modified version of the MIST and found differences between students with subclinical depression and a healthy control group. Overall, activation changes were rather similar in both groups but depression scores of the entire sample were positively related with changes in deactivation within the subgenual ACC. In a recent study using the MIST in adolescents, trait anxiety was associated with increased hippocampal and ventral striatal activation and decreased putamen activity (Corr et al., 2021). Moreover, trait anxiety in young, healthy adults was found to be positively associated with activity in mPFC, posterior cingulate cortex, and insula during the MIST stress condition (Wheelock et al., 2016).

Another useful imaging stress paradigm is Scan*STRESS* (Henze et al., 2020; Streit et al., 2014). It induces significant cortisol, heart rate and subjective stress responses and it alters activity in stress-related neural networks (Dimitrov-Discher et al., 2022; Henze et al., 2020; Liu et al., 2023; Streit et al., 2014). Recently, this paradigm has been used to study the effect of trait anxiety on resting state functional connectivity after stress exposure. The authors reported a positive relationship between trait anxiety and post-stress connectivity between right amygdala and pregenual ACC/ventromedial PFC (Nanni-Zepeda et al., 2022).

The present study explored the association between anxiety, depression, and acute neural stress responses in a subsample of the LawSTRESS project (Giglberger et al., 2022). In this project, we investigated, among others, anxiety, depression, and stress perception over more than one year in young and healthy students. For instance, we could recently show that momentary perceived stress in daily life in students experiencing a chronic stress phase lasting several months could be significantly predicted by neural acute stress responses in (pre)limbic regions assessed at baseline (Giglberger et al., 2023).

To assess anxiety and depression scores, the Hospital Anxiety and Depression Scale has been employed in the LawSTRESS project (Zigmond and Snaith, 1983). The HADS is an established and globally used brief self-rating scale with a high acceptance rate. Its design as a questionnaire with two subscales was supported by initial studies investigating the factor structure of the HADS (Bjelland et al., 2002). The German manual as well suggests a two-factor solution that closely aligns with the originally proposed two subscales (Herrmann-Lingen et al., 2011). However, the authors admitted that in different populations other factor structures,

e.g., uni- or three-dimensional solutions were found to have a better fit (Bjelland et al., 2002). In the following years, the uncertainty about the bidimensional structure has grown. Conceptually, anxiety and depression are highly associated and their symptoms strongly overlap making their differentiation often difficult (Kalin, 2020; Kessler et al., 2015).

The aim of the present study was to investigate whether neural responses to stress are associated with anxiety or depression in a young and healthy sample. Based on research in patient samples (Boehme et al., 2014; Dong et al., 2022b; Ming et al., 2017) and samples at risk (Corr et al., 2021; Dedovic et al., 2014; Wheelock et al., 2016), we hypothesized that HADS anxiety and depression scores are positively related to the overall neural stress response, especially in striato-limbic regions. In view of the unclear factor structure of the HADS, a confirmatory factor analysis was computed in our sample in order to test - if appropriate - an alternative factor structure for its association with neural stress responses as well. Lastly, taking advantage of our longitudinal study design, we explored the predictive value of our neural results on the trajectory of anxiety and depression scores.

5.3 Methods

5.3.1 Sample

We investigated law students in preparation for the first state examination (SG) – one of the most challenging and stressful exam periods in Germany – and law students in the mid-phase of their study program as CG. Participants were recruited via flyers, social media and presentations in universities as well as commercial law school courses and lectures. The current manuscript reports on data from a subsample of the LawSTRESS project comprising all 124 participants who underwent MRI (for a description of the entire study sample see Giglberger et al. (2022)). Of these, 13 were excluded due to pronounced motion artefacts despite motion correction (i.e., absolute movement > 3 mm during at least one run; n = 12) or due to poor image acquisition (n = 1), resulting in 111 participants (SG: 56, women: 33, hormonal contraceptives: 27; CG: 55; women: 37, hormonal contraceptives: 28; age: 21.96 ± 2.00). Women not using hormonal contraceptives were scheduled for the MRI sessions during the luteal phase of the menstrual cycle (Wolfram et al., 2011) determined by a chromatographic urinary ovulation test kit (gabmed GmbH, Köln, Germany). Individuals who met any of the following self-reported criteria were excluded: current psychiatric, neurological or endocrine disorders, treatment with psychotropic medications or any other medication

affecting central nervous system or endocrine functions, regular nightshift work or MRI-scanner contraindications (e.g., pregnancy, metal parts in the body).

The study was conducted in accordance with the Declaration of Helsinki, and it was approved by the local ethics committee. All participants provided written informed consent and received monetary compensation as well as individualized feedback on their study results.

5.3.2 General procedure

In the following, only aspects of the general procedure relevant for the present paper are reported. A detailed overall description of the LawSTRESS project can be found elsewhere (https://epub.uni-regensburg.de/51920/). The protocol comprised six sampling points (t1–t6) over 13 months. For the SG, t1 was one year, t2 three months, and t3 one week prior exam. T4 was at the middle of the 8-days exam period, t5 one week, and t6 one month thereafter. The same procedure, except the participation in the exam at t4, applied to the CG. Data collection was carried out in different waves and lasted from March 2018 until April 2021. At t1, an online questionnaire battery was submitted via SoSci Survey (https://www.soscisurvey.de; Leiner, 2021) to assess baseline data, psychometrics (i.a., anxiety and depression), physical health, health behavior and university studies related variables. Some of these data (including anxiety and depression) were collected again at t2, t3, t5, and t6. At baseline, an MRI scanning session (including the stress paradigm) took place for the MRI subsample.

5.3.3 Assessment of anxiety and depression

The HADS (Zigmond and Snaith, 1983) is a brief (14 items) self-assessment scale, originally designed as a screening questionnaire for clinically significant anxiety and depression in non-psychiatric hospital settings (Herrmann-Lingen et al., 2011). The anxiety (HADS-A) and depression subscales (HADS-D) each comprise seven items with a four-point Likert scale (0 – 3). The total score for each subscale is calculated by adding the raw scores (range 0 – 21). A score ≥ 8 is considered as borderline and ≥ 11 as clinically relevant for both subscales (Zigmond and Snaith, 1983). According to Bjelland et al. (2002), the cut-off of 8 or above provides an optimal balance between specificity and sensitivity (both around .80 for HADS-A and HADS-D). Since its development in 1983, the HADS was not only used in different medical settings (e.g., cardiology or oncology) but also in large samples of the general population (e.g., Breeman et al., 2015; Djukanovic et al., 2017). Its international distribution – it has now been

translated into more than 65 different languages – makes it a frequently used instrument worldwide. In the present study, the HADS was administered to all participants as part of an online questionnaire battery submitted via SoSci Survey (https://www.soscisurvey.de/; Leiner, 2014) and had to be filled out at least one day before the MRI measurement and at the sampling points t2, t3, t5, and t6. While five sampling points over 13 months do not allow a high-resolution course analysis, they can serve as a data basis for an exploratory analysis of anxiety and depression score trajectories.

5.3.4 Scan*STRESS*

5.3.4.1 Protocol

Scan*STRESS* (detailed description of the protocol in Henze et al., 2020; Streit et al., 2014) is a stress induction paradigm conceptualized for fMRI conditions primarily based on the psychological components uncontrollability and social-evaluative threat (Dickerson and Kemeny, 2004). The paradigm is composed of a block design with two runs containing two conditions (stress vs. control) each and a total duration of 24 minutes. During the stress blocks, the participants are instructed to perform visually presented computational and mental rotation tasks quickly and accurately via keystroke. Task speed and difficulty are adapted to the participant's performance, thereby forcing failure. After trials and between runs, two observers visible to the participants via video stream give standardized negative feedback on working speed and error frequency. During control blocks, simple figure and number matching tasks have to be performed in the absence of time pressure and negative feedback. In the present study, test sessions were scheduled between 1:00 and 5:00 p.m.

5.3.4.2 Salivary, heart rate, and subjective stress

Saliva samples for later cortisol assessment, heart rate, and emotional reactivity ratings have been repeatedly obtained throughout the test session. In the present analysis, these variables served as a manipulation check to document the occurrence of acute psychoendocrine stress responses. A total of ten saliva samples was collected - three before the start of the paradigm (75, 10, and 1 min before), one sample during the paradigm between the two Scan*STRESS* blocks, one sample immediately afterwards as well as 50, 65, 80, 95, and 110 min later. At the same sampling points, the participant's mood was surveyed using the German version of the Positive and Negative Affect Schedule (PANAS; Krohne et al., 1996; Watson et al., 1988). Due

to standardization, the entire PANAS was presented, but only the negative affect (NA) was evaluated. Saliva samples were assayed in duplicate using a time-resolved fluorescence immunoassay with fluorometric end-point detection (DELFIA) at the biochemical laboratory at the University of Trier (Dressendörfer et al., 1992). 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%. An MRI compatible finger oximeter (Nonin Medical, Model 7500 FO, Minnesota, USA) recorded the heart rate during Scan*STRESS* with a sampling rate of the highest heartbeat within four seconds.

5.3.5 Statistical analysis of salivary cortisol, heart rate, and subjective stress

Data were analyzed using R v4.0.3 (R Core Team, 2020) with the package rstatix (Kassambara and Alboukadel, 2021). All models were estimated with Maximum Likelihood and the significance level was set at $\alpha = .05$. For the acute stress response, log-transformed cortisol values (nmol/l) and NA served as within-subject factors in separate repeated measures analyses of variance (rANOVA). Participants with a cortisol increase of at least 1.5 nmol/l between the individual pre-stress level (sample -1) and the individual peak (sample +30, +50, or +65) were defined as cortisol responders (Miller et al., 2013). Regarding heart rate, an rANOVA for each run was computed. Greenhouse-Geisser corrections were applied where appropriate and only adjusted results are reported.

5.3.6 fMRI data acquisition

Participants were scanned in a Siemens MAGNETOM Prisma 3T MRI scanner (Siemens Healthcare, Erlangen, Germany) equipped with a 64-channel head coil. Functional images were acquired with a BOLD gradient EPI sequence covering 37 axial slices (3 mm thick, 1 mm gap, voxel size = 3 mm isotropic, interleaved, TR = 2000 ms, TE = 30 ms, flip angle = 90°, matrix size = 64 x 64 mm^2, FoV = 192 mm) and T1-weighted volumes with a 3D magnetization-prepared rapid gradient-echo (MP-RAGE) sequence (TR = 2400 ms, TE = 2.18 ms, flip angle = 9°, voxel size = 0.8 mm isotropic, distance factor = 50%). The complete MRI session included resting state and anatomical measurements after the stress paradigm, which are not reported in the present manuscript.

5.3.7 fMRI preprocessing and analysis

Data analyses were carried out with FSL 6.0 (Jenkinson et al., 2012; Smith et al., 2004) using FEAT version 6.0 (Woolrich et al., 2004, 2001). The first five EPI volumes were discarded to account for T1 saturation effects. Preprocessing procedure included MCFLIRT motion correction, slice timing correction, brain extraction using BET, spatial smoothing (Gaussian kernel, 8.0 mm FWHM), grand-mean intensity normalization and high-pass temporal filtering with 120.0 s cutoff. FLIRT and FNIRT were used for intraindividual coregistration and registration to the 2.0 mm MNI152 standard space (Henze, 2021). Time-series statistical analysis was performed using FILM. *Z* (Gaussianized *t/F*) statistic images were thresholded *a priori* non-parametrically using clusters determined by $z > 3.1$.

In a first step, GLMs were performed with six condition regressors (stress arithmetic subtraction, stress figure rotation, control numbers, control figures, and announcement of stress and control) and six motion regressors for each participant and each run (first level analysis, $z > 3.1$). Next, we analyzed mean responses for each participant over both runs (second level, $z > 3.1$). To study the overall group neural stress response, a mixed-effects group analysis (third level, $z > 3.1$) was conducted (Henze et al., 2020). For the main task effects (stress > control; control > stress), corrections via FWE for multiple comparison at a significance level of $p < .025$ (two-tailed combined test FWE $p < .050$) were applied.

The two GLMs, one including HADS-A and one HADS-D scores as continuous covariates (grand-mean centered) and sex as covariate of no interest, were conducted for the main task effect stress > control (third level, $z > 2.3$). Since sex differences in anxiety and depression are prominent (e.g., Altemus et al., 2014) and some studies also indicate sex differences in the neural stress response (e.g., Goldfarb et al., 2019; Henze et al., 2021), we performed unpaired two-group analyses with continuous covariate interaction. Whole-brain corrections were conducted with each contrast thresholded at FWE $p < .025$ (two-tailed combined test FWE $p < .050$).

To further explore the relationship between stress-responsive brain regions and anxiety or depression, a *post-hoc* ROI analysis was performed utilizing fslmaths and featquery. Therefore, a binary 5 mm spherical mask around the overlapping peak region of the preceding (sex-specific) HADS-A whole-brain analyses (comprising parts of the ncl. caudatus, pallidum and putamen) for each hemisphere (left: 51 67 37; right: 36 67 37) was generated. Mean beta values (extracted from second-level analysis) of the contrast stress > control were extracted

for each participant and were subsequently entered into one-way ANOVAs with sex as fixed factor and HADS-A and HADS-D as covariate.

5.3.8 Confirmatory factor analysis of the HADS

To explore the dimensionality of our HADS data, we conducted a confirmatory factor analysis (CFA) using the R lavaan package (Rosseel, 2012). We performed one-factor, two-factor, and bi-factor models (for further details see supplemental Methods section 'Confirmatory factor analysis').

5.3.9 Exploratory analyses: predictive value of neural responses on anxiety and depression score trajectories

To estimate a possible predictive value of our neural findings on anxiety and depression score trajectories until the exam (three assessments over one year, t1 – t3), we performed generalized linear mixed models (time nested within participants) using the R glmmTMB package (Brooks et al., 2017). The final models for both constructs contained the fixed effects *group* (0 = CG; 1 = SG), *time* in months as linear trend, its interaction with group and the covariate *sex* (0 = men; 1 = women). To account for dependencies in the data, random intercepts were estimated. In a next step, beta values of our precedingly described striatal *ROIs* (left and right) were included as main effect, in interaction with group and time trend and as three-way interactions (*group* x *time* x *ROI*). The conditional R squared was calculated for the overall explained variance and the marginal R squared for the variance explained by the fixed effects (Nakagawa and Schielzeth, 2013).

5.4 Results

5.4.1 Hospital Anxiety and Depression Scale

In line with findings of our overall sample (Giglberger et al., 2022), mean HADS-A and HADS-D scores at baseline (t1) were in the normal range (HADS-A: 6.44 ± 3.59; HADS-D: 3.41 ± 2.95) indicating that, on average, participants in our sample showed no/mild symptoms of anxiety and depression. Nevertheless, 35.1% (*n* = 39, women: 22) of the sample showed anxiety scores considered as borderline (scores ≥ 8) and 16.2% (*n* = 18, women: 10) exceeded the presumed clinically relevant score of 11. Regarding depression symptoms, 10.8% (*n* = 12, women: 9) of the participants reached the borderline score and 2.7% (*n* = 3, women: 3) a score considered as clinically relevant.

5.4.2 Associations of anxiety and depression scores with neural responses

First, regarding acute stress reactions during Scan*STRESS*, cortisol changes and whole-brain neural response patterns have been reported in Giglberger et al. (2023). Briefly, cortisol levels showed a significant mean rise following the stressor with a higher increase in men compared to women. Furthermore, a distributed network of neural activations and deactivations could be found in response to stress exposure. NA ratings rose significantly during the exposure to Scan*STRESS* ($F_{3.58,\ 386.69} = 55.27$, $p < .001$, $\eta_p^2 = .34$) and heart rates were significantly higher during stress compared to control blocks in both runs (run1: $F_{2.39,\ 264.96} = 156.22$, $p < .001$, $\eta^2 = .59$; run2: $F_{2.04,\ 226.45} = 209.09$, $p < .001$, $\eta^2 = .65$).

To analyze the association of anxiety and depression with the neural stress reaction, individual HADS-A and HADS-D scores (both grand-mean centered) were used as covariates in separate models (FWE-corrected $p < .050$), corrected for sex.

Adding HADS-A scores significantly predicted neural responses (stress > control) in one cluster including ncl. caudatus, thalamus, putamen, parahippocampal gyrus, and insula (Figure 9a; peak voxels can be found in supplementary Table 18). Moreover, an unpaired two-group analysis with HADS-A scores as continuous covariate revealed a significant sex difference (men > women) in striatal and temporal clusters comprising, among others, superior temporal gyrus, ncl. caudatus, putamen, middle cingulate gyrus, and insula (Figure 9b; peak voxels can be found in supplementary Table 19). *Post-hoc* ROI analyses for the chosen striatal region (Figure 9c) supported these findings by showing a significant main effect for HADS-A scores (left ROI: $F_{1,107} = 12.50$, $p < .001$, $\eta_p^2 = .10$; right ROI: $F_{1,107} = 12.90$, $p < .001$, $\eta_p^2 = .10$; both survive Benjamini-Hochberg correction) and a significant sex x HADS-A interaction (left ROI: $F_{1,107} = 8.60$, $p = .004$, $\eta_p^2 = .07$; right ROI: $F_{1,107} = 7.14$, $p = .009$, $\eta_p^2 = .06$; both survive Benjamini-Hochberg correction), but no main effect for sex (left ROI: $F_{1,107} = 0.78$, $p = .378$; right ROI: $F_{1,107} = 0.13$, $p = .724$, Figure 9d and 9e).

Entering depression scores to the neural analysis revealed no significant activations or deactivations associated with depression nor a sex x depression interaction.

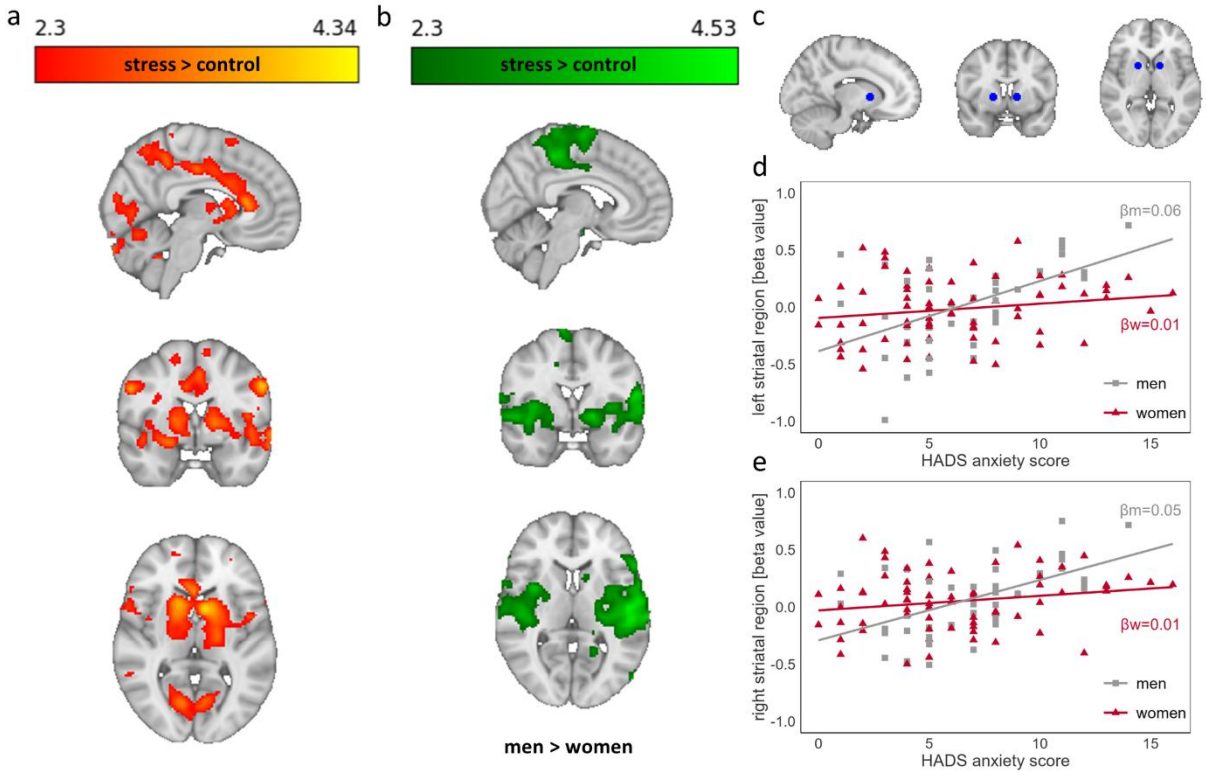

Figure 9. (9a) Significant cluster derived from the analysis for the main task effect stress > control with HADS-A scores (grand-mean centered) as covariates and corrected for sex; (9b) Sex-specific clusters obtained by an unpaired two-group analysis with continuous covariate interaction (HADS-A, grand-mean centered) describing a sex-specific relationship (men > women) between anxiety and neural responses (stress > control); (9c) Generated striatal mask around the overlapping peak region of the preceding whole-brain analyses comprising parts of the ncl. caudatus, pallidum, and putamen (left: 51 67 37; right: 36 67 37); (9d) and (9e) Sex-specific correlations of HADS-A scores with beta values of the main task effect stress > control in the left and right striatal ROI.

5.4.3 Confirmatory factor analysis of the HADS

The CFA revealed the best model fit for a bifactor model with twelve items (six items each from the original anxiety and depression scale). Highest factor loadings on the general factor (HADS-G) could be observed for items indicating frequent states of feeling tensed, nervous, and cheerless. Reliability indices supported the use of the HADS items in a composite total score (general factor: ω_H = .77, anxiety: ω_{HA} = .21, depression: ω_{HD} = .12). For a detailed description of the CFA results see supplemental results section 'Factor structure of the HADS'.

5.4.4 Associations of the HADS general factor with neural responses

Taking the results of the CFA into account, we took a further step and exploratively analyzed the relationship between the discovered general factor and neural stress responses (FWE-

corrected $p < .050$). Adding this factor as covariate revealed significant clusters (stress > control) including, among others, precuneus, middle cingulate cortex, putamen, ncl. caudatus, and the right amygdala which incorporates an activation peak (Figure 10; peak voxels can be found in supplementary Table 22). An unpaired two-group analysis with HADS-G as continuous covariate revealed no significant sex-specific clusters.

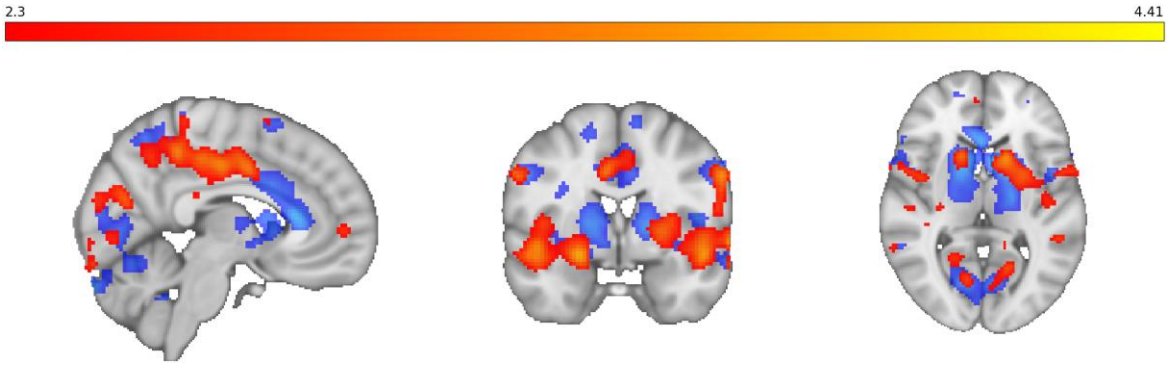

Figure 10. Significant clusters derived from the analysis for the main task effect stress > control (red to yellow) with the HADS general factor (composite score) as continuous covariate corrected for sex compared with the clusters from the analysis with the anxiety scores (blue).

5.4.5 Predictive value of neural responses on anxiety score trajectories

Regarding the trajectory of anxiety symptoms in our sample over one year, we could show the expected difference between SG and CG participants over time (time x SG $b = 0.04$, $p < .001$), but not at baseline (SG $b = 0.00$, $p = .976$), which is similar to our results in the entire study sample (Giglberger et al., 2022). In the SG, a steep rise of anxiety scores until the exam was observable, whereas the CG stayed relatively stable (see supplementary Figure 16). Adding beta values of our ROIs (left and right) to separate models confirmed their association with HADS-A scores at baseline (left_ROI: $b = 0.64$, $p = .007$; right_ROI: $b = 0.83$, $p = .002$), but could not reveal an impact on the trajectory (all $ps \geq .067$; see supplementary Table 23 for all model parameters). Since no activation patterns were found to be associated with HADS-D scores in our previous analyses, we refrained from further longitudinal analyses.

5.5 Discussion

The aim of the present analysis was to determine whether anxiety or depression scores in a young and healthy sample are associated with an altered response to acute psychosocial stress. Therefore, Scan*STRESS* was used as acute stressor. In our study, an elevated mean heart rate during stress vs. control blocks and a rise in negative affect during the task could be observed. Mean salivary cortisol levels - as previously reported (Giglberger et al., 2023) - did

also increase significantly during stress exposure with men exhibiting higher mean increases than women; an often reported sex difference in stress research (Kudielka and Kirschbaum, 2005; Zänkert et al., 2019). Regarding neural activation changes, a distributed network of activations and deactivations was found similar to previous findings (Henze et al., 2020; Streit et al., 2014). The observed affective, heart rate, and cortisol responses support the assumption that the paradigm elicits indeed psychosocial stress and not mere task-related mental load.

The rates of borderline and potentially clinically relevant HADS-A (borderline: 35.1%; clinically relevant: 16.2%) as well as HADS-D scores (borderline: 10.8%; clinically relevant: 2.7%) seem rather high in our sample. However, these frequencies are consistent with reports of higher prevalence rates of mental health problems in student cohorts compared to cohorts from the general population (Burger et al., 2014; Ibrahim et al., 2013). In a recent study with students from various disciplines, 33.8% reported depression symptoms and 40.2% anxiety symptoms (Dastan et al., 2023).

Regarding our main hypotheses, a significant relationship between anxiety scores and the neural stress response could be found. More specifically, the whole brain analysis with HADS-A scores as additional covariate revealed a significant striato-limbic cluster comprising, among others, ncl. caudatus, thalamus, putamen, parahippocampal gyrus, right amygdala, and insula. Limbic structures are known to be involved in reactivity to and processing of negative emotional stimuli (Kober et al., 2008; Phan et al., 2002). Furthermore, compared to healthy controls, patients with anxiety disorders were found to react to negative emotional situations with hyperactivity in limbic regions such as amygdala or insula (Etkin and Wager, 2007). Striatal regions are not necessarily discussed as key structures for stress or anxiety processing. However, in a meta-analysis, subregions of the striatum were found to be consistently activated by acute psychological stressors regardless of the used paradigm (Berretz et al., 2021). Additionally, the striatum plays an important role in reward sensitivity (Haber and Knutson, 2010; Liu et al., 2011), which is altered in patient samples (Admon et al., 2015; Cremers et al., 2015) and prone to stress in healthy participants (Oei et al., 2014; Porcelli et al., 2012). Hence, the striatum may be a critical structure where stress and reward processing are integrated. The positive relationship between anxiety and stress-related striatal activation might indicate a state of hypersensitivity during stress along with dysfunctional reward processing.

While sex differences in prevalence rates and severity of anxiety disorders have often been reported, their underlying neural basis is not well understood (Höglund et al., 2020; Merikangas and Almasy, 2020; Otten et al., 2021; Seedat et al., 2009). In the present study, we could detect a sex-specific association between anxiety and neural responses in temporal and striatal clusters comprising, among others, superior temporal gyrus, ncl. caudatus, putamen, middle cingulate gyrus, and insula. In men compared to women, higher anxiety scores were related to more activation within these clusters. Additionally, *post-hoc* ROI analyses for the chosen striatal regions confirmed the interaction of sex x HADS-A scores. Regions of the temporal lobe were found to be involved in all kinds of anxiety disorders (Kent and Rauch, 2003) and a meta-analysis on sex differences in response to emotional stimuli exhibited greater activation for negative stimuli in men than women containing structures like right superior temporal gyrus, right precentral gyrus, left middle frontal gyrus, and insula (Stevens and Hamann, 2012). Moreover, divergent striatal activation for men and women was shown for stress (Henze et al., 2021) and reward processing (Lighthall et al., 2012; Warthen et al., 2020), both possibly of significance for the development of anxiety disorders. Interestingly, a cluster with higher task-specific activation for women than men could not be detected. Thus, our findings suggest a stronger involvement of a set of temporal and striatal regions in men only and further emphasize the importance of investigating neural sex differences in psychological disorder reasearch.

Regarding depression scores, we failed to find significant associations with neural stress responses in our sample. To our knowledge, so far there is only one other study which investigated the link between neural stress responses and depression in a sample showing subclinical levels of depression (Dedovic et al., 2014). The authors reported a positive relationship between depression scores in the entire sample and the subgenual ACC deactivation. Several differences could explain the divergent findings in our analysis. First, the samples were selected differently. While we recruited healthy law students from the general student population, Dedovic et al. (2014) specifically selected a healthy and a subclinically depressed group based on their Beck Depression Inventory scores (Beck et al., 1987). Thus, the variability of depression levels in our sample was possibly too small to find associations with the stress response (HADS-D: 3.41 ± 2.95). This assumption is consistent with our finding that according to the HADS cut-off values, mean depression symptoms in our sample were less pronounced than anxiety symptoms. Second, the used stress paradigms, modified MIST

and Scan*STRESS*, might be similar but they are not the same. This can be illustrated, among other aspects, by the lack of significant mean cortisol stress responses following the MIST in the study by Dedovic et al. (2014) compared to a significant cortisol rise after stress exposure in the present study.

Since anxiety and depression disorders have a high comorbidity rate (Kalin, 2020; Kessler et al., 2015) and the factor structure of the HADS varies across studies (Bjelland et al., 2002; Cosco et al., 2012), we exploratively examined the HADS factor structure in our sample. The best fit achieved a bifactor model with a general factor characterized by frequent states of feeling tensed, nervous, and cheerless, while the original two-factor structure was on second place. This is in line with a meta-analysis by Norton et al. (2013) which included 21 studies and supported a bifactor structure as well. Consequently, we investigated the relationship between the discovered general factor and the neural stress response and could find a similar, but more circumscribed cluster as for the original anxiety scores. More circumscribed is meant in a sense of less white matter response related with mostly smaller (e.g., ACC, thalamus, or pallidum), but also larger (right amygdala) extents of responsiveness in individual structures. The association of right amygdala activation and anxiety scores fits perfectly in the already existing literature on fear and anxiety (Bruehl et al., 2014; Tovote et al., 2015). However, the amygdala seems to respond more strongly to specific components of anxiety as indicated by the pronounced association with the general factor, which is characterized by tension, nervousness, and cheerlessness. For the general factor, the activation peak of the cluster cannot only be localized in the right amygdala, but the extent of amygdala involvement is also increased compared to anxiety (HADS-G: 113 voxels; HADS-A: 64 voxels). On the other hand, also the anxiety score appears to incorporate information that could not be explained by the general factor (see structures like ACC, thalamus, or pallidum). Taking the CFA results into account, a certain worrying component of the HADS-A score might lead to a heightened responsiveness in structures like the ACC or thalamus. Further studies might elucidate this specific component-related differences.

Taking advantage of our longitudinal study design, we explored the predictive value of our neural results on the trajectory of anxiety symptoms. First, in line with our findings based on the entire LawSTRESS sample (Giglberger et al., 2022), we observed a significant group difference in anxiety over time with a mean increase of anxiety symptoms until the exam in the stress group whereas the participants of the comparison group stayed relatively stable.

Adding beta values of the striatal ROIs strengthened our whole-brain findings by showing a significant association with anxiety at baseline (t1) but had – neither in the CG nor in the SG – an influence on its expression over time. In our view, it is a promising approach to use brain activation patterns as predictor for subsequent experiences and behavior assessed longitudinally in everyday life. In a recent study, we could predict momentary perceived stress levels in daily life over one year by acute stress responses in (pre)limbic regions (Giglberger et al., 2023). However, we assume that our study design and our data were not optimal to predict future HADS anxiety scores. The statistical power of this exploratory analysis was limited as anxiety scores were assessed at only three times over one year until the exam. It could also be speculated, that neural responses to anxiety-specific paradigms, like the presentation of emotional pictures, might be conceptually closer to the perception of anxiety in everyday life than acute stress responses and, thus, more suitable in that specific case.

Our study has some further limitations that need to be addressed. First, although we were interested in the susceptibility of anxiety and depression in a healthy cohort, we could have strengthened our results and made it more generalizable with recruiting a more diverse and general-population based sample as supposed by the Research Domain Criteria (RDoC) framework (Insel et al., 2010). However, due to our specific study design (long-lasting, clearly predictable future stress period and control group) this was not feasible. Second, as already mentioned, the factor structure of the HADS is not certainly clarified. Future studies might use questionnaires specifically developed for anxiety or depression. However, we chose the HADS because it asks for rather mild symptoms without physical indicators which was assumed to better differentiate in an otherwise healthy cohort and to lead to more acceptance.

To conclude, anxiety, but not depression scores in a young and healthy cohort were found to be significantly associated with stress responses in a striato-limbic cluster giving rise to the assumption that interindividual differences in these regions may contribute to a certain vulnerability or resilience. The sex-specific anxiety related activation pattern emphasizes the importance to further investigate sex differences of the neural basis of mental disorders. After detailed examination of the HADS factor structure, a general underlying factor characterized by frequent states of feeling tensed, nervous, and cheerless could also be linked to a similar but more circumscribed cluster indicating potential anxiety component-related activation changes. A predictive value of acute stress responses in striatal ROIs on subsequent expression of anxiety symptoms during a chronic stress period could not be detected.

Chapter 6

6 General discussion

The primary objective of the present thesis was to examine responses to long-term stress in daily life and to identify potential neural markers for the prediction of psychobiological chronic stress outcomes. In our prospective-longitudinal, (quasi-)experimental LawSTRESS project, law students preparing for the first state examination were investigated over 13 months. *Study I* (Chapter 3) focused on assessing whether the extended preparation period and exposure to the examination influenced perceived stress levels, the CAR, and other stress-related measures. Furthermore, we explored correlations between the repeatedly assessed subjective stress ratings and the CAR, as well as the predictive value of several psychometric variables measured at the first sampling point. Taking a step further, *Study II* (Chapter 4) investigated whether stress-related activation changes in the amygdala, hippocampus, and mPFC served as predictors for the same chronic stress outcomes (perceived stress and the CAR). *Study III* (Chapter 5) examined associations between the neural acute stress response and anxiety as well as depression scores at baseline (t1). The following chapter provides a concise summary of the findings from the three studies and discusses their implications in relation to existing research.

6.1 Summary of main findings

6.1.1 The impact of chronic exam stress on psychological well-being

Within the LawSTRESS project, we demonstrated that academic stress, particularly the preparation for and conducting of major exams, significantly burdens students and profoundly impacts their health and well-being. Consequently, academic stress serves as a valuable model for chronic stress exposure.

Participants in the stress group reported an increase in stress-related psychometric variables, including symptoms of anxiety, depression, sleep disturbances, and facets of chronic stress. Although most students did not exhibit concerning levels of anxiety or depression symptoms or sleep disturbances in response to the exam, a considerable number exceeded clinically relevant scores for at least one sampling point. Regarding the AA stress scale, our most important self-report instrument, a steep rise until the exam was observable in the SG, whereas the levels of the CG remained relatively stable (Giglberger et al., 2022). These findings confirm and build upon previous research, emphasizing the

significant impact of academic stress on students' overall health and well-being (González-Cabrera et al., 2014; Koudela-Hamila et al., 2020). Remarkably, both the psychometric variables assessed via questionnaire and the subjective stress perception operationalized via the AA stress scale quickly normalized and returned to baseline levels four weeks after the examination. The stress-related psychological changes were accompanied by a gradual decrease in the cortisol awakening response, peaking during the exam days (t4) in the SG. Subsequently, cortisol concentrations in the morning rapidly recovered and returned to baseline levels within one week.

Research on the impact of academic stress on the CAR has yielded mixed results thus far, likely due to methodological differences, such as heterogeneous study samples or variations in the duration and intensity of exam periods (Duan et al., 2013; González-Cabrera et al., 2014; Hewig et al., 2008; Koudela-Hamila et al., 2020; O'Flynn et al., 2018; Weik and Deinzer, 2010). In a review, Law and Clow (2020) concluded that chronic stress exposure and the CAR are consistently negatively associated in longitudinal studies with reliable methodologies (e.g., Koudela-Hamila et al., 2020). The LawSTRESS project, featuring a longitudinal design, a baseline measurement, and a control group, provides robust support for this conclusion. Regarding stress-induced hypocortisolism, the theory of preceding increased activity of the HPA axis is frequently suggested (Fries et al., 2005; Miller et al., 2007). However, convincing evidence to substantiate this theory remains limited, and our data did not confirm any corroborating evidence. The impact of chronic stress on HPA axis activity appears to depend on several variables, including the timing and duration of the stressor and characteristics of the person experiencing it (Boggero et al., 2017; Miller et al., 2007).

Concerning individual characteristics, we found no indication that anxiety, depression, test anxiety, or facets of chronic stress predict the trajectory of the CAR. Additionally, we tested for associations between the stress-induced reduction of the CAR and the repeatedly measured perceived stress levels in everyday life. Previous research has reported either no relationship between subjective stress perception ratings and markers of cortisol regulation, or has yielded inconsistent results (M. Pruessner et al., 2003; Weekes et al., 2008). Thus, a lack of covariation is well-known (Campbell and Ehlert, 2012; Fahrenberg, 1979). Despite this, we hypothesized that the comprehensive assessment of perceived stress levels using AA in our study may enhance the likelihood of uncovering a relationship

between perceived stress and the CAR, as both theoretically measure the same concept of 'stress'. However, our results revealed no significant association. Further longitudinal studies are desirable to unravel the temporal trajectory of developing hypocortisolism and its influencing factors in greater detail.

In conclusion, our participants responded to long-term academic stress with changes in both psychological and biological stress indicators, supporting the assumption that academic stress is a good model for chronic stress exposure. Although only law students who were able to deal with the additional study burden, potentially resulting in a cohort of rather high-achieving individuals, participated, we suppose that the stress responses observed in our study are comparable to what individuals often face in educational settings or the workplace. This notion is further supported by the alarming number of participants exceeding clinically relevant scores for anxiety and depression. As these changes are temporary and return to baseline levels within four weeks after the exam period, it seems conceivable that these alterations represent adaptive responses to effectively cope with stress. Nevertheless, a sensitizing effect from such a long-lasting stress period should not be ruled out, especially in vulnerable individuals and should be addressed in future studies (e.g., by extreme group analyses).

6.1.2 Neural predictors of chronic stress outcomes

As acute and chronic stress responses as well as their associated increased risk for stress-related disorders strongly depend on neural processes, their investigation becomes crucial. First results from animal studies have highlighted the involvement of (pre)limbic brain regions, including the amygdala, hippocampus, and mPFC, in stress processing. Although directly applying animal models to humans is often limited, studies involving healthy participants and patient samples have confirmed the importance of these brain regions (Helm et al., 2018; Henigsberg et al., 2019; Henze et al., 2020; Holzschneider and Mulert, 2011; Liu et al., 2023; Pruessner et al., 2008). However, their relevance in the transition from acute stress to more enduring or chronic states remains largely unexplored.

Leveraging the advantages of the LawSTRESS project, we examined the predictive value of neural acute stress responses in the amygdala, hippocampus, and mPFC for subsequent chronic stress outcomes in daily life. To elicit an acute stress reaction, we applied Scan*STRESS*. Participants exhibited increased cortisol levels after the acute stressor and a

distributed network of activations and deactivations in response to psychosocial stress, consistent with previous studies (Henze et al., 2020; Streit et al., 2014). Regarding our ROIs, we observed decreased activation (higher activation during control than stress conditions) in all three brain structures. Analyzing perceived stress trajectories in daily life revealed a steep rise in stress levels until the exam in the SG, while CG levels stayed relatively stable. Individual stress-induced activation changes in the amygdala and hippocampus predicted the trajectory of the SG. Specifically, a stronger decrease of activation in these structures measured at t1 (12 months prior the exam) was associated with a more pronounced increase in perceived stress until the exam at t4. An exploratory *post-hoc* analysis revealed a similar relationship with the right mPFC. Our findings build upon patient studies that have linked amygdala activation and hippocampus volume to the prediction of depression and PTSD symptoms (Mattson et al., 2016; Rauch et al., 2000; Villarreal et al., 2002). Furthermore, we cautiously speculate that stress processes are somewhat temporally and contextually stable, as we found a significant association between responses to a laboratory stressor and an assessment of perceived stress in real life (context stability) one year later (temporal stability). Notably, the observed neural activation changes were unrelated to baseline stress levels in the entire sample and the trajectory of the CG. Thus, our results further support the idea that interindividual variations in stress processing are most evident under stress conditions.

Contrary to our hypothesis, the neural activation pattern elicited by Scan*STRESS* did not predict the blunted CAR observed in the SG during the examination days (t4). (Pre)limbic brain regions were previously shown to have a modulating effect on the CAR (Clow et al., 2010; Contreras and Gutierrez-Garcia, 2018). However, cortisol reactivity to experimentally-induced psychological stress and the CAR seem to be unrelated (Bouma et al., 2009), supporting the assumption that additional regulatory mechanisms (e.g., via the adrenal cortex of the suprachiasmatic nucleus) influence morning cortisol secretion (Clow et al., 2010).

Nonetheless, our study did not only show alterations of perceived stress levels and the CAR due to chronic stress exposure but also revealed a predictive value of neural stress response patterns in the amygdala, hippocampus, and right mPFC for subsequent perceived chronic stress levels. Based on our assumption of a certain stability over time

and over contexts in individual stress processing, these described neural stress responses may serve as useful stress vulnerability or resilience markers.

6.1.3 Neural stress responses and their associations with anxiety and depression

To further pursue our objective of identifying potential neural markers of stress vulnerability and tracing relevant temporal trajectories, we investigated associations between whole-brain acute stress responses and varying levels of anxiety and depression in healthy individuals. Baseline borderline and clinically relevant anxiety scores in our sample appeared to be quite high (borderline: 35.1%, clinically relevant: 16.2%). However, these rates align with prior research in student samples (Burger et al., 2014; Ibrahim et al., 2013).

Regarding our main research question, anxiety but not depression scores were significantly associated with the neural stress response in a striato-limbic cluster. Limbic structures play a decisive role in processing negative emotions, while the striatum is linked to reward sensitivity, a function exhibiting alterations in clinical populations and susceptible to stress in healthy samples (Admon et al., 2015; Cremers et al., 2015; Haber and Knutson, 2010; Kober et al., 2008; Liu et al., 2011; Oei et al., 2014; Phan et al., 2002; Porcelli et al., 2012). Thus, these regions may be responsible for integrating stress and reward processing, potentially contributing to a certain hypersensitivity during stress and dysfunctional reward processing. The lack of association with depression scores might be partially explainable by our study sample. We recruited healthy participants from the general student population. A study by Dedovic et al. (2014), which observed a relationship of depressive symptoms and task-based activity in the subgenual ACC, specifically selected healthy and subclinically depressed individuals. Thus, the observed range of depression scores within our sample may have been too limited to detect any significant relationship with the stress response. Additionally, the association between anxiety and neural responses was stronger in men than women in temporal and striatal clusters, emphasizing the importance of examining sex-related neural disparities in the study of psychological disorders.

Given the substantial comorbidity observed between anxiety and depression disorders (Kalin, 2020; Kessler et al., 2015), and considering the inconsistent findings regarding the factor structure of the HADS (Bjelland et al., 2002; Cosco et al., 2012), we exploratively examined the factor structure of the HADS in our dataset. The analysis revealed a bifactor

model with a general factor predominantly characterized by tension, nervousness, and lack of cheer. Entering that general factor into the whole-brain analysis yielded a comparable yet more circumscribed cluster than that associated with the initial anxiety scores. Hence, different aspects of anxiety, like tension or worrying, might activate different stress-sensitive brain regions. In future studies, the examination of specific symptoms rather than addressing anxiety as a singular construct may be worthwhile.

In a final step, we took advantage of the longitudinal design of the LawSTRESS project and employed the identified neural response pattern as a predictor for the anxiety level trajectories in SG and CG. While the anxiety levels of the SG rose until passing the exam, the levels of the CG remained relatively stable. The addition of the neural results to the longitudinal models had no significant impact on either the SG or CG trajectory but confirmed our previous findings by showing a significant association with anxiety scores at baseline.

Taken together, the observable association between anxiety scores (and also the HADS general factor) and stress responses in a striato-limbic cluster in our young and healthy participants indicates a potential role of these brain regions in vulnerability of or resilience for anxiety disorders. Notably, activation patterns differed by sex, highlighting the need for further research into the neural underpinnings of mental disorders across the sexes.

6.2 Developments in the investigation of brain-behavior associations

At the beginning of the current thesis, stress was defined as a stimulus perceived by an individual as exceeding its own coping strategies, along with the resulting behavioral and physiological responses (Lazarus and Folkman, 1984; Levine and Ursin, 1991). The brain plays a pivotal role in the interpretation process and orchestrates the responses of different stress systems. MRI studies aiming to uncover neural stress processes are crucial but are often unable to explain the brain-behavior link in its full extent. The artificial laboratory environment makes it difficult to directly apply MRI results to real-life behavior. In contrast, AA can gather data in an individual's real-life and natural environment. Thus, the two methodologies MRI and AA may complement each other and are able to further expand our knowledge on brain-behavior links.

6.2.1 Digital phenotyping

As indicated in the introduction, the number of studies using that combined approach is rising (Gadassi Polack et al., 2021). However, the research discussed thus far has predominantly focused on employing smartphone queries as ambulatory assessments. The rapid spreading of personal wearable electronic devices has significantly enhanced the feasibility of collecting physiological and behavioral data with minimal or no user input (Insel, 2017; Torous et al., 2016). Fitness trackers or smart watches, for instance, enable the acquisition of physiological data, including heart rate, blood pressure, and sleep parameters. Smartphones serve not only as tools for responding to queries but also as repositories for various background data, encompassing media/app usage, communication patterns, and geolocation information of an individual. That so called digital phenotyping enables the investigation of large-scale and continuous data collected in naturalistic environments (Onnela, 2021; Torous et al., 2016). Thus, digital phenotyping offers numerous opportunities for robust data collection; however, it also introduces challenges, like the need for effective data analysis strategies and the necessity to address privacy and data security concerns (Onnela, 2021).

Nonetheless, as early as 2009, the first studies integrating digital phenotyping data and MRI results were conducted. A strong increase in interest in this research approach has only become apparent since 2018 (Triana et al., 2024). The opportunities for integrating different MRI techniques with digital data sources are extensive. This integration encompasses among others the utilization of structural images, diffusion tensor imaging, and BOLD-dependent measures (both resting state and task-based) on the imaging side; the phenotyping front can benefit from a diverse array of data collection methods, including behavioral (e.g., physical activity, AA) and physiological parameters (e.g., heart rate, blood pressure). To date, the most frequent used approach is fMRI combined with physical activity parameters, followed by sleep and AA queries (Triana et al., 2024). For instance, in an intervention study, functional connectivity patterns predicted the changes in sedentary behavior measured with accelerometers in a walking group (Morris et al., 2022). According to the authors, the combination with physiological measures, such as heart rate or blood pressure, is still underexplored and might be promising for future research.

6.2.2 Methodological advances in brain imaging research

A further development of the combined approach is using brain imaging data as predictor for behavior. As stated by Berkman et al. (2013), one critical requirement for this approach entails the careful selection of a neural predictor a priori. The chosen predictor must accurately reflect the mental process driving the behavior under investigation. The determination of a suitable neural predictor is informed by psychological theories and/or prior brain-mapping findings, emphasizing the need for brain-mapping results that are both reproducible and reliable (Berkman and Falk, 2013). Methodological enhancements employed to address the prevailing replication crisis in psychology, such as the standardization of analysis procedures, the adoption of preregistration practices, and the promotion of sharing data and code, are increasingly being adopted in MRI research (Marek et al., 2022; Munafò et al., 2017). Meanwhile, several abilities could be accurately correlated with specific brain structures and functions (Bullier, 2001; Rugg and Vilberg, 2013).

However, when attempting to find associations between brain measures and complex human behavior in so called brain-wide association studies (BWAS), the replication rates tend to be low. According to Marek et al. (2022), small sample sizes, along with lower as expected effect sizes, lead to statistically underpowered studies, inflated effect sizes, and difficulties in replication. These challenges are also well-known in genome-wide association studies, which have responded with a substantial increase in sample sizes (Hong and Park, 2012). Thus, the authors concluded that BWAS should rely on large datasets with at least thousands of datapoints (Marek et al., 2022). In the last years, the establishment of neuroimaging consortia and data sharing initiatives (e.g., Human Connectome Project (HCP; Van Essen et al., 2012), Enhancing Neuro Imaging Genetics Through Meta Analysis (ENIGMA; Thompson et al., 2014), or UK Biobank (Littlejohns et al., 2020; Palmer, 2007)) has enabled the acquisition and integration of an unprecedented amount of imaging data, which has led and is expected to lead to more robust associations between neural markers and complex human behavior, such as behavioral stress responses or mental disorders (Marek et al., 2022). Moreover, these findings exhibit greater generalizability due to their data collection across multiple sites, different research groups, and diverse populations (Dwyer et al., 2018). Another critical aspect to consider is that the brain experiences substantial transformation over time. Both structural and functional alterations occur as

we age (Hedman et al., 2012; Persson et al., 2014; Thambisetty et al., 2010). Additionally, learning experiences and interactions with our environment are pivotal in shaping the brain and its diverse functions (Crone and Elzinga, 2015). Thus, imaging studies would greatly benefit from employing within-subject designs (Marek et al., 2022). In recent years, there has been an increase in longitudinal imaging studies, with imaging consortia also playing a significant role in this effort (e.g., Lifespan Connectome Data of the HCP). Considering these advancements, the chances of connecting neural markers to intricate human behavior are more promising than ever.

6.2.3 Machine learning as promising analyzing technique

In addition to the developments in AA tools and initiatives for MRI data sharing, the role of statistical methods is of great importance. As indicated in the introduction, the majority of studies employing a combined imaging and AA approach have predominantly utilized relatively simplistic analytical techniques, such as bivariate correlations or regression analyses (Gadassi Polack et al., 2021; McGowan et al., 2023). The studies encompassed in the present thesis used linear mixed models to examine the association between neural markers and longitudinal changes in chronic stress outcomes. This represents a methodological progression, reflecting the unique data structure inherent in AA data and the study's objective of identifying predictors for stress trajectories. Nevertheless, with the continuous evolution of analytical approaches, the potential for more sophisticated analysis will undoubtedly expand in the future.

Machine learning methodologies have undergone significant development, testing, and application in the fields of health and social sciences. This term encompasses computational strategies that autonomously discover methods and parameters to achieve an optimal solution for a problem, rather than relying on pre-programmed instructions from a human (Bishop, 2006). Machine learning algorithms have become an integral part of our daily lives, e.g., for personalized marketing, web searches, or translation software (Jordan and Mitchell, 2015). In healthcare, the utilization of these algorithms has experienced a rapid growth for pattern recognition in various diseases, like the detection of several types of cancer or eye disease (Agarwal et al., 2023; Bataineh, 2019; Nuzzi et al., 2021). Dwyer et al. (2018) identified four principal domains within clinical psychology where machine learning can be optimally applied, namely diagnosis, prognosis, treatment

prediction, and the identification and monitoring of potential biomarkers. Encouraging results have emerged from research in these domains. For instance, studies employing machine learning methodologies reported reduced misdiagnosis rates in depression (Redlich et al., 2014) and improved prognostic accuracy for first-episode psychosis (Koutsouleris et al., 2016).

In addition to the utilization of questionnaire, demographic, and clinical data, there is increasing interest in integrating neuroimaging data in machine learning approaches (Woo et al., 2017). Machine learning algorithms enable the development of integrated models that capture activity patterns across multiple brain regions, facilitating the prediction of psychological outcome variables. Consequently, diverse brain modalities – such as activity, structural features, and functional connectivity – can be harmoniously combined to jointly predict the variable of interest (Rashid and Calhoun, 2020; Woo et al., 2017). Currently, research is still at the exploratory stage, utilizing different methods, algorithms, and models to identify the most promising techniques. The subsequent phase of establishment involves the validation of models exhibiting optimal performance, a process that emphasizes the necessity of large and heterogeneous samples, e.g., from imaging consortia and data sharing initiatives (Woo et al., 2017).

Thus far, most studies have focused on neural markers derived from machine learning approaches to differentiate between patients and healthy controls, thereby attempting to establish direct associations between neural markers and clinical outcomes such as diagnosis or symptom severity. An alternative approach incorporates the investigation of intermediate features (e.g., cognitive processes or stress regulation mechanisms) that exhibit altered patterns in diverse combinations across different disorders. This approach is already recognized in genetics, where it is supposed that intermediate phenotypes are more closely related to the biological function of genes than categorical and heterogenous clinical diagnoses (Gottesman and Gould, 2003; Meyer-Lindenberg and Weinberger, 2006). Applying this principal to neuroimaging data may facilitate to unravel neural markers and provide deeper insights into the mental processes pertinent to the development of specific functional impairments across disorders.

Taken together, machine learning approaches have the potential to significantly advance our understanding of mental processes and their impact on behavior. However, it is critical to acknowledge that research in this area is still in its early stages, and the inherent

limitations of these methodologies have to be considered (for a summary see Dwyer et al., 2018).

6.3 Final conclusion

The acute stress response entails the interplay of multiple systems, which is usually well-orchestrated (Godoy et al., 2018; Hermans et al., 2014). However, the accumulation or prolonged presence of stressors may result in chronic stress, potentially culminating in the onset of various disorders (Agorastos and Chrousos, 2022). Furthermore, individual differences in disposition can significantly influence vulnerability or enhance resilience. The brain is able to identify events, assess them as actual or potential threat, and subsequently regulate the stress response (Godoy et al., 2018; McEwen, 2007). Thus, the investigation of neural stress processes and their impact on the shift from a state of acute to a state of chronic stress is critical.

In the LawSTRESS project, a study design was implemented allowing both the examination of prolonged stress exposure and the investigation of neural predictors associated with the transition from acute to chronic stress. The findings of the current thesis demonstrate that academic stress is a great burden for students and that it serves as an appropriate model for the examination of chronic stress. Significant disparities between the SG and the CG were identified in perceived stress levels, the CAR, and several other stress-related variables. Moreover, the observed rise in perceived stress assessed through AA was predicted based on neural markers, specifically regarding activation changes in response to acute psycho-social stress in amygdala, hippocampus, and right mPFC. In a further analysis of the MRI data, a striato-limbic activation cluster was associated with anxiety levels at the beginning of the study. However, a predictive value of the anxiety-related activation pattern for the trajectory of anxiety levels until passing the exam was not observable.

Despite acknowledging certain limitations, our study design exhibits several notable strengths: a prospective-longitudinal approach with a clearly defined stress period and a baseline measurement, a quasi-experimental design, and a comprehensive multimodal data collection. Moreover, with Scan*STRESS*, a robust psycho-social stress paradigm for the MRI environment was employed to identify neural markers of stress regulation mechanisms. Additionally, we assessed outcome variables for different stress systems, including extensive psychometric assessments, cortisol, and an accurate measure of

current stress perception through AA. Thus, the current findings contribute to our understanding of chronic stress trajectories and the impact of specific neural markers on vulnerability and/or resilience. The developments in digital phenotyping, MRI data collection and analysis are thereby particularly promising. Gaining deeper insight into neural stress mechanisms together with other important vulnerability factors (e.g., genetic markers) has the potential to elucidate the relationship between stress and disease, resulting in a facilitated identification of individuals at risk, stratification of patients, and development of enhanced, individualized preventions and interventions.

7 References

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8 Appendix

8.1 Supplementary methods

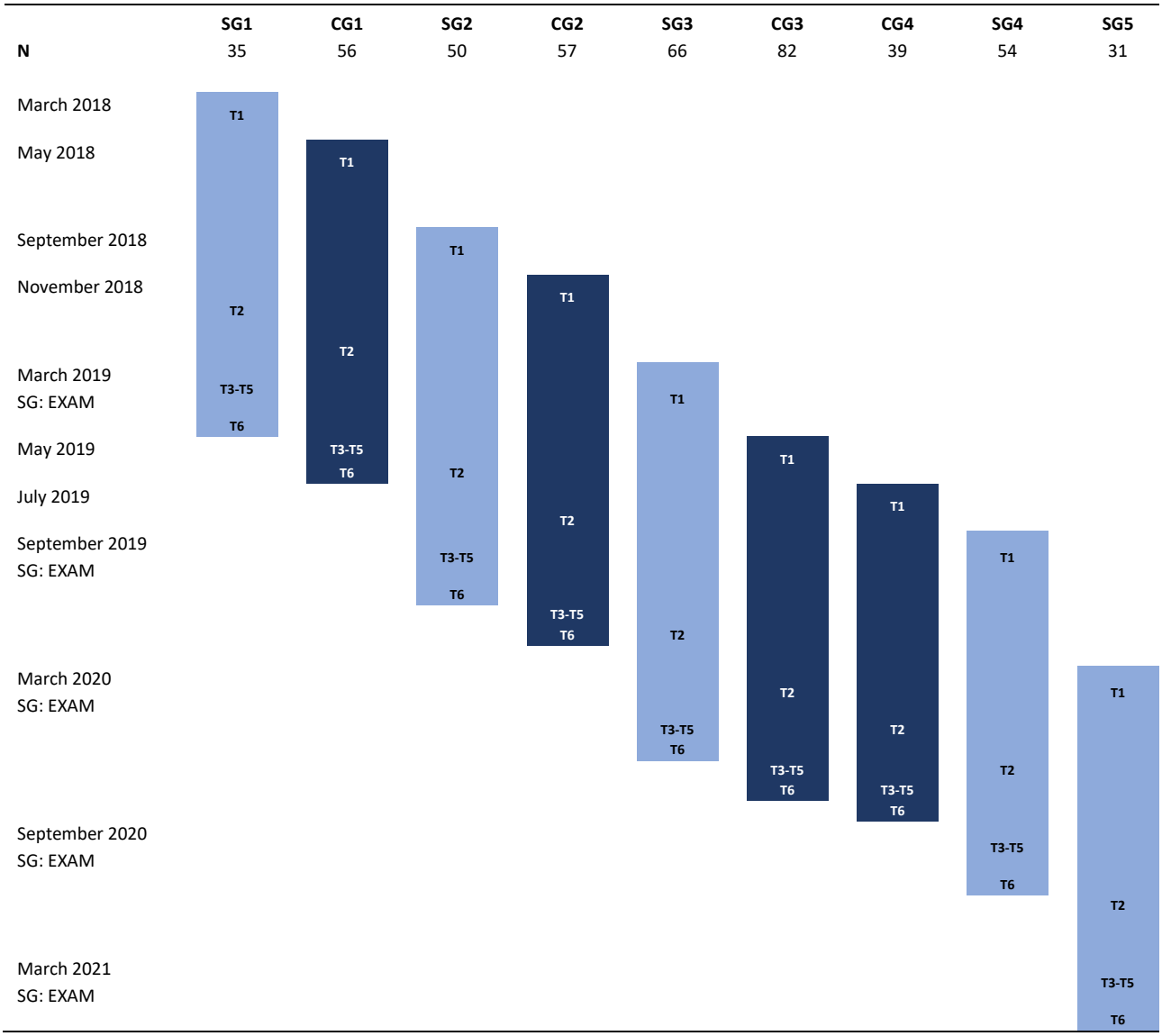

Figure 11. Nested data collection of the LawSTRESS project.
Note. CG: Control group; SG: Stress group.

8.1.1 AA stress scale

To measure momentary perceived stress, 18 items covering positive/negative mood (e.g., 'I am happy'), calm/restlessness (e.g., 'I am tense'), concern/confidence (e.g., 'I am worried'), self-satisfaction/shame (e.g., 'I am disappointed'), overload (e.g., 'I am under time pressure'), anxiety (e.g., 'I am afraid') and somatic symptoms (e.g., 'I am in pain') were assessed. The response format was a seven-point Likert scale ('strongly disagree' to 'strongly agree'). Item wording and selection, respectively, were predominantly based on existing questionnaires

(State-Trait Anxiety Inventory, Laux et al., 1981; Multidimensional Mood State Questionnaire, Steyer et al., 1997; Positive and Negative Affect Schedule, Watson et al., 1988) or research papers (Powell and Schlotz, 2012).

Exploratory factor analyses of 10.0% of these surveys (systematically covering all timepoints) in cohort A (1613 out of 16430) revealed a one-factor-solution with the items 'time pressure', 'relaxed', 'tense', 'overstrained' and 'I am disappointed with my performance' (for the 18-items-version see supplementary Table 9). Building upon this, confirmatory factor analyses of all surveys were conducted, and all showed good fit indices (all comparative fit indices (CFI) > .99; all root mean square errors of approximation (RMSEA) < .15).

Table 9. Items of the AA questionnaire.

German items	English translation
Ich bin wach	I am alert
Ich bin traurig	I am down
Ich bin gereizt	I am irritable
Ich bin entschlossen	I am determined
Ich bin zufrieden	I am content
Ich bin angespannt	I am tense
Ich bin unter Zeitdruck	I am under time pressure
Ich bin froh	I am happy
Ich bin besorgt	I am worried
Ich bin enttäuscht von meiner Leistung	I am disappointed with my performance
Ich bin überfordert	I am overstrained
Ich bin entspannt	I am relaxed
Ich bin ängstlich	I am afraid
Ich habe Schmerzen	I am in pain
Mir ist übel	I feel nauseous
Ich fühle mich gesund	I feel healthy
Ich bin erschöpft	I am exhausted
Ich fühle mich einsam	I feel lonely

8.1.2 Scan*STRESS* protocol

Scan*STRESS* is a stress induction paradigm for fMRI conditions developed by our group, predominantly aiming at inducing social-evaluative threat and uncontrollability as stress-inducing psychological components (Henze et al., 2020; Streit et al., 2014) .

Participants arrived in the laboratory 75 minutes prior stress onset. After receiving instructions, a relaxation phase was implemented during which a neutral movie was presented. Forty-five minutes prior stress onset, a sugary drink (75 g glucose in 200 ml herbal

tea) was given and approximately 20 minutes later a brief training session of the Scan*STRESS* control blocks was completed.

The paradigm was implemented in Presentation® software (version 12.9, www.neurobs.com) and consisted of two different conditions: a stress and a control condition. During stress blocks, participants had to solve visually presented arithmetic and spatial mental rotation tasks under time pressure. Task speed and difficulty were adapted to the participant's performance ensuring frequent failure and uncontrollability. Furthermore, a previously introduced observation panel gave feedback regarding working speed ("work faster") and accuracy ("error") via life stream. During the control condition, simple figure and number matching tasks had to be performed in the absence of time pressure and negative feedback (indicated by a black cross over the video stream).

Scan*STRESS* comprised two runs with a duration of 11:20 minutes each. The first one started with the control condition, the second one with the stress condition. In each run, stress and control blocks were presented in an alternating order. Each task block lasted 60 seconds and was preceded by a five seconds announcement phase and followed by a 23 seconds break. During the break between the two runs, the MRI scanner was paused, and subjects received additional feedback that the committee was unsatisfied with their performance and that they needed to increase their efforts. Furthermore, biological (salivary cortisol, heart rate) and psychological (emotional stress reactivity) responses were repeatedly collected. The complete MRI session included two resting state sequences after the stress paradigm and an anatomical scan, which are beyond the scope of this manuscript. After completion of the MRI scan, participants remained in the laboratory for another 45 minutes to fill out questionnaires. Test sessions were scheduled between 1:00 and 5:00 p.m.

8.1.3 Confirmatory factor analysis

Although numerous studies have already examined the underlying dimensionality of the HADS, findings are still inconsistent (Bjelland et al., 2002; Cosco et al., 2012). Therefore, we decided to conduct a CFA using the R lavaan package (Rosseel, 2012). The models were estimated with Maximum Likelihood and the significance level was set at $\alpha = .05$. We evaluated the goodness-of-fit using the χ^2/df ratio, CFI, Tucker-Lewis index (TLI), the RMSEA, and the standardized root mean square residual (SRMR). A good fit index is considered to be close to or higher than a CFI and TLI score of .95 and close to or lower than a χ^2/df ratio of 3,

RMSEA score of .06 and SRMR score of .08 (Hu and Bentler, 1999). We performed one-factor, two-factor, and bi-factor models. In case of the decision for a bi-factor model, the relevance of the general and the specific factors was evaluated examining the hierarchical omega indices (ω_Hs). A good fit is indicated by a $\omega_H \geq .70$ for the general factor and $\omega_{HS} \geq .30$ for specific factors (Rodriguez et al., 2016).

8.2 Supplementary results

Table 10. Parameter estimates for overall effects for the final model with perceived stress as dependent variable.

Fixed Effects	Estimate	Std. Error	*p*
Intercept	2.52	0.03	**< .001**
Timepoint	.05	0.01	**< .001**
Timepoint^2	-.01	0.01	.327
Timepoint^3	.00	0.00	.519
SG	.09	0.05	.059
Timepoint x SG	.39	0.02	**< .001**
Timepoint^2 x SG	-.19	0.01	**< .001**
Timepoint^3 x SG	.02	0.00	**< .001**
Random Effects	*SD*	Correlation (Intercept)	
Subject (Intercept)	0.32		
Timepoint	0.06	-.25	

Note. SG: Stress group; *SD*: Standard deviation; Std. Error: Standard error.

Table 11. Parameter estimates for overall effects for the model within the SG with perceived stress as dependent variable and cohort as fixed effect.

Fixed Effects	Estimate	Std. Error	*p*
Intercept	2.60	0.03	**< .001**
Timepoint	.44	0.01	**< .001**
Timepoint^2	-.20	0.01	**< .001**
Timepoint^3	.02	0.00	**< .001**
Cohort B	.16	0.05	**< .001**
Timepoint x Cohort B	-.02	0.03	.574
Timepoint^2 x Cohort B	.01	0.02	.366
Timepoint^3 x Cohort B	.00	0.00	.288
Random Effects	*SD*	Correlation (Intercept)	
Subject (Intercept)	0.32		
Timepoint	0.07	-.35	

Note. SG for cohort A (*n* = 97) with 100 queries per participant; SG for cohort B (*n* = 128) with 12 queries per participant. SG: Stress group; *SD*: Standard deviation; Std. Error: Standard error.

Table 12. Parameter estimates for overall effects for the model within the CG with perceived stress as dependent variable and cohort as fixed effect.

Fixed Effects	Estimate	Std. Error	*p*
Intercept	2.52	0.03	**< .001**
Timepoint	.05	0.01	**< .001**
Timepoint^2	-.01	0.01	.315
Timepoint^3	.00	0.00	.532
Cohort B	.22	0.04	**< .001**
Timepoint x Cohort B	-.09	0.03	**.006**
Timepoint^2 x Cohort B	.05	0.02	**.006**
Timepoint^3 x Cohort B	-.01	0.00	**.007**
Random Effects	*SD*	Correlation	
		(Intercept)	
Subject (Intercept)	0.31		
Timepoint	0.06	-.18	

Note. CG for cohort A (*n* = 107) with 100 queries per participant; CG for cohort B (*n* = 119) with 12 queries per participant. CG: Control group; *SD*: Standard deviation; Std. Error: Standard error.

Table 13. Parameter estimates for overall effects for the model of the stress group (SG.model) with the cortisol awakening response as dependent variable.

Parameter	Estimate	Std. Error	*p*
(Intercept)	.80	0.04	**<.001**
30 min	.33	0.03	**<.001**
45 min	.34	0.04	**<.001**
T2 x 0 min	-.05	0.03	.075
T2 x 30 min	.01	0.03	.702
T2 x 45 min	.02	0.03	.474
T3 x 0 min	.00	0.03	.988
T3 x 30 min	-.05	0.03	.073
T3 x 45 min	-.06	0.03	.075
T4 x 0 min	.01	0.03	.794
T4 x 30 min	-.12	0.03	**<.001**
T4 x 45 min	-.16	0.03	**<.001**
T5 x 0 min	-.02	0.03	.466
T5 x 30 min	.00	0.03	.917
T5 x 45 min	-.01	0.03	.781
T6 x 0 min	-.02	0.03	.427
T6 x 30 min	.01	0.03	.860
T6 x 45 min	.00	0.03	.896
Covariates			
Women using HC	.03	0.04	.444
Women using HC x 30 min	-.12	0.03	**.001**

Women using HC x 45 min	-.11	0.04	**.004**
Men	.01	0.05	.803
Men x 30 min	-.08	0.04	**.035**
Men x 45 min	-.12	0.04	**.007**
Awakening time	.08	0.02	**<.001**
Awakening time x 30 min	-.06	0.02	**.009**
Awakening time x 45 min	-.09	0.03	**.001**
Random Effects	*SD*	Correlation	
		(Intercept)	30 min
Subject (Intercept)	0.13		
30 min	0.09	-.69	
45 min	0.10	-.79	.99
Timepoint (Intercept)	0.17		
30 min	0.14	-.59	
45 min	0.18	-.66	1
Residual	0.09		

Note. Min: Minutes after awakening; SG: Stress group; T: Timepoint; HC: Hormonal contraception; *SD*: Standard deviation; Std. Error: Standard error.

Table 14. Fit indices of the model of the stress group (SG.model) with the cortisol awakening response as dependent variable and the different predictor models.

Predictor	df	AIC	BIC	logLik
SG.model	40	-1545.56	-1324.97	812.78
AA stress scale	58	-1533.42	-1213.57	824.71
Depression symptoms at t1	58	-1535.91	-1216.05	825.96
Anxiety symptoms at t1	58	-1528.89	-1209.03	822.45
Test anxiety at t1	58	-1534.86	-1215.00	825.43
TICS scales at t1				
Work overload	58	-1534.89	-1215.03	825.45
Excessive demands at work	58	-1524.44	-1204.58	820.22
Chronic worrying	58	-1529.56	-1209.70	822.78

Note. Anxiety and depression symptoms measured by the HADS; test anxiety estimated by the PAF; work overload, excessive demands from work and chronic worrying assessed by the TICS.

Table 15. Activated and deactivated structures under psychosocial stress (two tailed combined FWE-corrected for whole brain, threshold < .05) including z- and p-values as well as the localization of peak voxels.

Brain structure		**statistics**			**MNI coordinates**		
		k	***p***	**z**	**X**	**Y**	**Z**
Insula	**left**	**100415**	**< .001**	**6.92**	**-32**	**16**	**6**
	right			6.83	30	30	8
Superior frontal gyrus	right			6.81	22	-2	50
Middle frontal gyrus	right			6.80	30	4	48
Lateral occipital cortex	left			6.76	-18	-72	40
Middle frontal gyrus	left			6.73	-30	2	54
Medial frontal cortex	**right**	**31342**	**< .001**	**-7.26**	**2**	**40**	**-20**
	left			-6.92	-6	34	-26
Subcallosal cortex	left			-6.60	-2	10	-14
Inferior frontal gyrus, orbital	right			-6.42	20	26	-18
Precuneus	right			-6.32	0	-54	24
	left			-6.08	0	-66	28
Angular gyrus	**left**	**2361**	**.008**	**-6.79**	**-56**	**-66**	**34**
	left			-6.65	-54	-64	42
	left			-6.59	-54	-72	34
Inferior parietal gyrus	left			-5.30	-40	-80	46

Note. k = cluster size in voxels; MNI = Montreal Neurological Institute; global cluster maxima are in boldface.

Table 16. Parameter estimates for overall effects for the final perceived stress model within the fMRI sample.

Fixed Effects	**Estimate**	***SE***	***p***
Intercept	2.51	0.04	**< .001**
Timepoint	0.03	0.02	.077
Timepoint^2	0.01	0.01	.522
Timepoint^3	-0.00	0.00	**.030**
SG	0.10	0.06	.074
Timepoint x SG	0.39	0.03	**< .001**
Timepoint^2 x SG	-0.20	0.01	**< .001**
Timepoint^3 x SG	0.02	0.00	**< .001**
Random Effects	*SD*	Correlation	
		(Intercept)	
Subject (Intercept)	0.31		
Timepoint	0.06	-.20	

Note. SE = Standard error; *SD* = Standard deviation; SG = Stress group.

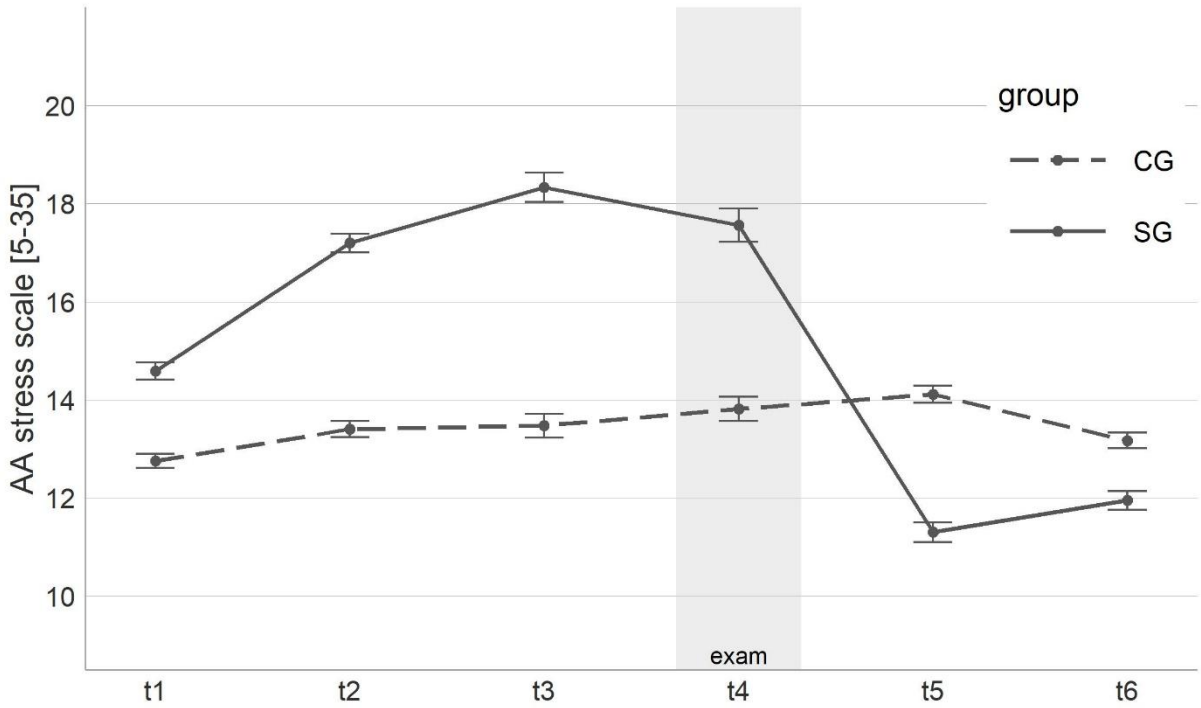

Figure 12. Time course of mean perceived stress levels (±SEM) in the stress (SG) and control group (CG) within the fMRI sample over the study period.

Note. t = timepoint; t1 = one year before the exam, t2 = three months prior exam, t3 = one week prior exam and t4 = in the middle of the exam period.

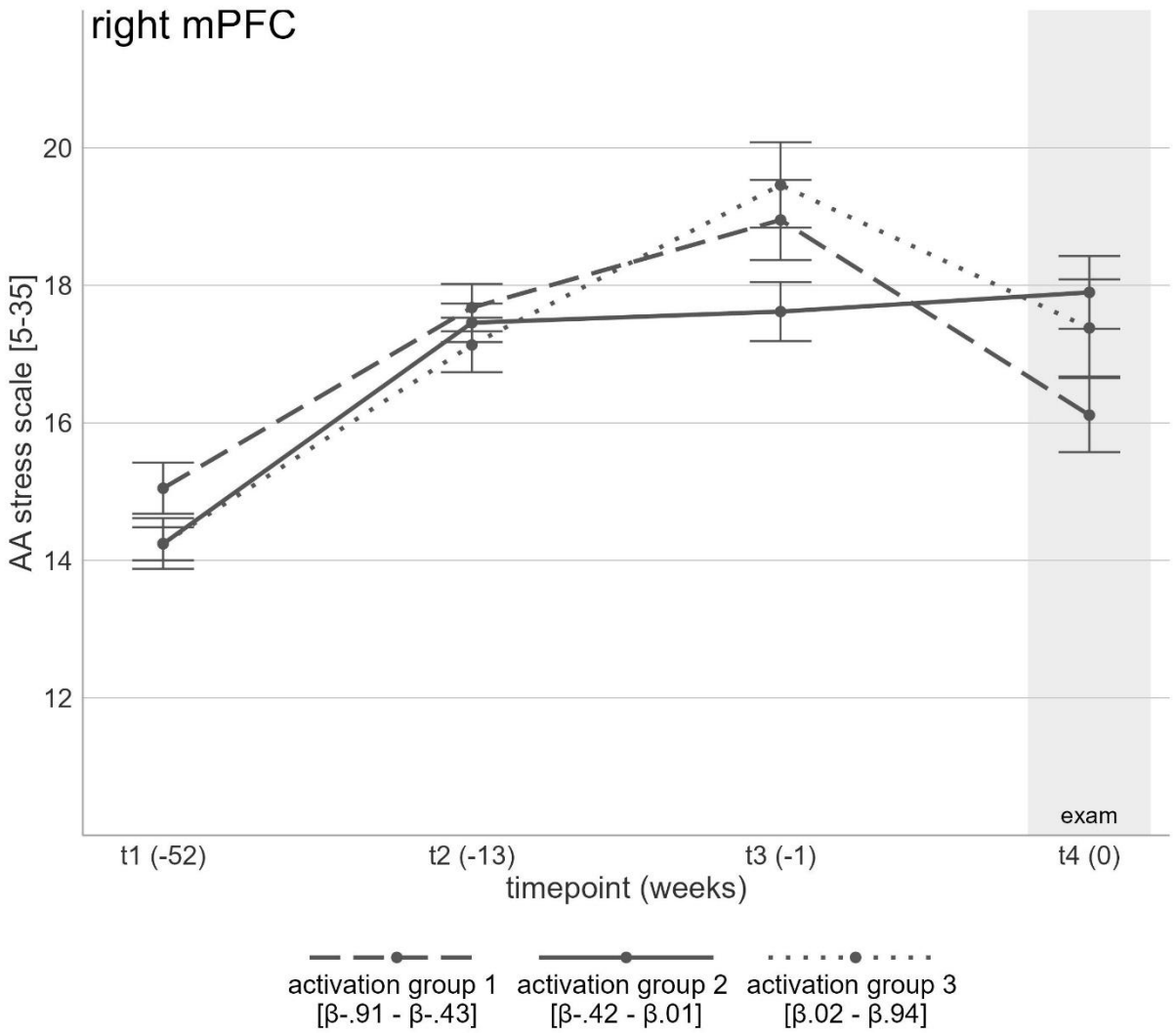

Figure 13. Time course of the *AA stress scale* (±SEM) in the stress group. For illustrative purposes, participants were divided into three groups of equal size according to their right mPFC response.

Note. t = timepoint; t1 = one year before the exam, t2 = three months prior exam, t3 = one week prior exam and t4 = in the middle of the exam period.

Table 17. Parameter estimates for overall effects of the final model for the cortisol awakening response in the stress group of the fMRI sample.

Fixed Effects	**Estimate**	***SE***	***p***
Intercept	0.73	0.06	**< .001**
30 min	0.39	0.05	**< .001**
45 min	0.42	0.06	**< .001**
T2	-0.04	0.04	.337
T3	0.01	0.04	.792
T4	0.03	0.04	.434
T5	0.00	0.04	.944
T6	0.01	0.04	.865
T2 x 30 min	-0.02	0.04	.597
T2 x 45 min	-0.00	0.04	.991
T3 x 30 min	-0.06	0.04	.088
T3 x 45 min	-0.07	0.04	.083
T4 x 30 min	-0.12	0.04	**.002**
T4 x 45 min	-0.18	0.04	**< .001**
T5 x 30 min	-0.01	0.04	.725
T5 x 45 min	-0.03	0.04	.443
T6 x 30 min	-0.02	0.04	.654
T6 x 45 min	-0.02	0.04	.587
Covariates			
Women using HC	0.07	0.07	.296
Women using HC x 30 min	-0.15	0.05	**.004**
Women using HC x 45 min	-0.17	0.06	**.005**
Men	0.05	0.07	.475
Men x 30 min	-0.13	0.05	**.015**
Men x 45 min	-0.18	0.06	**.003**
Awakening time	0.10	0.03	**.002**
Awakening time x 30 min	-0.06	0.03	.060
Awakening time x 45 min	-0.08	0.04	**.036**

Random Effects	*SD*	Correlation	
		(Intercept)	30 min
Subject (Intercept)	0.12		
30 min	0.08	-.62	
45 min	0.09	-.78	.99
Timepoint (Intercept)	0.18		
30 min	0.15	-.62	
45 min	0.18	-.68	1.00
Residual	0.10		

Note. SE = Standard error; *SD* = Standard deviation; Min = Minutes after awakening; T = Timepoint; HC = Hormonal contraception.

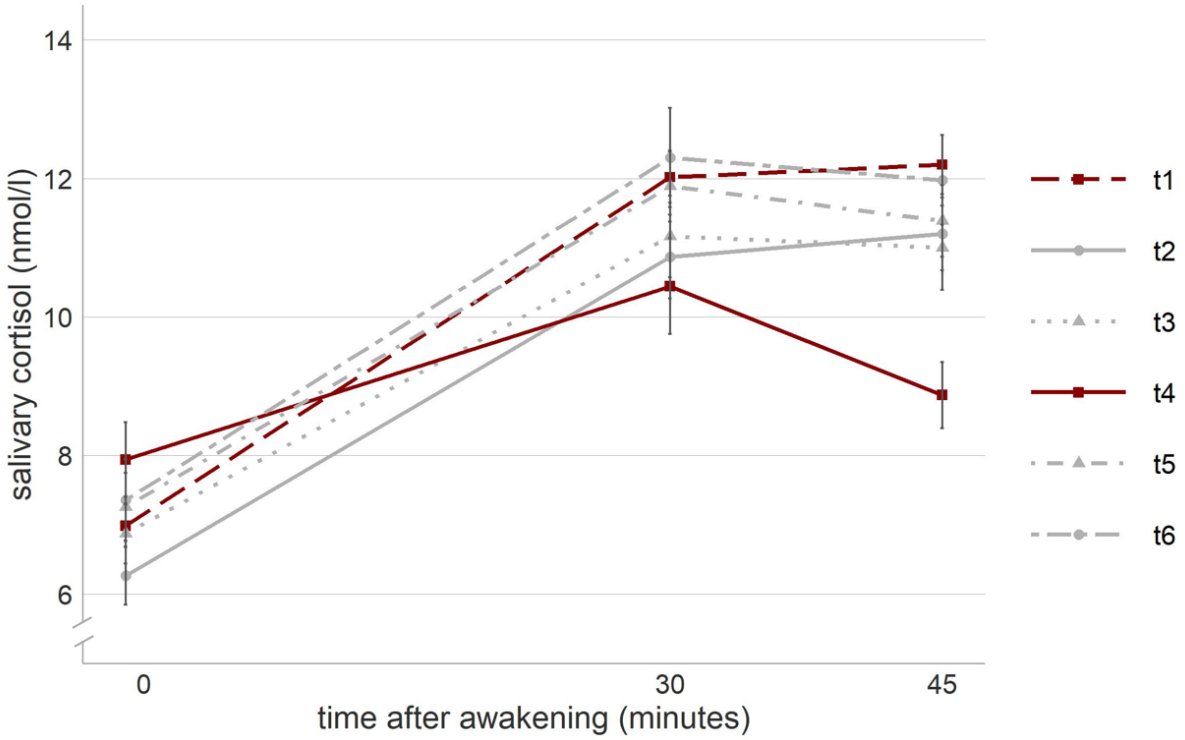

Figure 14. Mean cortisol values (±SEM) for the stress group of the fMRI sample over the study period.

Note. t = timepoint; t1 = one year before the exam, t2 = three months prior exam, t3 = one week prior exam, t4 = in the middle of the exam period, t5 = one week after the exam, and t6 = one month after the exam.

Table 18. Activated structures under psychosocial stress with anxiety scores as covariates and corrected for sex including z- and p-values as well as the localization of peak voxels.

Brain structure		**statistics**			**MNI coordinates**		
		k	***p***	***z***	**X**	**Y**	**Z**
Nucleus caudatus	**left**	**25195**	**< .001**	**4.27**	**-8**	**10**	**8**
Superior temporal gyrus	left			4.15	-62	8	-6
Superior parietal gyrus	left			4.14	-20	-46	50
Precentral gyrus	left			4.07	-56	-2	36
Thalamus	left			3.95	-18	-12	0
Postcentral gyrus	left			3.89	-46	-10	40

Note. k = cluster size in voxels; MNI = Montreal Neurological Institute; global cluster maxima are in boldface.

Table 19. Activated structures under psychosocial stress for men > women with anxiety scores as covariates including z- and p-values as well as the localization of peak voxels.

Brain structure		**statistics**			**MNI coordinates**		
		k	***p***	***z***	**X**	**Y**	**Z**
Superior temporal gyrus	**left**	**7779**	**< .001**	**4.45**	**-52**	**-16**	**10**
	left			4.35	-60	-8	2
	left			4.21	-54	-24	8
Postcentral gyrus	left			4.11	-60	4	14
Middle temporal gyrus	left			3.98	-44	-24	-10
Pallidum	left			3.96	-18	-2	-4
Middle cingulate gyrus	**right**	**5732**	**< .001**	**4.06**	**12**	**-28**	**40**
	right			3.92	10	-36	48
Postcentral gyrus	left			3.52	-24	-34	72
Supplementary motor area	right			3.50	6	-8	68
Postcentral gyrus	left			3.43	-30	-38	66
Supplementary motor area	left			3.40	-12	-8	72
Insula	**right**	**4449**	**< .001**	**3.79**	**36**	**4**	**4**
Rolandic operculum	right			3.70	62	-14	14
Superior temporal gyrus	right			3.67	42	-24	0
Insula	right			3.53	40	2	-8
	right			3.49	40	4	-12
Superior temporal gyrus	right			3.48	54	-4	-4

Note. k = cluster size in voxels; MNI = Montreal Neurological Institute; global cluster maxima are in boldface.

Factor structure of the HADS

No missing data were obtained since item responding was compulsory. For CFA, a one-factor model, a two-factor model, and a bifactor model was used. Due to a better model fit, one item of the anxiety (item HADS11) and one of the depression subscale (item HADS10) were excluded (Table 19). The model fit indicated that the bifactor model with 12 items provided the best fit (Table 20). As presented in Figure 14, all the remaining items significantly loaded

onto the factors. Reliability indices supported the use of the HADS items in a composite total score.

Table 20. Items of the HADS.

Item	Item text
HADS01 (A)	I feel tense or 'wound up'.
HADS02 (D)	I still enjoy the things I used to enjoy.
HADS03 (A)	I get a sort of frightened feeling as if something awful is about to happen.
HADS04 (D)	I can laugh and see the funny side of things.
HADS05 (A)	Worrying thoughts go through my mind.
HADS06 (D)	I feel cheerful.
HADS07 (A)	I can sit at ease and feel relaxed.
HADS08 (D)	I feel as if I am slowed down.
HADS09 (A)	I get a sort of frightened feeling like 'butterflies' in the stomach.
HADS10 (D)	I have lost interest in my appearance. (not included in the 12-items models)
HADS11 (A)	I feel restless as I have to be on the move. (not included in the 12-items models)
HADS12 (D)	I look forward with enjoyment to things.
HADS13 (A)	I get sudden feelings of panic.
HADS14 (D)	I can enjoy a good book or radio or TV program.

Note. A = anxiety subscale; D = depression subscale.

Table 21. Results of the confirmatory factor analysis.

Factor structure	χ^2	df	χ^2/df (*p*)	CFI	TLI	RMSEA	SRMR
One factor (14 items)	147.47	77	1.92 ($p < .001$)	.84	.82	.09	.08
One factor (12 items)	109.26	54	2.02 ($p < .001$)	.86	.83	.10	.08
Two factors (14 items)	140.80	76	1.85 ($p < .001$)	.86	.83	.09	.08
Two factors (12 items)	104.25	53	1.97 ($p < .001$)	.87	.84	.09	.08
Bifactor (14 items)	92.60	63	1.47 ($p = .010$)	.93	.91	.07	.07
Bifactor (12 items)	61.17	42	1.46 ($p = .030$)	.95	.93	.06	.06

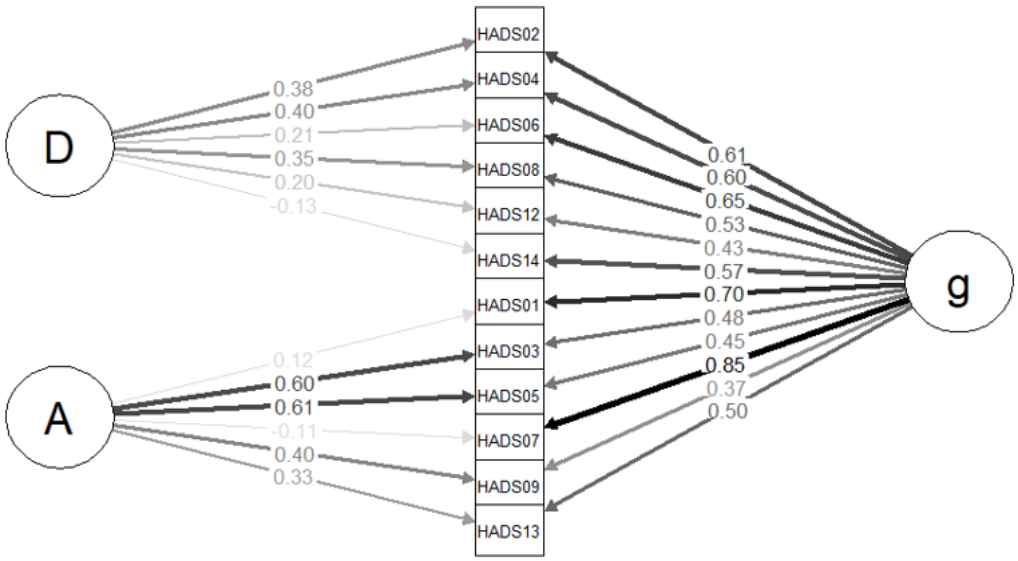

Figure 15. Bifactor CFA model with 12 items.
A: anxiety symptoms; D: depression symptoms; g: general factor.

Table 22. Structures of the clusters of the main effect stress > control with HADS general factor scores as covariates corrected for sex including *z*- and *p*-values as well as the localization of the of peak voxels.

Brain structure		**statistics**			**MNI coordinates**		
		k	***p***	***z***	**X**	**Y**	**Z**
Precuneus	**left**	**3763**	**< .001**	**3.92**	**-8**	**-48**	**52**
Middle cingulum	right			3.80	16	-40	40
Precuneus	left			3.49	-18	-46	50
Middle cingulum	left			3.42	-14	-40	40
	right			3.37	6	-18	40
	right			3.31	8	-26	44
Superior temporal pole	**left**	**3478**	**< .001**	**4.03**	**-62**	**10**	**-6**
Superior temporal gyrus	left			3.91	-50	4	-10
Postcentral gyrus	left			3.77	-54	-6	36
	left			3.70	-48	-10	40
Superior temporal gyrus	left			3.49	-64	4	-2
Nucleus caudatus	left			3.45	-14	12	6
Amygdala	**right**	**2564**	**= .005**	**3.70**	**24**	**-2**	**-12**
Superior temporal gyrus	right			3.46	52	4	-10
	right			3.44	46	0	-8
Putamen	right			3.32	16	12	-2
Nucleus caudatus	right			3.23	14	14	10
Superior temporal gyrus	right			3.18	58	-20	-4
Cuneus	**right**	**2106**	**= .013**	**3.49**	**14**	**-70**	**22**
Calcarine sulcus	left			3.34	-6	-70	16
Lingual gyrus	right			3.18	12	-70	2
	left			3.06	-4	-96	-18
Calcarine sulcus	left			3.05	0	-94	-16
Cuneus	left			3.03	-8	-78	22

Note. k = cluster size in voxels; MNI = Montreal Neurological Institute; global cluster maxima are in boldface.

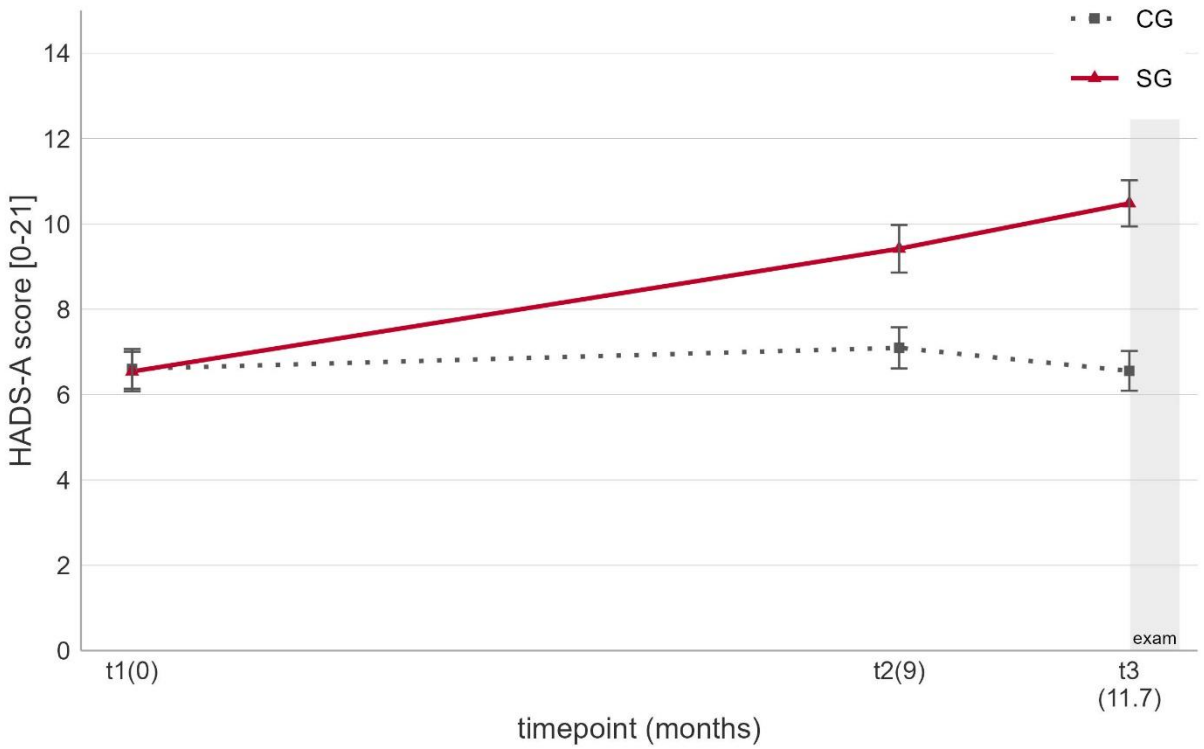

Figure 16. Time course of mean anxiety (HADS-A) scores (± SEM) for stress (SG) and control group (CG).

Table 23. Parameter estimates for overall effects of the model with anxiety symptoms as dependent variable (basic.model) and the models with beta values of the left (left.ROI.model) and right (right.ROI.model) striatal ROI as predictor.

	basic.model			**left.ROI.model**			**right.ROI.model**		
	Estimates	***SE***	***p***	**Estimates**	***SE***	***p***	**Estimates**	***SE***	***p***
Intercept	1.78	0.08	**< .001**	1.78	0.08	**< .001**	1.70	0.08	**< .001**
ROI				0.64	0.24	**.007**	0.83	0.27	**.002**
SG	0.03	0.10	.745	0.00	0.10	.977	0.07	0.11	.481
Time	0.00	0.01	.811	0.00	0.01	.802	0.01	0.01	.426
SG x Time	0.04	0.01	**< .001**	0.04	0.01	**< .001**	0.04	0.01	**< .001**
ROI x SG				-0.11	0.33	.742	-0.36	0.37	.333
ROI x Time				-0.02	0.02	.225	-0.04	0.02	.067
ROI x SG x Time				-0.01	0.03	.760	0.01	0.03	.711
Covariate									
Sex	0.02	0.04	.728	0.02	0.05	.614	0.02	0.05	.610
SD: participant (Intercept)	0.35			0.35			0.36		
Marginal R^2	0.137			0.195			0.190		
Conditional R^2	0.583			0.586			0.587		

Note. SE = Standard error; *SD* = Standard deviation; SG = stress group.