Association between genetic factors and chronic stress responses in daily life

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Foreword

In the present dissertation three studies investigating chronic stress responses in daily life and genetic factors are presented.

Chapter 3 was written in close cooperation and in joint first authorship with my project partner Marina Giglberger and will be used in her dissertation as well. Chapter 4 and 5 were composed especially for this thesis. All chapters are based on original research articles that are already published (Chapter 3 and 4) or are currently under review (Chapter 5). For *Study I* and *II* permission to include the article in my dissertation is granted by *Elsevier* ('use of one's own article in a dissertation for non-commercial purposes'). *Study III* is currently submitted and under review in the journal *Genes, Brain and Behavior* (*Wiley*). The studies are listed below in order of appearance. Additional information regarding the contributions of the respectively co-authors is provided. As already stated, *Study I* will be used in the dissertation of Marina Giglberger as well. Chapter 4 and 5 are currently not used or designated for use in other dissertations.

To improve readability, tables and figures of the manuscripts were numbered continuously and reference style was adjusted. The appendix and the reference section were combined and are presented at the end of this dissertation. Regarding the outcome variables different figures have been created for each article as study sample sizes varied slightly. Please note that only one exemplary figure for each outcome variable is presented in the appendix. Furthermore, supplementary material B (tables of the gene-wide single marker analyses) of *Study II* was not included and references to the tables have been removed. Besides, no other changes have been made to the articles. Original graphs and original supplementary material can be found with the respective article or at the repository of the University of Regensburg at https://epub.uni-regensburg.de/view/projects/LawSTRESS_project.html. Further information and graphs, including questionnaire trajectories over measurement timepoints can be found in the project repository 'The LawSTRESS project: Additional information'.

The studies were part 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). Hannah L. Peter received additional funding by the DGPs section 'Biological Psychology and Neuropsychology' (research fellowship during corona pandemic), the 'Financial Incentive System to Promote Gender Equality' (FAS) of the University of Regensburg, and the 'Bavarian Programme to Realize Equal Opportunities for Women in Research and Teaching 2021'. Additionally the co-autor Marcella Rietschel acknowledges support by the German Federal Ministry of Education and Research (BMBF) through ERA-NET NEURON, 'SynSchiz - Linking synaptic dysfunction to disease

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Study I

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Hannah L. Peter, Marina Giglberger, Stefan Wüst, Peter Kirsch, Marcella Rietschel and Brigitte M. Kudielka developed the study concept and study design. Hannah L. Peter and Marina Giglberger performed data collection, project administration, data analysis, and drafted the manuscript. Elisabeth Kraus, Ludwig Kreuzpointner and Sandra Zänkert supported data analysis. Elisabeth Kraus, Ludwig Kreuzpointner, Gina-Isabelle Henze, Christoph Bärtl, Julian Konzok, Peter Kirsch, Marcella Rietschel, Brigitte M. Kudielka and Stefan Wüst provided critical revisions.

Study II

Peter, H.L., Giglberger, M., Frank, J., Streit, F., Zänkert, S., Kreuzpointner, L., Rietschel, M., Kudielka, B.M., Wüst, S., 2022a. The association between genetic variability in the NPS/NPSR1 system and chronic stress responses: a gene-environment-(quasi-) experiment. Psychoneuroendocrinology 144, 105883. https://doi.org/10.1016/j.psyneuen.2022.105883.

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Study III

Peter, H.L., Giglberger, M., Streit, F., Frank, J., Kreuzpointner, L., Rietschel, M., Kudielka, B.M., Wüst, S., 2022b. Association of polygenic scores for depression and neuroticism with perceived stress in daily life during a long-lasting stress period. [Manuscript submitted for publication].

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List of abbreviations

5-HTTLPR	5-HT transporter-linked polymorphic region
AA	Ambulatory assessment
ACTH	Adrenocorticotropic hormone
AIC	Akaike information criteria
ANOVA	Analyses of variance
AUCg	Area under the curve with respect to the ground
AUCi	Area under the curve with respect to the increase
BIC	Bayesian information criteria
CAR	Cortisol awakening response
CG	Control group
cGxE	Candidate gene-environment interaction
CRH	Corticotropin-releasing hormone
CRHR1	Gene coding for receptor of CRH
CTQ	Childhood Trauma Questionnaire
DELFIA	Dissociation-enhanced lanthanide fluorescence immune-assay
DEP-PGS	Polygenic score for depression
FKBP5	Gene coding for FK506-binding protein 51
fMRI	Functional magnetic resonance imaging
GABRA2	Protein coding gene gamma-aminobutyric acid type A receptor subunit alpha2
GC	Glucocorticoids
GPC	Genetics of Personality Consortium
GPS	Global positioning system
GR	Glucocorticoid receptor
GSA	Gene-set analyses
GWAS	Genome-wide association study
GWEIS	Genome-wide environment interaction study
GxE	Gene-environment interaction
HADS	Hospital Anxiety and Depression Scale
HC	Hormonal contraception
HPA	Hypothalamic-pituitary-adrenocortical
HWE	Hardy-Weinberg equilibrium
IL-6	Interleukin-6
LC	Locus coeruleus
MAF	Minor allele frequency
MAOA	Gene coding for monoamine oxidase A
MEMS	Medication event monitoring systems
min	Minutes
MR	Mineralocorticoid receptor
NEU-PGS	Polygenic score for neuroticism
NPS	Neuropeptide S
NPSR1	Neuropeptide S receptor
NR3C1	Gene coding for GR
NR3C2	Gene coding for MR
p.a	Previously associated
PAF	Test anxiety questionnaire (Prüfungsangstfragebogen)
PC	Principal component
PGC	Psychiatric Genomics Consortium
PGS	Polygenic scores
PROSPER	Supporting role of specialist services

P_T	*p*-value threshold
PVN	Paraventricular nucleus
QC	Quality control
R^2	R squared
RIS	Regensburg Insomnia Scale
SAM	Sympathetic–adrenal–medullary
SCI	Stress and Coping Inventory
SCL-90-R	Symptom-Check-List-90-R
SCZ-PGS	Polygenic score for schizophrenia
SD	Standard deviation
SE	Standard error
SEM	Standard error of the mean
SG	Stress group
SMS	Short message service
SNP	Single nucleotide polymorphism
STREGA	Strengthening the reporting of genetic association studies'
t1-t6	Sampling timepoint 1 – 6
TICS	Trier Inventory of Chronic Stress

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Summary

Although it is widely accepted that chronic stress is a substantial risk factor for several disorders, little is known about the biopsychological mechanisms underlying this stress-disease link. Furthermore, there is substantial evidence that individuals differ regarding their stress responses and their susceptibility to stressful life events. As genetic factors contribute significantly to these differences, the investigation of genetic factors and their interaction with the environmental factor 'chronic stress exposure' was of special interest in the present thesis. The LawSTRESS project, a study with a prospective-longitudinal (quasi-) experimental design and an in-depth phenotyping may helps to unravel mechanisms underlying the interplay between genetic factors and stress.

In total, 471 law students from Bavarian universities were recruited, 452 students were included in the following analyses as they completed at least the first sampling timepoint. The sample was divided in two different cohorts. Cohort A comprised 204 students mainly from the University of Regensburg. Cohort B consisted of 248 law students from other Bavarian universities who underwent a modified examination protocol without laboratory visits in Regensburg. Each cohort consisted of a stress group (SG; 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 (CG; cohort A: n = 107 and cohort B: n = 119) consisting of law students in earlier semesters, experiencing normal study-related workload.

Students have been studied over a 13-months period. The study protocol included six sampling points with the first assessment (t1) taking place twelve months, the second (t2) three months and the third (t3) one week prior to the exam. Timepoint four (t4) took place during the exam and timepoints five and six (t5, t6) one week and one month after the exam. To assess chronic stress responses a multimodal and multidimensional approach was applied. The main outcome variable was stress perception in daily life which was measured at high frequency and in an ecological valid manner with repeated ambulatory assessments (AA) on each sampling point. In cohort A the assessment of perceived stress was combined with the measurement of the cortisol awakening response (CAR) which is a well-established marker of cortisol regulation in psychoneuroendocrinology. Further stress-related variables as for example anxiety and depression symptoms were assessed via online-questionnaires at each timepoint except t4.

In *Study I* (Chapter 3) the effects of the chronic exam stress on psychological well-being of the students were investigated. Furthermore, the association between daily life stress and the CAR over this long-lasting stress phase was analyzed. As hypothesized, significant differences were observed between the SG and the CG. The SG showed increases in perceived stress levels, anxiety and depression symptoms, sleep disturbances as well as in facets of perceived chronic stress.

Furthermore, the SG showed a blunted CAR at t4. No association was found between repeatedly measured perceived stress levels and the alterations in the CAR. Moreover, none of the examined psychometric predictors, namely anxiety and depression symptoms, test anxiety, and several facets of perceived chronic stress, predicted the decrease of the morning cortisol concentration.

In *Study II* and *III* gene-environment interaction (GxE) effects were examined. To overcome well known limitations of previous candidate gene and single marker studies, two methods analyzing the effects of multiple genetic variants simultaneously were applied. The main objective of *Study II* (Chapter 4) was to investigate the relevance of the a priori selected candidate gene system neuropeptide S (NPS) and its receptor (NPSR1). Based on previous research highlighting the importance of the NPS/NPSR1 system for stress-related disorders, this system was selected as target system. Gene-set analyses (GSA) examining the joint association of 936 SNPs of *NPS* and *NPSR1* were conducted. Contrary to our assumption no association was found between genetic variability in the NPS/NPSR1 system and changes in perceived stress levels and anxiety symptoms. However, a significant GxE was found regarding the CAR. The association between genetic variability in the NPS/NPSR1 system and the CAR seems to be stress-sensitive and only becomes visible under the environmental condition 'chronic stress exposure'. In *Study III* (Chapter 5) explorative polygenic score (PGS) analyses were conducted. We investigated the genetic disposition to depression and neuroticism, respectively. Both phenotypes are moderately heritable and of high relevance in stress research. Contrary to our assumptions, no association between the PGS for depression and the stress-related variables, namely perceived stress, depression symptoms as well as parameters of the CAR, were found. However, regarding the PGS for neuroticism a significant GxE was found for perceived stress levels. Individuals with a higher genetic disposition to neuroticism reported higher perceived stress and showed greater increases over the stressful period, thus they were more sensitive to stress.

In conclusion, the present dissertation showed that the preparation for and exposure to the first state examination was a considerable burden for law students and was accompanied by alterations of psychological well-being as well as a blunted CAR. The presented design seems to constitute a good model to investigate chronic stress responses. Regarding the identification of genetic factors related to chronic stress responses, we found an association between the NPS/NPSR1 system and alteration of the CAR as well as an association between perceived stress levels and the genetic disposition to neuroticism. Besides the relatively small sample size, we conclude that our study contributed to the research on the interaction of genetic factors and stress regulation in humans. However, replication and more research are needed to evaluate our approach. We assume that the combination of in-depth phenotyping, quasi-experimental

design, and the application of GSA and PGS analyses is a promising approach complementing large-scale genome-wide association and genome-wide environment interaction studies. Especially the growing digitalization enabling more researchers to use AA combined with the PGS approach offers great potential to reveal genetic underpinnings of chronic stress responses.

Chapter 1

1 Introduction and outline of the thesis

Psychological stress is an unavoidable part of our everyday life, for example in form of social demands or excessive demands at work (Almeida et al., 2002). While occasional moderate stress is considered to have no detrimental impact on health, or in the case of successful coping can even strengthen resilience (Jin et al., 2014), chronic or excessive 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). Although biological mechanisms underlying this stress-disease link are still not fully understood, it is generally accepted that dysregulations of stress-relevant systems, as for example in the hypothalamic–pituitary–adrenal (HPA) axis and concomitant alterations in cortisol secretion, probably play an important role (O'Connor et al., 2021; Tsigos and Chrousos, 1994). Moreover, there is substantial evidence that some individuals are more strongly affected by stressful life events than others (Galatzer-Levy et al., 2018; Nillni et al., 2013; Uher and Zwicker, 2017). These differences in vulnerability can be partly explained by genetic factors. Genetic factors and their interaction with the environment are likely to contribute to interindividual differences in stress response patterns and ultimately to differences in disease vulnerability (Caspi et al., 2003; Normann and Buttenschøn, 2019; Pluess, 2015). Uncovering gene-environment interactions (GxE) can help to identify individuals at risk, to develop personalized prevention as well as intervention programs, and to elucidate molecular mechanism of stress regulation (Dick et al., 2018; Visscher et al., 2017).

Significant heritabilities have been documented for stress-related disorders (Howard et al., 2019; Sullivan and Geschwind, 2019), for personality traits related to stress regulation (Kendler et al., 2008; Nagel et al., 2018a; Polderman et al., 2015; Raevuori et al., 2007) as well as for indicators of HPA axis activity (Federenko et al., 2004; Wüst et al., 2000b). Moreover, genome-wide association studies (GWAS) demonstrated that most complex traits and common disorders are highly polygenic (Visscher et al., 2017). The genetic disposition for stress-related disorders as well as related traits is composed of thousands of variants, each characterized by a very small effect size (Howard et al., 2019; Nagel et al., 2018a). Numerous GxE studies related to stress research have been conducted. Most studies investigated single genetic markers of specific candidate genes (Sharma et al., 2016; Smoller, 2015). However, due to lack of replication and potential false discovery, candidate gene findings and candidate GxE (cGxE) findings are massively criticized (Duncan and Keller, 2011; Harden, 2021). Furthermore, it seems unlikely that genetic effects in stress research are solely based on one or a few single nucleotide polymorphisms (SNP) within a gene system. Therefore, it was proposed to integrate high quality genotyping in stress research as well as to apply new genetic approaches analyzing the variability of a wide

range of SNPs simultaneously, as for example gene-set analyses (GSA) and polygenic scores (PGS; Harden and Koellinger, 2020).

Besides an improved genotyping, studies investigating GxE effects would benefit from detailed phenotyping and an optimized study design. In this context the conduction of GxE experiments with a systematic variation of the environmental variable was proposed. The experimental design decreases measurement error within the environment component and reduces gene-environment correlation, which hinder the discovery of true gene-environment interactions (Bakermans-Kranenburg and van IJzendoorn, 2015; Leighton et al., 2017; van IJzendoorn et al., 2011). To meet the criteria of an experimental or quasi-experimental design a cohort that experiences a long-lasting stress period in the future is required. Furthermore, a recruitment of an adequate control group should be feasible. The preparation for the first state examination for law students fulfils these prerequisites and constitutes a robust manipulation of the environment facilitating the investigation of GxE effects on chronic stress responses. This state examination ranks among the most stressful academic exam periods in the German university system. In Bavaria, students absolve six written exams of several hours each within eight days. Because of the high failure rate (up to 30%), the limited possibility to repeat the exam, and the major importance of the final mark for the future career perspective, students prepare intensively for this exam for about one year. Previous studies already showed that academic stress, above all during exam phases, results in strong increases in perceived stress levels and alterations in several biological systems, as for example the HPA axis (Burger et al., 2014; Koudela-Hamila et al., 2020). It has to be noted that investigated stress periods in previous studies were significantly shorter than in the present project.

In our project, law students were studied over a 13-months period. The prospective and longitudinal design allowed to assess the variables of interest under resting conditions and to investigate individual trajectories across long-term stress exposure. Students preparing for the state examination have been assigned to the stress group (SG), whereas law students in earlier semesters, with normal study-related stress load, constituted the control group (CG). Besides the prospective-longitudinal (quasi-) experimental design, we pursued a multimethod and multidimensional approach combining laboratory methods with ecologically valid methods of field research assessing biological and psychological stress responses repeatedly over the observation period. Perceived stress levels were measured by ambulatory assessment, a method measuring the participants' experience and behavior in everyday life at high frequency and proximal in time to the immediate experience (Trull and Ebner-Priemer, 2014). The AA method results in a reduction of retrospectiv bias, higher reliability, and higher ecological validity, thus potentially reducing the measurement error of the outcome variable (Trull and Ebner-Priemer, 2014). The

assessment of perceived stress levels was combined with measurements of cortisol regulation, more specifically the cortisol awakening response (CAR), a well-known marker of HPA axis activity.

The main aims of the present work were to examine the effects of chronic exam stress, to unravel GxE effects and to identify genetic as well as psychometric predictors of biopsychological chronic stress responses in daily life. Chapter 2 provides a brief overview of the research topics stress and genetic factors in stress regulation. In addition, Chapter 2 comprises a brief introduction to experimental GxE studies and novel genetic methods, namely gene-set and polygenic score analyses. Chapter 3 presents the first study. *Study I* aimed at answering the question whether the long-term stress exposure, defined as preparation for the first state examination for German law students, results in alterations of perceived stress levels, the cortisol awakening response as well as in other stress-related psychometric variables. Furthermore, associations between CAR and changes in perceived stress were investigated (Giglberger et al., 2022). Chapter 4 comprises *Study II*, investigating the association between the genetic variability within the candidate gene system of neuropeptide S (NPS) and its receptor (NPSR1) and chronic stress responses. To capture genetic variability within the NPS/NPSR1 system, GSA were performed investigating the joint effect of 936 SNPs within *NPS* and *NPSR1* (Peter et al., 2022a). Subsequently, Chapter 5 presents the third study which encompasses exploratory PGS analyses. The association between genetic disposition to depression or neuroticism and stress regulation under chronic exam stress was investigated (Peter et al., 2022b). Finally, in Chapter 6 a general discussion, integrating the findings of the presented results is provided.

Chapter 2

2 Theoretical background

2.1 Psychobiology of stress

Stress arises when an individual is confronted with internal or external demands, which are potentially threatening or perceived as threatening for the individual's physical health or emotional well-being (Ulrich-Lai and Herman, 2009). According to transactional stress theories, a demand is perceived as stressful if the affected individual assesses it as exceeding his or her own coping strategies (Lazarus and Folkman, 1984). When experiencing psychological and physical stress a variety of systems is activated in order to overcome the challenges and maintain and reestablish homeostasis (McEwen, 2019). Two key systems regulating the stress response are the sympathetic–adrenal–medullary (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, is activated via the hypothalamus which triggers the locus coeruleus (LC) in the brain stem, resulting in an increase of noradrenaline within the brain and the stimulation 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 trigger the production and release of the catecholamines adrenaline and noradrenaline of the adrenal medulla. Within seconds noradrenaline and adrenaline cause the so-called *fight-or-flight response* (Cannon, 1929) characterized by an elevated vigilance, arousal and focused attention (Chrousos and Gold, 1992), the acceleration of breathing, the elevation of heart rate and blood pressure through excitation of the cardiovascular system as well as vasodilatation in muscles (Gunnar and Quevedo, 2007; Osborne et al., 2020).

The HPA axis is stimulated via the release of corticotropin-releasing hormone (CRH) in the paraventricular nucleus in the hypothalamus. CRH acts on the pituitary gland and induces the release of adrenocorticotropic hormone (ACTH). ACTH in turn stimulates the adrenal cortex to synthesize and release glucocorticoids (GC). The main GC in humans is the steroid hormone cortisol which has a variety of effects throughout the whole organism including the regulation of the immune system, brain function, behavior as well as the modulation of metabolic and cardiovascular processes (de Kloet et al., 2019; McEwen, 1998; Sapolsky et al., 2000). GC exert their effect via two receptors, the high affinity mineralocorticoid receptor (MR) and the low affinity glucocorticoid receptor (GR). Recently, they were shown not only to function as transcription factors that alter gene transcription, but also to mediate rapid non-genomic actions (de Kloet et al., 2019; Joëls et al., 2018; Mourtzi et al., 2021). The GR controls the effects of GC, when concentrations are elevated (Sapolsky et al., 2000) and possibly regulates the dampening of the initial stress reaction, via a negative feedback loop suppressing the further release of GC (de Kloet et

al., 2019; Reichardt et al., 1998). The balance in MR- and GR-mediated actions seems to be essential for the restoration of homeostasis through the proper initiation and termination of the stress response (de Kloet et al., 2019; Mourtzi et al., 2021).

The stress response can be described as a highly coordinated interaction between various biological systems including the HPA axis, the SAM, and the immune system, preparing the organisms to cope with the changing demands on a behavioral, cognitive and affective, as well as on a physiological level (de Kloet et al., 2019; Joëls et al., 2018; Mueller et al., 2022). Additionally, stress response patterns show large intra- and interindividual variability (Kudielka and Wüst, 2010; Rab and Admon, 2021; Zänkert et al., 2019). The investigation of this variability is inevitable to reveal mechanisms of stress-related psychopathology. The stress response is influenced by characteristics of the individual, as for instance the genetic disposition, personality traits, prior experiences, and behavioral habits (Joëls and Baram, 2009; Zänkert et al., 2019) as well as by certain properties of the stressful event or demand (e.g., novelty, unpredictability and uncontrollability, frequency, intensity and duration; Dickerson and Kemeny, 2004; Mason, 1968). Due to its complexity and the large intra- and interindividual variability, it remains a major challenge to accurately characterize the stress response (Rab and Admon, 2021).

2.2 Pathophysiology of stress

Although the stress response is substantial for an organism to adapt to changing environments and demands, it can be potentially harmful in the context of excessive or prolonged stress (Joëls and Baram, 2009; McEwen, 2004). It was shown that chronic psychological stress is a significant risk factor for several disorders, including psychiatric disorders, as for example, depression, anxiety, and sleep disorders as well as cardiovascular diseases and diseases resulting from dysregulated immune functions (Chrousos, 2009; Cohen et al., 2007; Cohen et al., 2019; Dar et al., 2019; Hammen, 2005; Kendler et al., 1999; Porcelli et al., 2016; Rohleder, 2014). Findings indicate that prolonged stress exposure can cause changes in central nervous, endocrine, metabolic and immunological functions and concomitant alterations in the synthesis and release of stress mediators, either leading to an up- or downregulation both potentially with implication for mental and physical health (McEwen, 2004). The exact biopsychological mechanisms underlying stress-induced pathology are not fully understood, however a dysregulation of the HPA axis is probably a key factor in mediating the stress-disease relationship (Normann and Buttenschøn, 2019; O'Connor et al., 2021; Tsigos and Chrousos, 1994). Therefore, it is crucial to understand how the HPA axis and the secretion of cortisol are affected by stress. Besides the investigation of cortisol reactivity to acute stress (Nater et al., 2013; Skoluda et al., 2015) , prospective studies

measuring inter- and intraindividual alterations of basal HPA axis activity and circadian regulation of cortisol secretion over a chronic stress period are needed (Stalder et al., 2016).

The regulation of the HPA axis is highly complex and measurement of the different markers is challenging. For the assessment of the final product cortisol several methodologies are available (Hellhammer et al., 2009). Cortisol can be measured in hair, urine, blood and saliva each representing different time intervals of cortisol release. Hair cortisol for example enables to determine cortisol concentrations of the last months whereas measurement in salivary represents the current concentration (Hellhammer et al., 2009; Kirschbaum et al., 2009). Furthermore, assessment methods capture different states of biological availability of cortisol. Salivary cortisol reflects the concentration of free, biological active cortisol (Kirschbaum and Hellhammer, 1994). It further has to be noted that cortisol release occurs in a pulsatile and circadian manner which complicates its assessment (Levine et al., 2007). Diurnal cortisol release follows a specific pattern characterized by an increase in the morning and a decline over the day, reaching its nadir around bedtime. However, with the implementation of the measurement of salivary cortisol different assessment protocols easily assessable in field studies emerged to examine cortisol regulation in daily life. In this context the CAR is of substantial interest (Stalder et al., 2016).

2.2.1 The cortisol awakening response

The sharp increase of cortisol levels in the first 30 to 45 minutes after morning awakening is known as the CAR (Pruessner et al., 1997; Stalder et al., 2016; Stalder et al., 2022). The CAR underlies complex and distinct regulatory mechanisms compared to basal diurnal secretion pattern of cortisol and is stimulated by morning awakening (Clow et al., 2010; Wilhelm et al., 2007). The assessment of the samples can be conducted at home in a naturalistic setting. Furthermore, it combines characteristics of a reactivity index and aspects related to circadian regulation. Therefore, the CAR is often used as an index for HPA axis (re)activity (Stalder et al., 2016). Evidence suggested that the CAR probably serves as preparation for the upcoming demands of the day (Powell and Schlotz, 2012). In twin studies a moderate heritability of the CAR was consistently found (Wüst et al., 2000b). However, situational factors as for example the awakening time and menstrual cycle phase have substantial impact on the magnitude of the CAR on a particular day and should be controlled (Hellhammer et al., 2007; Law et al., 2013; Stalder et al., 2016). The CAR is associated with various psychosocial, physical and mental health parameters and it is assumed that 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). An altered CAR is associated with stress-related disorders as well as with physical health as for example systemic hypertension (Wirtz et al., 2007) and coronary artery disease (Bhattacharyya et al., 2008). Posttraumatic stress disorder seems to be related to a blunted CAR (Wessa et al.,

2006), whereas depression and the risk for developing a depression were found to be related to a heightened CAR (Adam et al., 2010) albeit results have been mixed (Fries et al., 2009; Kudielka and Wüst, 2010). Studies investigating the association between the CAR and perceived chronic stress also yielded mixed results (Chida and Steptoe, 2009). While chronic work overload and worrying were associated with an increased CAR (Schlotz et al., 2004), a blunted CAR was observed 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 encompass a sampling time verification, elaborate statistical analyses including confounding variables (Stalder et al., 2016) and a longitudinal design (O'Connor et al., 2021).

It can be concluded that the CAR is an important indicator for HPA axis activity and it was shown that both, a blunted as well as an elevated CAR seem to be associated with adverse health effects (O'Connor et al., 2021). Future research ought to incorporate prospective-longitudinal designs and repeated assessments to broaden the knowledge about regulatory function of the CAR during chronic stress.

2.2.2 Chronic stress in students as model for chronic stress

Although in general it is assumed that students constitute a resilient and healthy part of the population, findings are accumulating that psychological stress as well as depression and anxiety symptoms are widespread among students (Bunevicius et al., 2008; Grützmacher, 2018; Heilmann et al., 2015; Herbst et al., 2016; Sheldon et al., 2021; Weber et al., 2020). Moreover, the prevalence of mental health problems appears to be higher in the student population than in the general population (Auerbach et al., 2016; Burger et al., 2014; Ibrahim et al., 2013; Rabkow et al., 2020; Rotenstein et al., 2016) suggesting increased vulnerability (Grützmacher, 2018). University studies and academic exams represent a significant source of stress across disciplines. For example, in 530 students of human medicine in the preclinical study period, increased scores were found for depression and anxiety symptoms (Burger et al., 2014; Scholz et al., 2014). Another study found that about 50% of medical students develop “burnout” symptoms in the course of their university studies (IsHak et al., 2013). Compared to a comparison group, 366 students from various disciplines reported significantly increased impairment from physical and psychological symptoms (Heilmann et al., 2015). Rabkow et al. (2020) examined 306 German law students from different semesters and found an increased frequency of depression symptoms compared to general population. Moreover, in a large-scale study across all disciplines of the University of Berlin, law students were in the upper range in regard to subjective stress experience (Grützmacher, 2018). In terms of biological markers, academic stress was

found to be related to salivary and hair cortisol levels (Koudela-Hamila et al., 2020; Loft et al., 2007; Stetler and Guinn, 2020). Regarding the association of stress due to academic exams and the CAR results have been mixed. In a longitudinal study, Duan et al. (2013) examined male students in preparation for an important exam and found a blunted CAR in the exam group. This effect was more pronounced in students with higher perceived stress levels. Other studies examining the CAR could find no impact of the exam (González-Cabrera et al., 2014; O'Flynn et al., 2018) whereas some studies found an increased CAR (Hewig et al., 2008; Weik and Deinzer, 2010). Furthermore, alterations in cardiovascular as well as in immunological parameters were found to be associated with exam stress (Kamezaki et al., 2012; Koudela-Hamila et al., 2020; Loft et al., 2007). For example, the LPS-stimulated production of the proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α in monocytes was increased compared to baseline measure one month before the exam period (Maydych et al., 2017). Given the methodological heterogeneity, especially regarding the baseline measurements, the duration and intensity of the exam period as well as the different structures of the examined study disciplines across the countries, it is not surprising that the findings on academic stress and exam periods were not consistent.

However, the findings show that students suffer from substantial stress load accompanied by alterations in biological systems, especially during exam periods. Hence, it can be concluded, that the exam period in the academic context serves as a good model to examine chronic stress. It furthermore should be noted that the preparation for the first state law examination is one of the longest and most learning-intensive exam periods in the German university system. The investigated exam periods in previous studies were significantly shorter and less-continuous than in the present project.

2.3 Genetics in stress research and stress-related disorders

Psychiatric disorders, as for example depression and anxiety disorder are considered complex phenotypes and are partly hereditary (Sullivan and Geschwind, 2019). At the same time there is substantial evidence that the exposure to stressful life events and adversity increases the risk for psychiatric disorders (Chrousos, 2009; Cohen et al., 2019; Musci et al., 2019). Stress regulation itself is a complex phenotype and can be regarded as dynamic manifestation of the interaction between the genetic constitution of individuals and their environment. More research is needed to investigate how stress and genetic factors interact to impact different aspects of the stress response ultimately leading to dysregulations, detrimental health outcomes, and psychiatric conditions in some individuals (Dalvie et al., 2021).

2.3.1 Polygenicity of complex traits and 'missing heritability'

Although the pathophysiology of psychiatric disorders is still not fully understood, genetic factors play a substantial role. Twin studies revealed heritability estimates ranging from 37% in depression, 30% to 40% in anxiety disorder, and 46% in post-traumatic stress disorder to 81% and 85 % in schizophrenia and bipolar disorders, respectively (Craske et al., 2017; Stein and Gorman, 2001; Sullivan and Geschwind, 2019; Sullivan et al., 2003; Sullivan et al., 2000). However, these estimates did not provide information about how many and which genetic variants contribute to the heritability. It was not until the conduction of GWAS that empirical data confirming the hypothesized polygenic nature of complex traits and psychiatric disorders was provided (Maier et al., 2018; Visscher et al., 2017). GWAS revealed that the genetic basis for psychiatric disorders comprises a substantial number of variants, each characterized by a small effect size. Building upon these disease-associated variants, many candidate gene studies emerged and new hypotheses about diseases mechanisms could be developed. However, the predictive power of these variants has limited utility because of their small effect sizes (Fang et al., 2020). Additionally, the new knowledge and data obtained through GWAS led to the development of new methodological and statistical approaches (Visscher et al., 2017).

Despite the increasing sample sizes and advance of GWAS also in the field of psychiatric phenotypes, the phenomenon of 'missing heritability' still exists (Assary et al., 2018; Manolio et al., 2009; Wray and Maier, 2014). The 'missing heritability' refers to the discrepancy between the heritability estimates of twin studies and the lower heritability estimates of common SNPs revealed by GWAS (Sullivan and Geschwind, 2019; Uher, 2014). Despite de novo mutations and rare SNPs, GxE effects are possibly an explanation for this discrepancy (Manolio et al., 2009; Manuck and McCaffery, 2014). As already stated, most complex diseases and behaviors are influenced by genetic as well as environmental factors (Musci et al., 2019; Smoller, 2015). Thus, to reveal the pathogenetic mechanisms of stress-related disorders the study of gene-environment interactions seems to be inevitable.

2.3.2 Genetic factors in stress regulation

There is evidence for a substantial heritability of personality traits which are related to stress and stress regulation, as for example anxiousness, neuroticism as well as self-esteem (Kendler et al., 2008; Polderman et al., 2015; Raevuori et al., 2007; Vukasović and Bratko, 2015). However, findings from quantitative and molecular genetics are also available on biopsychological markers of stress regulation. Twin studies and GWAS found modest heritabilities for different markers of basal HPA axis activity (Bartels et al., 2003; Bolton et al., 2014; Rietschel et al., 2017; Velders et al., 2011; Wüst et al., 2000a). Furthermore, there is some evidence suggesting a moderate heritability of the cortisol stress response to acute psychological stress (Federenko et al.,

2004; Ouellet-Morin et al., 2008; Steptoe et al., 2009) as well as of subclinical constructs such as self-reported chronic stress exposure or affective reactivity to negative events (Federenko et al., 2006; Rietschel et al., 2014; Venables et al., 2017).

Although first genome-wide by environment interaction studies (GWEIS; Arnau-Soler et al., 2018; Coleman et al., 2020; Werme et al., 2021) as well as multi-locus approaches, as for example polygenic score analyses (see section 2.3.5.2) emerged in the last decade, the majority of studies examining the interaction between stress and genetic factors investigated single candidate genes or single SNPs, respectively (Assary et al., 2018; Musci et al., 2019; Sharma et al., 2016; Smoller, 2015). Candidate gene and cGxE findings based on the investigation of single genetic markers within a gene-system have to be regarded with caution as reliability of the findings is questionable (see section 2.3.3; Border et al., 2019; Duncan and Keller, 2011; Harden, 2021). However, they still have some value in the sense of *proof-of-concept*, thus a small overview of the numerous studies is presented.

Several studies investigating candidate genes potentially involved in HPA axis pathway and regulation have reported significant associations with cortisol regulation and other stress-relevant phenotypes such as brain activity and heart-rate in response to acute stress as well as psychological stress responses (DeRijk et al., 2006; Kudielka et al., 2009; Kumsta et al., 2013; Li et al., 2019a; Mahon et al., 2013; Zänkert et al., 2019). For example, variants of the gene coding for FK506-binding protein 51 (*FKBP5*), an important protein modulating GR activity, seem to be associated with cortisol stress reactivity (Ising et al., 2008; Luijk et al., 2010) and stress-related brain activity such as threat-induced hippocampal activation (Fani et al., 2013). First cGxE studies indicate that *FKBP5* SNPs in interaction with childhood trauma are related to cortisol reactivity in response to acute stress (Buchmann et al., 2014; Luijk et al., 2010; Zannas and Binder, 2014). Moreover, variants in genes coding for GR (*NR3C1*) and MR (*NR3C2*) as well as for the receptor of CRH (*CRHR1*) were associated with cortisol responses to the Trier Social Stress Test, an acute stress paradigm for laboratory (DeRijk et al., 2006; Kumsta et al., 2007; Mahon et al., 2013; Wüst et al., 2004). Additionally, it was found that SNPs in *NR3C1* were associated with the CAR as well as with self-perceived stress levels (Li-Tempel et al., 2016). Further examples for interesting candidate genes associated with stress regulation are genes within the serotonergic (e.g., 5-HT transporter-linked polymorphic region, *5-HTTLPR*) and the dopaminergic system (catechol-O-methyl- transferase gene, dopamine D4 receptor gene) as well as genes coding for brain-derived neurotrophic factor, alpha-2B adrenergic receptor, and monoamine oxidase A (*MAOA*; Alexander et al., 2009; Alexander et al., 2010; Allen et al., 2017; Kumsta et al., 2013; Miller et al., 2013; Sun et al., 2020b). Variations in *5-HTTLPR* and *MAOA* for example were associated with differences in brain activation in response to the Montreal Imaging Stress Task, an acute stress

paradigm for the fMRI environment (Sun et al., 2020a; Sun et al., 2020b). The *5-HTTLPR* was furthermore associated with negative stress effects of exam stress on mood and perceived stress (Verschoor and Markus, 2011). In addition, evidence is accumulating that also immunological genes should be considered. It was shown that a SNP on the promoter gene of the IL-6 gene was associated with a stress-induced increase of the proinflammatory cytokine IL-6, especially in the context of chronic stress (Slavich and Cole, 2013). Moreover, based on findings from animal models and genetic association studies in humans, it can be assumed that the genes coding for NPS and NPSR1 play an important role in stress regulation and stress-related disorders, including anxiety and panic disorder (see Chapter 4; Ghazal, 2016; Tobinski and Rappeneau, 2021). In humans, mainly the functional *NPSR1* sequence variant rs324981 was investigated. First results suggest an association between the minor and more active T allele and cortisol regulation during acute stress (Kumsta et al., 2013; Streit et al., 2017).

Most of the studies investigated the impact of genetic variation on acute stress responses. In regard to chronic stress during adulthood, stressful life events as for example caregiver stress and work-related stress have been examined. A lot of research focused on the *5- HTTLPR*. Carriers of the short allele seem to be more susceptible to stressful life events than carriers of the long allele, possibly via altered HPA axis activity (Caspi et al., 2003; Miller et al., 2013; Uher and McGuffin, 2008; van IJzendoorn et al., 2012). Regarding caregiver stress, first findings suggest that negative health outcomes or alteration in HPA axis activity due to the burden of carrying for a relative with chronic illness may be related to genetic variability, for example within the *MAOA* (Brummett et al., 2008; Wolf and Middleton, 2018). Another study investigating teachers, a profession repeatedly described as stressful (Guglielmi and Tatrow, 1998), found that MR haplotypes were associated with perceived chronic stress levels (van Leeuwen et al., 2011). Results, however, should be interpreted with caution because of lacking replication, cross-sectional designs and retrospective assessment of stressful life events.

Besides the criticism of current cGxE studies, there is general agreement that genetic variation is associated with the individual reaction to stress, probably on a psychological as well as on a neurobiological level, which in turn can increase vulnerability to psychopathology (Bogdan et al., 2016; Caspi et al., 2010; DeRijk et al., 2008; Normann and Buttenschøn, 2019; Zannas and Binder, 2014). The uncovering of genetics underlying individual differences in response to stressful life events (stress sensitivity) is crucial for a further understanding of disease-related pathology and can improve identifying individuals at high risk and developing individualized treatments as well as prevention programs (Arnau-Soler et al., 2018; Dick, 2018).

2.3.3 Challenges in GxE studies

As already mentioned, there has been increasing criticism regarding candidate gene and cGxE studies in the last decade, mainly because of lack of replication and potential false discovery (Harden, 2021). These critics are related to publication bias, insufficient power, and the inaccurate adjustment for multiple testing. Given the small effect sizes of single genetic variants most psychological cGxE studies investigating individual genetic markers were severely underpowered to detect true effects (Caspi and Moffitt, 2006; Dick et al., 2011; Duncan and Keller, 2011). Several researchers stated that most of the published findings are probably wrong (Border et al., 2019; Duncan and Keller, 2011; Hewitt, 2012). Furthermore, it seems not adequate to investigate complex highly polygenic traits by only examining single loci (Schmitz and Conley, 2017; Visscher et al., 2008). Besides the lack of power, examining GxE effects even more obstacles arise (Bakermans-Kranenburg and van IJzendoorn, 2015; Gauderman et al., 2017; Monroe, 2008), above all the questions of how to measure the environment and how to avoid confounding due to gene-environment correlation (Kendler and Baker, 2007; Plomin et al., 1977). Due to the complexity of the environment, studies differ strongly regarding the assessment of environmental factors and stressful life events (e.g., with reference to severity, duration or type of the stressor). Thus, comparisons between studies are difficult, in particular because it is likely that different types of stress have diverse effects (Dick et al., 2015). Moreover, environmental factors are often assessed retrospectively. These measurements are probably liable to heritable response biases, mood-congruency effects, and recall biases (Colman et al., 2016; Hardt and Rutter, 2004; Monroe and Reid, 2008; Zammit and Owen, 2006). Besides the assessment of the environment, the precise measurement of the phenotype also is of great importance because the measurement error in genetic and environmental component reduces the ability to detect real associations. The need for large sample sizes in genetic studies commonly results in poor characterization of the environment and phenotypes (Moffitt et al., 2005; Uher, 2014).

Although modern cGxE studies can probably still make valuable contributions to research under certain conditions, these challenges highlight the need for new methods capturing the genetic variability of multiple SNPs simultaneously (see section 2.3.5). Additionally, GxE studies on stress regulation would benefit from prospective-longitudinal approaches with a precise assessment of phenotype and environmental factors (Dick et al., 2018; Leighton et al., 2017).

2.3.4 Gene-environment experiment

In order to overcome some of the limitations, experimental designs with a systematic variation of the environmental factor have been proposed (van IJzendoorn et al., 2011). Gene-environment experiments with randomized assignment to different groups have three major advantages compared to correlational studies (Bakermans-Kranenburg and van IJzendoorn, 2015;

Schmitz and Conley, 2017; van IJzendoorn et al., 2011). One advantage is that an experimental design enables to reduce the uncontrollable influence of unmeasured genetic effects on environmental exposure. Genetic and environmental factors are mostly not independent because the genetic predisposition shapes the environment an individual is exposed to in an active, passive and reactive manner. Therefore, specific environmental conditions are more common in certain genotypes (Mills et al., 2020b; Plomin et al., 1977). Such correlations between genes and the environment can hinder the discovery of true GxE effects and invalidate the conclusion (Bakermans-Kranenburg and van IJzendoorn, 2015; Leighton et al., 2017; van IJzendoorn et al., 2011). The manipulation of the environment in a causal manner and the randomization of an experimental design break the potential gene-environment correlation (van IJzendoorn et al., 2011). Moreover, causal interpretation is facilitated conducting an experiment showing that genotype associations can be moderated by alterations in the environment (Schmitz and Conley, 2017). Furthermore, GxE findings are largely dependent on accurate assessment of genotype and environment (Bakermans-Kranenburg and van IJzendoorn, 2015). Instead of cross-sectional or self-reported retrospective assessments of the environment, an active manipulation can reduce the measurement error and lead to more control of the environment component. It was proposed that a better measurement of the environment may for certain research questions be more crucial than larger sample sizes (Vrshek-Schallhorn et al., 2019; Wong et al., 2003). The third advantage is the increased power of experimental studies. The authors stated that randomized GxE experimental studies are at least 10 times more powerful than correlational GxE studies (Bakermans-Kranenburg and van IJzendoorn, 2015; McClelland and Judd, 1993).

Several experimental candidate gene studies, particularly in the context of genotype-intervention, were conducted and successfully revealed that the genotype matters when examining intervention efficacy (Albert et al., 2015; Belsky and van IJzendoorn, 2015). For example, the PROSPER project, a large-scale randomized intervention study on alcohol misuse in adolescents, revealed associations between intervention efficacy and the SNP rs279845 within the protein coding gene gamma-aminobutyric acid type A receptor subunit alpha2 (*GABRA2*; Russell et al., 2018). It was shown that the intervention reduced alcohol misuse in adolescents with the TT genotype whereas no effect was observed in A allele carriers. Furthermore, another randomized controlled trial investigating behavioral problems of children with attention deficit/hyperactivity disorder found that the intervention efficacy was genetically moderated by a polymorphism in the dopamine transporter gene (van den Hoofdakker et al., 2012). Although studies already yielded interesting results, it has to be noted that they mostly focused on single or only a few SNPs.

In summary, it can be stated that the experimental manipulation of a specific dimension of the environment can improve the power of G×E analyses and possibly leads to more consistent results than correlational studies.

2.3.5 New methods in psychiatric genetics

Although candidate gene studies investigating single genes or *a priori* defined gene systems based on GWAS results may have some value for psychological stress research, it appears unlikely that the effects of genes can be adequately explained by an individual functional sequence variant. Hence, a sufficient number of SNPs should be investigated to capture the genetic variability within a gene system or a single gene. New methods and approaches are needed to examine GxE at a multi-gene level, especially given the polygenic nature of psychiatric and stress-related phenotypes (Harden and Koellinger, 2020; Winham and Biernacka, 2013).

In the following two promising methods that go beyond the single SNP approach are presented. When studying complex, polygenic traits or diseases, the effect of single genetic markers is often too weak to be detected. Statistical methods, such as gene set analysis and polygenic score analysis, remedy this problem and determine the joint effect of several genetic markers on a particular phenotype (Winham and Biernacka, 2013; Wray et al., 2014). Furthermore, the joint analysis of multiple markers reduces the multiple testing burden and consequently improves power to detect genetic factors associated with complex diseases (de Leeuw et al., 2015; Mooney and Wilmot, 2015).

2.3.5.1 Gene-set analysis

In GSA multiple markers are aggregated to the level of whole genes and these in turn are aggregated to groups of genes, so called gene sets, based on shared biological or functional characteristics (de Leeuw et al., 2015). The gene sets sharing common property are then tested for their association with the phenotype of interest. Hence, GSA provides an insight into biological mechanisms, as for example specific pathways or cellular functions, associated with the phenotype (Das et al., 2020). Furthermore, effects composed of multiple weaker associations that would likely be missed may be detected by aggregation in GSA (Fridley and Biernacka, 2011; Winham and Biernacka, 2013). There are two types of GSA, the competitive analysis investigating whether the gene set of interest shows a stronger association with the phenotype compared to genes outside the predefined gene set and the self-contained analysis testing whether the gene set is associated at all with the phenotype (de Leeuw et al., 2015). The gene-set analysis is often used as secondary analysis of GWAS data sets and its application has led to new hypotheses about biological processes involved in polygenic disorders (Das et al., 2020; Mooney and Wilmot, 2015).

Moreover, the GSA provides a novel opportunity to investigate cGxE effects as a promising alternative to single SNP analyses (Windhorst et al., 2016). Conducting GSA, the combined effect of various genetic markers across a gene or a gene system can be examined and the genetic variability of the whole system of interest would be adequately covered. Furthermore, some tools already implemented a feature to add environmental factors to examine gene-environment interactions (de Leeuw et al., 2015). The GSA approach has already been successfully used in a study investigating the interaction between the genetic variability across the dopaminergic system and harsh parenting on children's externalizing behavior (Windhorst et al., 2016). The authors found associations between dopamine genes and externalizing behavior only in children without harsh parenting. The genetic variance of dopamine genes seems not to explain differences in externalizing behavior in children experiencing harsh parenting. They conclude that the GSA represents a promising approach to complement single SNP analyses and GWEIS studies.
In the context of stress regulation, the NPS/NPSR1 system seems to be a promising candidate gene system (Tobinski and Rappeneau, 2021). Thus, in Chapter 4 the GSA approach is applied to examine the association between chronic stress responses and the NPS/NPSR1 system.

2.3.5.2 Polygenic score analysis

PGS are estimates of an individual's genetic disposition to a specific trait or liability to disease (Choi et al., 2020; Wray et al., 2021) assessing the cumulative effect of multiple effect or susceptibility alleles. PGS are computed as the sum of effect alleles of an individual, weighted by the allele's effect size estimated by an independent GWAS on the targeted phenotype. Shortly summarized, summary statistics of a GWAS (base data) are used to calculate PGS for each subject in an independent study sample (target data) to ultimately perform PGS-phenotype associations within the target sample and predict the outcome variable (Choi et al., 2020). When performing PGS analyses, certain factors have to be considered as they may impact the results, the number of SNPs to include, how to control for linkage disequilibrium (LD) between the SNPs and if applicable the shrinkage of the estimated effect size to approximate true effect size of the SNP (Choi et al., 2020; Mills et al., 2020a, c). The decision regarding the number of SNPs for example leads to a trade-off between prediction and the inclusion of noise and noncausal SNPs and depends on the specific research question (Mills et al., 2020c).
The PGS approach is the only one aggregating the effects of many SNPs into a single score providing a quantitative measure of genetic disposition for a phenotype at the individual level. To date, no currently available PGS can predict the behavioral outcome of any specific individual accurately (Martin et al., 2019a). However, PGS explain more variance in disease risk than single SNPs and predictive accuracy improves with increasing sample size of the underlying GWAS (Belsky and Harden, 2019; Dudbridge, 2013; Maier et al., 2018; Wray et al., 2014). Furthermore,

the approach has several useful applications despite prediction (Harden and Koellinger, 2020; Janssens, 2019; Kullo et al., 2022), including the identification of shared genetic architecture among traits (Andersen et al., 2017), the investigation of gene-environment and gene-gene interactions (Agerbo et al., 2015; Colodro-Conde et al., 2018; Iyegbe et al., 2014), the studying of mediating effect of environmental factors on gene-phenotype associations (Wertz et al., 2019) and the conducting of patient stratification and sub-phenotyping (Mavaddat et al., 2019; Werner et al., 2022).

Particularly in the context of GxE studies, the PGS approach augmented the power to detect GxE effects (Iyegbe et al., 2014). Related to stress, the usage of depression and neuroticism as phenotypes seem particularly promising. Stress exposure, as for example negative family relationships, child maltreatment, and low socioeconomic status, was identified as one of the main risk factors for depression (Dahl et al., 2017; Dunn et al., 2015; Hammen, 2005). Regarding neuroticism, there is evidence that it is highly correlated to stress sensitivity (McCrae, 1990) and it was shown to be a significant risk factor for several stress-related disorders (Kotov et al., 2010; Schneider et al., 2012). Moreover, research confirmed a considerable genetic component for both phenotypes (Polderman et al., 2015; Sullivan et al., 2000; Vukasović and Bratko, 2015) and large scale GWAS with sufficient power are available (Howard et al., 2019; Nagel et al., 2018a).

In stress research, several studies have already applied PGS to unravel the interplay between genetic and environmental factors, mainly childhood trauma and stressful life events, underlying stress-related disorders albeit with inconsistent results (Arnau-Soler et al., 2019; Colodro-Conde et al., 2018; Lehto et al., 2018; Li et al., 2019b; Mullins et al., 2016). Regarding the genetic disposition to depression, studies investigated the predictive value of the interaction between a PGS for depression and stressful life events for depression symptoms. Some studies reported significant PGS-stress interactions (Arnau-Soler et al., 2019; Coleman et al., 2020; Fang et al., 2020; Mullins et al., 2016), whereas others failed to find a meaningful interaction (Musliner et al., 2021; Musliner et al., 2015; Peyrot et al., 2018). Again, these mixed results can probably be explained by methodological differences, above all due to the retrospective assessment of the environmental variable as well as the mostly poor characterization of the phenotype of interest and the cross-sectional design. First longitudinal studies investigating the predictive value of a PGS for depression on depression symptoms under chronic stress conditions or after a traumatic event revealed significant associations (Domingue et al., 2017; Fang et al., 2020; Lobo et al., 2021). In regard to the genetic disposition to neuroticism, it was found that Neuroticism-PGS was associated with depression symptoms and depression diagnosis (Li et al., 2019b; Rietschel et al., 2017). The latter was partially mediated by stressful life events (Li et al., 2019b). Another GxE study revealed that Neuroticism-PGS was associated with depression only in twins who

were reared together and not in twins who were reared apart (stress condition; Lehto et al., 2018). Only few attempts were made to predict other phenotypes than depression or depression symptoms as for example sleep difficulties, hair cortisol or stress-related alterations in biological markers (Guffanti et al., 2019; Rietschel et al., 2017; Stephan et al., 2020).

The possibility to use PGS for the prediction in richly phenotyped, smaller data sets in contrast to the commonly performed prediction of categorial disease status seems promising to unravel genetics underlying the stress response (Harden, 2021). Especially the combination of PGS and AA provides and interesting approach to investigate whether individuals with genetic disposition to depression or neuroticism show distinct stress reactions (see section 2.4). The PGS approach is applied in Chapter 5 to examine the association between chronic stress responses in daily life and the genetic disposition for depression and neuroticism, respectively.

To sum up, the GSA aims at uncovering genes that are associated with the phenotype by testing the joint effect of multiple SNPs that are biologically or functionally related. PGS provide estimates for the genetic disposition to a specific trait or disease at the individual level which can be applied easily in further analyses. These approaches can complement previous candidate gene studies and large-scale GWEIS and GWAS to unravel the genetic underpinnings of stress regulation.

2.4 Ambulatory assessment

Methods such as ecological momentary assessment (Stone and Shiffman, 1994), experience sampling method (Larson and Csikszentmihalyi, 2014) or ambulatory assessment (AA; Trull and Ebner-Priemer, 2014) serve to examine individuals in their natural environment. These methods often comprise a multimodal data collection, as for example the assessment of self-reported experience and behavior, as well as biological and physiological parameters (Ebner-Priemer and Kubiak, 2010). Studying individuals in real-life is not a new idea, however, the increasing digitalization opened up new opportunities and allowed the AA to become increasingly popular (Trull and Ebner-Priemer, 2020). Trull and Ebner-Priemer (2020) summarized several important characteristics of the AA methodology, which highlight the main differences to traditional assessment methods (e.g., questionnaires, interviews, and laboratory tasks). One of the main advantages is that AA designs are characterized by intensive measurement repetition resulting in higher reliability and the possibility to examine within-processes and dynamic changes of mental states over time (Shiffman et al., 2008). Furthermore, due to real-time measurements the AA captures momentary experiences and reduces retrospective bias. Thus, AA enables to examine not only dynamic mood processes but also potential influencing factors (Reichert et al., 2021).

Finally, as AA is conducted during the daily life of the individuals, it is characterized by an increased ecological and external validity (Conner, 2015; Trull and Ebner-Priemer, 2014).

Several studies illustrating the usefulness of the AA design for behavioral stress research are already available (Schlotz, 2019). However, AA methods measuring the so called 'experiencing self' cannot substitute questionnaires assessing retrospective experiences and behavior. Both methodologies measure different facets of the same construct and the decision for one or the other method depends on the specific research question (Conner and Barrett, 2012; Shiffman et al., 2008). It was shown that momentary assessments were more strongly associated with stress-related biological processes than retrospective and trait self-report measures (Conner and Barrett, 2012). The lack of significant covariation of stress indicators, as for example perceived stress and cortisol concentration, is a well-known phenomenon (Campbell and Ehlert, 2012; Fahrenberg, 1979). However, first AA studies found that salivary cortisol levels collected throughout the day were associated with momentary negative affect (Jacobs et al., 2007; Schlotz, 2019). We hypothesized that reliable associations between stress indicators can be found when appropriate methods are applied. In Chapter 3 we investigated the association between self-reported momentary stress assessed via AA and the CAR during a long-lasting stress phase.

Furthermore, the AA method was proposed to serve as intermediate phenotype in the search for genetic factors associated with psychopathology (Assary et al., 2018; Fox and Beevers, 2016). Intermediate phenotypes are assumed to have a closer proximity to biological gene function compared to categorial and heterogenous psychiatric diagnoses. Thus, they probably show stronger genotype-phenotype association and might facilitate the uncovering of genetic effects (Gottesman and Gould, 2003; Meyer-Lindenberg and Weinberger, 2006). First studies applying AA in single candidate gene studies (Cristóbal-Narváez et al., 2017; Gunthert et al., 2007; Pishva et al., 2014; Russell et al., 2016; Sicorello et al., 2020; Simons et al., 2009; van Roekel et al., 2018; van Winkel et al., 2014; Wichers et al., 2008), twin studies (Jacobs et al., 2006) as well as in combination with the PGS approach (Monninger et al., 2022; Pries et al., 2020; Schick et al., 2022) already yielded promising results and revealed interesting insights into the possible value of the AA approach in uncovering genetic underpinndings. The possibility to measure behavioral and stress response patterns in daily life allows to examine potential underlying behavioral mechanisms of stress-related psychopathology (Reichert et al., 2021). The investigation of within-person associations and alterations over the time period as well as daily fluctuations can complement cross-sectional studies investigating associations between genetic factors and psychopathology assessed with questionnaires or clinical diagnoses. Furthermore, the AA method potentially reduces the measurement error in the phenotype of interest as the psychological

state is assessed at high frequency and proximal in time to the immediate experience (Trull and Ebner-Priemer, 2014; van Winkel et al., 2014). Additionally, the effective sample size is increased due to the repeated measurements probably resulting in an enhanced power (Assary et al., 2018). Schick et al. (2022) investigated whether the association between momentary stress and psychotic experiences were modified by a PGS for schizophrenia in three different groups, namely subjects with enduring non-affective psychotic disorder, first-degree relatives of these subjects, and a control group. They found that PGS was associated with psychotic experiences in response to minor daily stressors depending on the group. The results show that genetic disposition seems to be related to stress response patterns in daily life and might elucidate mechanisms ultimately leading to psychopathology. Another study found that the genetic risk for schizophrenia and the quantity of social contacts were associated with positive affect in daily life (Monninger et al., 2022). Participants with a low PGS seem to profit from social contacts during the COVID pandemic whereas participants with a high genetic disposition showed no benefit regarding their positive affect.

These findings illustrate how the combination of AA methods with different genetic approaches may advance our knowledge of the interplay between genes and the environment in momentary mental well-being and behavior and provide new insights into the mechanism and development of stress-related disorders. Despite the advantages and possibilities of the AA method, only few studies have been conducted and mainly single candidate genes were investigated. More studies are needed combining AA with genome-wide approaches, as for example PGS analyses.

To sum up, the AA method enables an improved depiction of phenotypic architecture, including ecologically valid assessment of current stress-relevant experience, biological indicators as well as within variation during daily life and can thus, possibly serve excellently as an intermediate phenotype in genetic studies (Fox and Beevers, 2016).

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 (e.g., O'Connor et al., 2021; 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 stress group (SG), while law students, who did not experience this specific

stress period, constituted the control group (CG; see https://doi.org/10.5283/epub.51920 for additional information).

In the present manuscript, we focus on the cortisol awakening response (CAR) 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), post-traumatic 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 (AA), 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 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 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

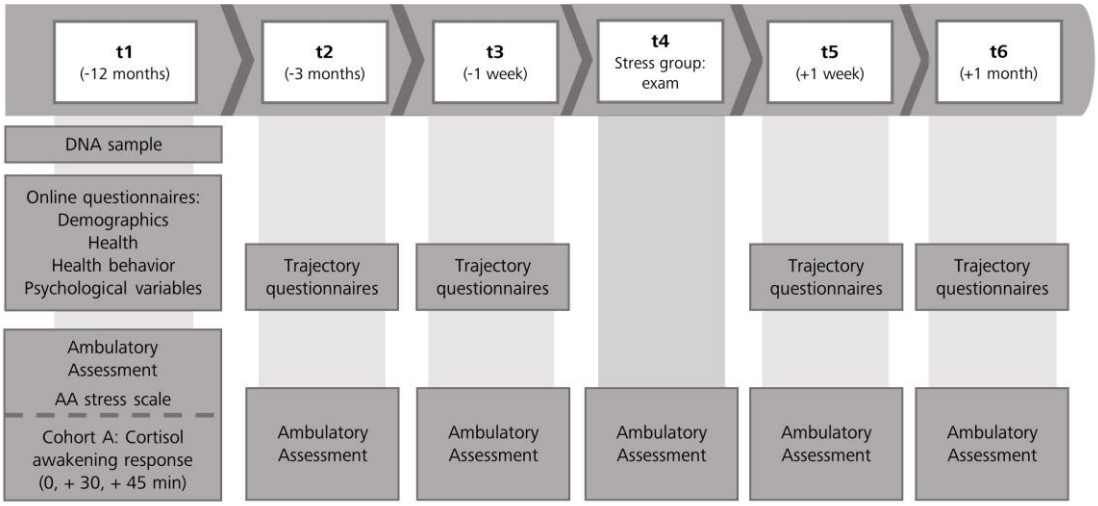
work. The study was approved by the local ethics committee. All participants provided written informed consent and received monetary compensation as well as a feedback report on their individual study results.

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 8 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, 2014) 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 functional magnetic resonance imaging (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 3.3.3). In the present manuscript, only AA data (including the CAR) and questionnaire data are presented (Figure 1).

Figure 1. Timing of data collection.



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 05:00 and 07:30 a.m. and the last one at 09:00 p.m. The remaining queries were presented at pseudo-randomized times between 08:30 a.m. and 08:00 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 07:30 a.m. and in the evening at 09:00 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 8.1.1 and Table 8). Additionally, in the first query after awakening, four items related to sleep (e.g., 'The quality of sleep last night was good ') 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 and the significance level was set at $\alpha = .05$. 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 et al., 2021) and MuMIn (Bartoń, 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 1. 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 1. 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 HC	*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. 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. HC = Hormonal contraception; *SD* = Standard deviation. * 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 2).

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 et al., 2008; Moreira de Sousa et al., 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 2; figures can be found on https://doi.org/10.5283/epub.51920).

Table 2. 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

Note. HADS = Hospital Anxiety and Depression Scale; RIS = Regensburg Insomnia Scale; TICS = Trier Inventory of Chronic Stress.

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 ($\pm$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 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 2 & supplementary Table 9). 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 2 (see supplementary Table 10 and 11).

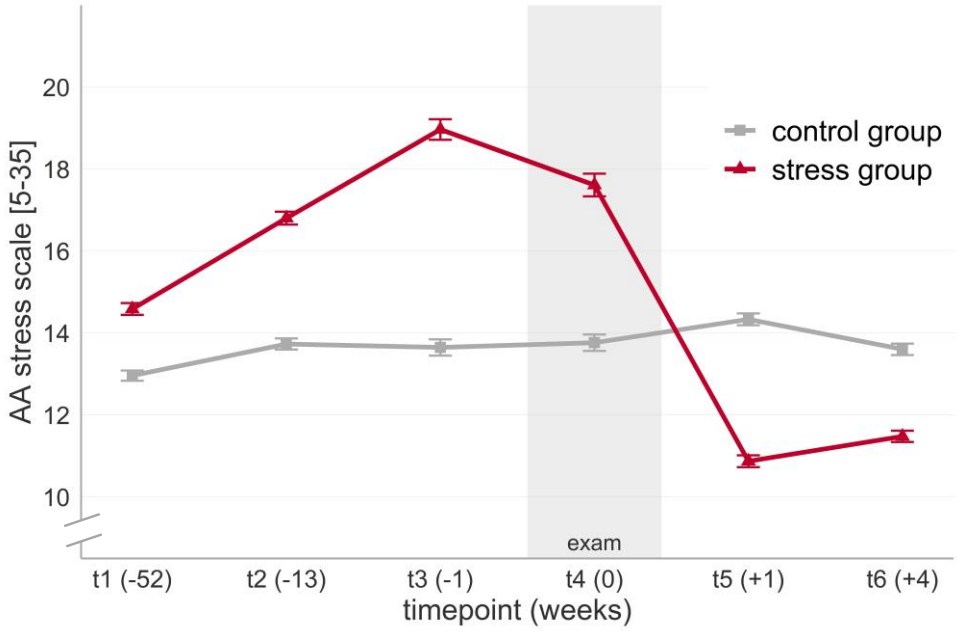


Figure 2. Time course of mean perceived stress levels (± SEM) in the stress group (SG) and the control group (CG) over the study period (cohort A).

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 3 & Table 3). 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 12 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 3. Parameter estimates for overall effects for the final group model (model 2).

Fixed Effects	Estimate	*SE*	*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. SE = Standard error; *SD* = Standard deviation; Min = Minutes after awakening; SG = Stress group; T = Timepoint; HC = Hormonal contraception.

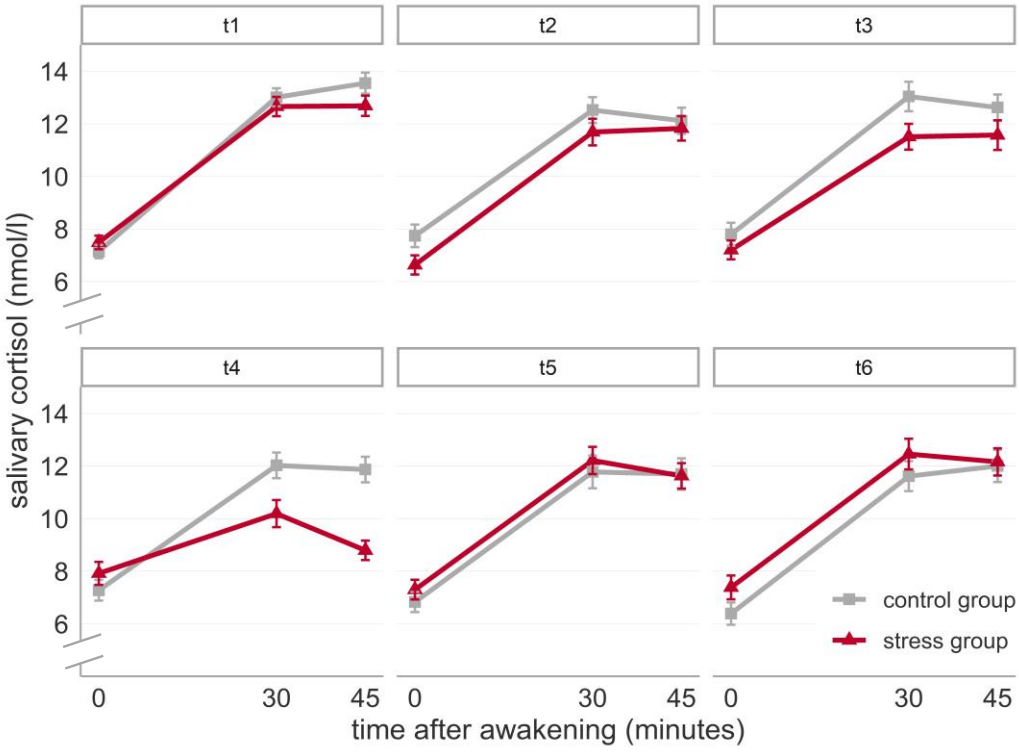


Figure 3. Mean cortisol values (± SEM) for the stress group (SG) and control group (CG) over the study period. *Note.* Timepoints: t1 (1 year before the exam), t2 (-3 months), t3 (-1 week), t4 (during exam in the stress group), t5 (+1 week), t6 (+1month).

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 13).

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 temporary 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., heterogenous 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 et al. (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 (Pruessner et al., 2003b; 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 The association between genetic variability in the NPS/NPSR1 system and chronic stress responses: a gene-environment-(quasi-) experiment

4.1 Abstract

The neuropeptide S (NPS) and its receptor (NPSR1) have been implicated in stress regulation and stress-related disorders. The present study aimed at investigating the association between overall genetic variability in the NPS/NPSR1 system and psychological and cortisol stress regulation in everyday life. Our study was conceptualized as a gene-environment-(quasi-) experiment, a design that facilitates the detection of true GxE interactions. As environmental variable, we used the preparation for the first state examination for law students. In the prospective and longitudinal LawSTRESS project, students were examined at six sampling points over a 13-months period. While students who prepared for the exam and experienced long-lasting and significant stress, formed the stress group, law students experiencing usual study-related workload were assigned to the control group.

As phenotypes we assessed changes over time in the cortisol awakening response (CAR; n = 176), perceived stress levels (n = 401), and anxiety symptoms (n = 397). The CAR was assessed at each sampling point immediately upon awakening and 30 as well as 45 minutes later. Perceived stress levels in daily life were measured by repeated ambulatory assessments and anxiety symptoms were repeatedly assessed with the anxiety subscale of the Hospital Anxiety and Depression Scale. With gene-set analyses we examined the joint association of 936 *NPS/NPSR1* single nucleotide polymorphisms with the phenotypes to overcome well known limitations of candidate gene studies.

As previously reported, we found a blunted CAR during the exam as well as significant increases in perceived stress levels and anxiety symptoms until the exam in the stress group, compared to the control group. The gene-set analysis did not confirm associations between genetic variability in the NPS/NPSR1 system and changes in perceived stress levels and anxiety symptoms. Regarding the CAR, we found a significant GxE interaction for the area under the curve with respect to the ground (p = .050) and a trend towards a significant effect for the area under the curve with respect to the increase (p = .054). When the analysis was restricted to the SG, associations for both CAR parameters were significant (ps < .050). This finding suggests that the association between genetic variability in the NPS/NPSR1 system and the CAR becomes visible under the environmental condition ‘chronic stress exposure’.

We conclude that the present study complements findings from animal models and that it provides novel evidence for a modulatory influence of the NPS/NPSR1 system on cortisol regulation in humans.

4.2 Introduction

Neuropeptide S (NPS) and its receptor (NPSR1) were suggested to play an important role in stress regulation and stress-related disorders, including anxiety and panic disorder (Ghazal, 2016; Tobinski and Rappeneau, 2021). NPS is predominantly synthesized in brainstem neurons (Xu et al., 2004). Projections of NPS neurons to the hypothalamic paraventricular nucleus (Grund et al., 2017) as well as the amygdala have been revealed (Clark et al., 2011). NPS exerts its effects via the G-protein-coupled NPSR1 (Xu et al., 2004) which is expressed throughout the brain including cortex, amygdala, thalamus and hypothalamus (Clark et al., 2011; Grund and Neumann, 2019; Xu et al., 2004). In animal models, NPS has strong anxiolytic and fear-attenuating effects (Jüngling et al., 2008; Xu et al., 2004). Investigating rodent strains selectively bred for high vs. low anxiety-related behavior illustrated the importance of genetic variability of the NPS/NPSR1 system (Slattery et al., 2015). The authors concluded that *Nps* and *Npsr1* sequence differences between the strains as well as expression differences partly underlie the high vs. low anxious behavioral phenotype. Moreover, the NPS/NPSR1 system modulates the neuroendocrine stress response (Tobinski and Rappeneau, 2021). NPS induced the release of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and corticosterone (Smith et al., 2006; Zhu et al., 2010). Reversely, NPS levels in the amygdala increased in response to an acute stressor (Ebner et al., 2011). Furthermore, NPS administration reduced stress-induced anxiety in rodents (Chauveau et al., 2012).

The NPS/NPSR1 system consists of two genes, namely *NPS* (chromosome 10q26.2) and *NPSR1* (chromosome 7p14.3). While medium-sized genetic case-control studies suggested the involvement of the NPS/NPSR1 system in anxiety disorders (Donner et al., 2010), this view could not be confirmed in recent large genome-wide association studies (Levey et al., 2020). In smaller studies on anxiety and stress-related phenotypes, particularly the functional *NPSR1* variant rs324981, a coding single nucleotide polymorphism (SNP; A>T Asn107Ile), was investigated. The more active T allele was linked to panic disorder (Domschke et al., 2011), increased anxiety sensitivity (Klauke et al., 2011), and an increased amygdala response to fear-relevant faces (Dannlowski et al., 2011). Furthermore, an association with salivary cortisol responses to acute stress was detected in male participants (Kumsta et al., 2013). Similar results were found by our group in a haplotype-based analysis (Streit et al., 2017). Overall, evidence from animal models and genetic association studies suggest that the NPS/NPSR1 system is a promising target for the investigation of neurobiological determinants of interindividual differences in chronic stress regulation (Tobinski and Rappeneau, 2021). However, while previous conclusions were often based

on only a few SNPs or even on only one, capturing the entire genetic variability would allow a more reliable assessment.

Moreover, improved genotyping should be combined with detailed phenotyping and an optimized study design. As stress regulation is the dynamic manifestation of a complex interaction between individuals and their environment, it appears plausible to consider gene-environment interactions (GxE). A design particularly suitable to find such effects is the GxE experiment as it potentially decreases measurement error within the environment component and reduces influences of unmeasured genetic effects on environmental exposure, which hinder the discovery of true GxE interactions (Bakermans-Kranenburg and van IJzendoorn, 2015; Leighton et al., 2017; van IJzendoorn et al., 2011). Additionally, GxE studies on stress regulation would benefit from prospective-longitudinal approaches and ecologically valid assessments of everyday life experiences. Fulfilling the prerequisites of an experimental or quasi-experimental design (i.e., with a group assignment based on preexisting differences) requires a cohort that will experience a long-lasting significant stress period in a predictable future period and an appropriate control group. Here, we propose that the preparation for the first state examination for law students constitutes a robust environment manipulation facilitating the investigation of GxE effects on chronic stress response indicators. This exam is considered one of the most stressful exam periods in the German university system. It consists of six written exams within eight days and students usually prepare for it for about one year. It can be repeated only once, has a failure rate of about 24% to 30% and the final mark is of crucial importance for the future career.

In the LawSTRESS project, students were investigated over a 13-months period enabling repeated assessments of our dependent variables. Perceived stress was assessed by ambulatory assessments (AA) allowing an ecologically valid recording of momentary stress-relevant experiences and a high reliability due to repeated real-time and real-life measurements (Trull and Ebner-Priemer, 2014). These measurements were combined with the collection of saliva samples after awakening to measure the cortisol awakening response (CAR). The CAR represents a distinct increase of cortisol levels in the first 30 to 45 minutes after awakening (Pruessner et al., 2003a; Stalder et al., 2016). The regulatory mechanisms of the CAR differ from basal diurnal secretion pattern, since it is evoked by morning awakening (Wilhelm et al., 2007). Twin studies consistently found a moderate heritability of the CAR (Wüst et al., 2000a). Various stress-related disorders, including major depression (Adam et al., 2010) and post-traumatic stress disorder (Wessa et al., 2006) are related to the CAR. Regarding the association between the magnitude of the CAR and perceived chronic stress, studies yielded mixed results (Chida and Steptoe, 2009). However, Law and Clow (2020) concluded that studies with more reliable methodologies predominantly found chronic stress to be related to an attenuated CAR. The present analysis aimed

at investigating the association between genetic variability in the NPS/NPSR1 system and psychological and endocrine stress regulation in everyday life over a 13-months stress period. More specifically, we expected an interaction effect of genetic variability and the environmental factor stress exposure (stress vs. control group) on the CAR, perceived stress levels, and anxiety symptoms. We assumed that genotype-phenotype associations should be particularly pronounced under stressful environmental conditions. We computed gene-set analyses (GSA) to test if SNPs in the NPS/NPSR1 system are jointly associated with the phenotype in a gene-environment analysis. GSA analyses do not allow for conclusions about single variants, therefore additional – and purely explorative – gene-wide single marker analyses for *NPS* and *NPSR1* were calculated. We stringently restricted our analyses to our *a priori* hypothesized outcome variables of interest (mentioned above) to limit the number of computed models and to thereby minimize the risks related to multiple comparisons.

4.3 Methods

4.3.1 Sample

In the LawSTRESS project, 470 law students from Bavarian universities were recruited. For the present analysis students taking part at least until timepoint 3 could be included (n = 420). Subsequently, during the quality control (QC) steps of the genetic data (see section 2.5.2) another 17 participants were excluded resulting in a total sample of 403 students. A detailed description of the recruitment process and the overall project was reported elsewhere (Giglberger et al., 2022 and https://doi.org/10.5283/epub.51920).

Students were recruited in two different cohorts. Each cohort consisted of a stress group (SG), experiencing a long-lasting and significant stress period, namely the preparation for the first state examination for law students, and a control group (CG) experiencing usual study-related workload. For the present analysis, cohort A comprised 182 students (SG: n = 84 and CG: n = 98) mainly from the University of Regensburg. Cohort B consisted of 221 students (SG: n = 110 and CG: n = 111) from other Bavarian universities. Cohort B underwent a less elaborate study protocol without laboratory visits to Regensburg; no CAR data was assessed, and a less extensive AA was applied (see section 2.3.1.). Apart from that, there were no further differences in study design or procedure between cohort A and B. Exclusion criteria in the LawSTRESS project were: self-reported current psychiatric, neurological, or endocrine disorders, treatment with psychotropic medications or any other medication affecting central nervous system or endocrine functions, or regular night-shift work. The study was approved by the local ethics committee. All participants provided written informed consent prior to participation and received monetary compensation and individual feedback.

4.3.2 General procedure

The study protocol comprised six sampling timepoints (t1 – t6) over 13 months (Figure 1). T1 for the SG took place one year before the exam since a pilot study revealed that most students begin their intensive preparation phase about a year prior to the exam date. The remaining timepoints were scheduled three months (t2) and one week (t3) prior to the exam, on the weekend during the eight days exam period (t4), as well as one week (t5) and one month (t6) after the exam. Except for the exam at t4, the same procedure applied to the CG. Data collection lasted from March 2018 until April 2021. At t1, exclusion criteria were checked and informed consent was signed. To obtain baseline data, psychometrics, physical health, health behavior and university studies-related variables an online questionnaire battery was sent out. Additionally, a buccal swab for DNA analysis was collected and the material and detailed description for the first ambulatory assessment (AA) was handed out. AA was also conducted at t2-t6. Moreover, a trajectory questionnaire was assessed at all timepoints except for t4 (Figure 1).

4.3.3 Acquisition of behavioral and endocrine data

4.3.3.1 Ambulatory assessment

A detailed description of the AA can be found in section 3.3.3. In brief, for cohort A (*n* = 182) the AA comprised an assessment of a five items stress scale (AA stress scale; Giglberger et al., 2022) 10 times a day (movisensXS; Versions 1.3.2 to 1.5.13; Karlsruhe, Germany) and the collection of three saliva samples after awakening. AA was conducted on two consecutive working days at t1, t2, t5 and t6. At timepoints t3 and t4, close to or during the examination period, the AA took place only on one day. The CAR was assessed on both sampling days at t1 and on the first day of each AA phase at the remaining timepoints. Saliva samples were collected immediately after waking as well as 30 and 45 minutes later using cortisol Salivettes® (Sarstedt, Nümbrecht). Saliva samples were stored at −20°C until analysis and analyzed 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).

In cohort B (*n* = 221), the AA was performed on one day per measurement timepoint. Perceived stress levels were measured with the AA stress scale in the morning at 07:30 a.m. and in the evening at 09:00 p.m. Questionnaires were presented online and had to be answered within 90 minutes.

4.3.3.2 Questionnaires

Anxiety symptoms were assessed online at t1, t2, t3, t5 and t6 with the anxiety subscale of the Hospital Anxiety and Depression Scale (HADS; Herrmann-Lingen et al., 2011). Demographic

variables (age, gender, etc.) were measured at t1. For assessed variables not included in the present report, please see Giglberger et al. (2022) and https://doi.org/10.5283/epub.51920.

4.3.4 Statistical analysis

4.3.4.1 Analysis of the trajectory of the variables over the time period

To examine the change in the CAR, perceived stress levels and anxiety symptoms over the time period we computed linear mixed models in order to account for the hierarchical structure of the data. A detailed description can be found in the supplementary methods (section 8.1.2). The results of the analyses described in this paragraph have already been presented in Giglberger et al. (2022). However, it should be noted that sample sizes for some of the models differ between the two reports. For the present report, CAR analyses contained a sample of *n* = 182, whereas the whole sample of *n* = 403 was included for the analysis of the AA stress scale and anxiety symptoms. In our previous paper, changes in the AA stress scale have been analyzed only in cohort A (*n* = 204; i.e., all participants with CAR data).

4.3.4.2 Phenotype variables for the genetic analyses

To assess changes in the CAR, the AA stress scale, and anxiety symptoms across measurement timepoints difference scores were computed. For each timepoint, the CAR was calculated with raw values as the area under the curve with respect to the increase (*AUCi*), representing the time-dependent change of cortisol in the morning, and the AUC with respect to the ground (*AUCg*), serving as measurement of the total hormonal output (Pruessner et al., 2003a). For the first measurement timepoint, we computed a mean of the *AUCi* and *AUCg* of day one and two. The alteration of *AUCi* and *AUCg* was then computed as the difference between t4 and t1 (*AUCi delta* and *AUCg delta*). For perceived stress levels, we computed a mean of the AA stress scale for each timepoint. To increase the homogeneity of AA stress scale measurements in cohorts A and B, for cohort A only the first query after awakening and the last query at 09:00 p.m. were used for the present analysis. The increase was defined as the difference between the individual peak (t3 or t4) and the baseline at t1 (*AA stress scale delta*). Since anxiety symptoms were not measured at t4, the increase in anxiety symptoms was defined as the difference between the value of the anxiety subscale of the HADS at t3, one week prior the exam, and t1 (*anxiety delta*).

4.3.5 Genetic analyses

4.3.5.1 DNA sampling and genotyping

We used buccal swabs (Analytik Jena GmbH, Jena) to brush exfoliated cells from the oral mucosa of the participants for a non-invasive DNA sampling. DNA isolation was carried out at the Genetic

Psychology Lab at the University of Bochum using a salting out procedure (Miller et al., 1988) with the Master Pure™ DNA Purification Kit (Epicentre). Buccal Swabs and DNA were stored at −20°C. Genotyping was performed in one batch using the Illumina Infinium™ Global Screening Array 3.0 with Multi-disease drop in (Illumina, San Diego, CA, USA) at the Life & Brain facilities, Bonn, Germany.

4.3.5.2 Quality control and imputation

Quality control of the data was performed with PLINK 1.9 (see www.cog-genomics.org/plink/1.9/; Chang et al., 2015). SNPs with minor allele frequency (MAF) of < .01, deviating from Hardy-Weinberg equilibrium (HWE) with a *p*-value of < 1e-6, and with missing data > .02 were removed. Participants were excluded in case of missingness > .02, sex-mismatch, and heterozygosity rate > |.20|. Filtering for missing rate of samples and SNPs was conducted iteratively. On a SNP set filtered for high quality (HWE $p > .02$, MAF > .20, missing rate = 0) and linkage disequilibrium pruning ($r^2 = .10$), filtering for relatedness and population structure was performed. In the case of relatedness (pi-hat > .20), one participant was excluded at random. To adjust for population stratification, principal components (PC) were computed. Outliers on any of the first 20 PCs (|z| > 4.5) were excluded. Genotyping was performed in 451 participants, in total 19 participants were excluded in these steps, 4 because of missing data, 3 due to sex discrepancy and cryptic relatedness, and 12 participants were detected as ancestry outliers and were removed. In a last step, data was checked for duplicate SNPs and one was retained at random. After quality control, we performed genotype imputation. Imputation was carried out with Eagle v2.4.1 (Loh et al., 2016) and Minimac4 (Das et al., 2016) using 1000 Genomes Phase3 v5 (Auton et al., 2015) as reference panel. For further analysis the estimated most likely genotype and only SNPs with an info score ≥ .90 were used (see supplementary methods section 8.1.3).

4.3.5.3 Gene-set analysis

To examine the association between the genetic variability in the NPS/NPSR1 system and alterations in the CAR, perceived stress levels, and anxiety symptoms, we performed gene-set analyses using MAGMA v1.08a (de Leeuw et al., 2015). For GSA, single variants were annotated to genes based on NCBI (37.3; Coordinators, 2017) including markers within 20 kilobase up- and downstream of the transcription region yielding 247 SNPs annotated to *NPS* and 689 SNPs to *NPSR1* (in total: 936 SNPs). In a gene-set analysis, multiple genetic markers are analyzed simultaneously to examine their joint association with the phenotype. First, MAGMA performs a gene-based analysis by aggregating the information of multiple individual markers assigned to the same gene and testing their joint effect on the phenotype. Secondly, single genes are

aggregated to sets of genes. We computed self-contained GSA, investigating whether the genes in the gene-set (*NPS* and *NPSR1*) are associated significantly at all with the examined phenotype, in contrast to default competitive analysis, comparing different gene sets.

In a first step, we performed a GxE GSA analysis (de Leeuw et al., 2015). Gene based analysis was performed on individual-level genotype data using the PC regression model with default settings. We added the interaction-term group (flag: --interact = group) and as covariates *sex* and the first five PCs (*PC 1-5*). Subsequently, self-contained GSA for the gene-set containing the two genes *NPS* and *NPSR1* were computed, followed by several *post-hoc* analyses. In the case of a significant GxE GSA, we computed separate self-contained GSA for SG and CG to break down the GxE interaction. Additionally, we performed a GSA with both groups together without the interaction term *group* for each phenotype to test if the genetic variability in the NPS/NPSR1 system was associated with the phenotype, independent of particular stress exposure. All gene analyses were carried out on the individual-level genotype data using the PC regression model with default settings and with the covariates *sex* and *PC 1-5*.

4.3.5.4 Gene-wide single marker analyses

While a gene-set analysis has substantial advantages, namely the reduced number of tests needed and the increased power, it is also a rather conservative approach that does not provide any information about individual variants. To extend the results of the GSA, explorative gene-wide single marker analyses were subsequently conducted associating the single *NPS*/*NPSR1* SNPs and changes in the measured stress parameters (the CAR, perceived stress levels, and anxiety symptoms). We computed the analyses using PLINK 1.9 (flags: -- assoc and -- linear) for the whole sample and for both groups separately. As covariates we added *sex* and *PC 1-5* in all analyses.

Additionally, we investigated whether some of the SNPs of the NPS/NPSR1 system, already mentioned in the literature, namely rs324981, rs727162, rs2530547 (Anedda et al., 2011; Streit et al., 2017), rs2530548, rs2530566, rs990310, and rs11018195 (Donner et al., 2010) showed an association with the phenotype (in the following referred to as 'previously associated (p.a.)' SNPs).

In accordance with the guidelines of 'Strengthening the reporting of genetic association studies' (STREGA; Little et al., 2009), we report detailed information on genotyping, quality control steps, how we dealt with population stratification and adequate descriptions of the sample and statistical methods. Investigated genes and variants have been selected *a priori*.

4.4 Results

4.4.1 Demographics

Demographic information of the sample can be taken from Table 4. Except for age ($t(401) = -10.91$, $p < .001$), none of the demographic variables differed significantly between SG and CG.

Table 4. Demographic characteristics of the total sample.

	Stress group	Control group
n	194	209
Age (Mean ± standard deviation)	22.93 (± 1.72)	21.03 (± 1.78)
Women	n = 146 (75.3%)	n = 161 (77.0%)
Women using hormonal contraception	n = 94	n = 101

4.4.2 Phenotype variables

As previously reported, the trajectories of the CAR, perceived stress levels, and anxiety symptoms differed significantly between SG and CG (Giglberger et al., 2022). As expected, the present analysis, which is partly based on different sample sizes (see section 2.4.1), yielded very similar results. Briefly, we found a significant increase of cortisol after awakening (*0 min b* = .79, $p < .001$; *30 min b* = .35, $p < .001$; *45 min b* = .36, $p < .001$) with no difference between the groups at the first timepoint (*SG x minutes ps* ≥ .278) and significant alterations in the SG compared to the CG over the 13-months period. During the exam, significantly lower mean cortisol values 30 and 45 minutes after awakening were observed in the SG (*SG x t4 x 30 min b* = -.07, $p = .046$; *SG x t4 x 45 min b* = -.10 $p = .004$). The trajectory of the AA stress scale was best represented by the model containing a cubic time trend (compared to the preceding model: linear model ΔAIC = 875.31; quadratic model ΔAIC = 500.70; cubic model ΔAIC = 65.25; see supplementary Table 15). At t1 no difference could be found between the SG and the CG (*SG b* = -.02, $p = .507$; see supplementary Table 14), whereas a significant difference was found between the trajectories of perceived stress levels (*timepoint x SG b* = .45, $p < .001$; *timepoint*^{2} *x SG b* = -.20, $p < .001$; *timepoint*^{3} *x SG b* = .02, $p < .001$). The SG showed an increase in mean perceived stress levels until t3 and a decrease thereafter. Regarding anxiety symptoms, the model with a quadratic trajectory represented the best fit for the data (linear model ΔAIC = 30.13; quadratic model ΔAIC = 122.29; see supplementary Table 17). No difference in anxiety symptoms at t1 could be found between the CG and the SG (*SG b* = -.03, $p = .627$), however they differed significantly over the observation period (*timepoint x SG b* = .30, $p < .001$; *timepoint*^{2} *x SG b* = -.06, $p < .001$). We observed an increase in anxiety symptoms in the SG until t3 and a decrease after the exam to

similar levels measured at t1 and in the CG (supplementary Figure 9 and Table 16). See Table 5 for descriptive statistics of the delta variables used in the gene-set and the gene-wide single marker analyses.

Table 5. Descriptive statistics of the aggregated variables for the cortisol awakening response, perceived stress levels, and anxiety symptoms.

	Stress group			Control group		
	Mean	*SD*	Min; Max	Mean	*SD*	Min; Max
AUCg delta (*n* = 176)	-77.67	200.82	-725.14; 498.65	-28.66	167.23	-450.86; 435.56
AUCi delta (*n* = 176)	-99.08	178.87	-759.61; 335.84	-37.53	158.56	-566.36; 407.91
AA stress scale delta (*n* = 401)	6.11	6.19	-7.50; 25.00	1.90	5.28	-14.00; 16.50
Anxiety delta (*n* = 397)	3.30	3.98	-6.00; 17.00	0.22	3.96	-13.00; 13.00

Note. *AUCi* and *AUCg* were only assessed in cohort A, whereas AA stress scale and anxiety symptoms were assessed in the whole sample (cohort A and B). *SD* = standard deviation; *AUCi/g* = area under the curve with respect to the increase/ground.

4.4.3 Genetic analyses

In the GxE gene-set analysis, the decrease in the cortisol awakening response from t1 until the exam at t4 was significantly associated with the NPS/NPSR1 gene-set for the *AUCg* (p = .050) and the association showed a trend towards significance for *AUCi* (p = .054). Subsequently, GSA were conducted separately for the CG and the SG to further elucidate this GxE interaction. These analyses revealed a significant association of the NPS/NPSR1 gene-set with *AUCi delta* and *AUCg delta* within the SG (*AUCi*: p = .006; *AUCg*: p = .029) but not within the CG (*AUCi*: p = .710; *AUCg*: p = .489). When both groups were tested without the environmental factor *group* as interaction term, only a trend for a significant effect was detected for *AUCg* (p = .075; *AUCi*: p = .116).

Explorative gene-wide single marker analysis for *AUCi delta* revealed several SNPs with low *p*-values for *NPSR1* in the analysis that included both groups (supplementary Figure A4) and especially within the SG (Figure 4), suggesting that these SNPs may show a tentative association with *AUCi delta*. Additionally, the 'previously associated' SNP rs324981 was suggestive of association (both groups: p = .001; SG: p = .017; Figure 4 and supplementary Figure 10). Again, due to missing power for single maker analysis and only marginally low *p*-values, these observations can just serve as illustration. Please see supplementary Tables of the original article for *p*-values of the SNPs of the gene-wide single marker analyses of both groups together, and for CG and SG separately. In *NPS* scarcely any SNP showed a noteworthy association with *AUCi delta*. 'P.a.' SNPs rs990310 and rs11018195 in *NPS* reached nominal significance in the stress group (both p =

.039). The remaining 'p.a.' SNPs showed no association with the change in *AUCi* (both groups: *ps* ≥ .276; SG: *ps* ≥ .260; CG: *ps* ≥ .127).

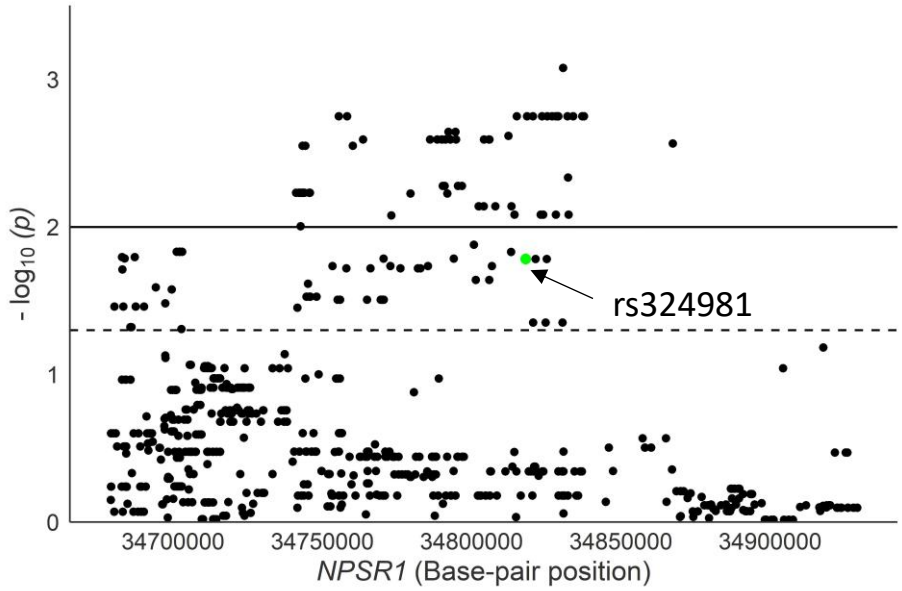


Figure 4. Plotted results of gene-wide single marker analysis for *NPSR1* and *AUCi delta* in the stress group (SG). *Note.* On the x-axis genomic coordinates of the single nucleotide polymorphisms (SNPs) are depicted and, on the y-axis, the negative logarithm of the corresponding *p*-value is displayed for each SNP. The horizontal lines indicate association thresholds: solid line: $p < .01$ and dashed line: $p < .05$. The SNP rs324981 is marked in green.

Regarding *AUCg delta*, the gene-wide single marker analysis in both groups and in the SG revealed few SNPs in *NPSR1* showing an association (Figure 5). In the CG, as well as in the analyses of the SNPs in *NPS* only very few variants were associated with the phenotype (supplementary Figure 11). None of the 'p.a.' SNPs showed an association with *AUCg delta* (both groups: *ps* ≥ .270; SG: *ps* ≥ .127; CG: *ps* ≥ .299).

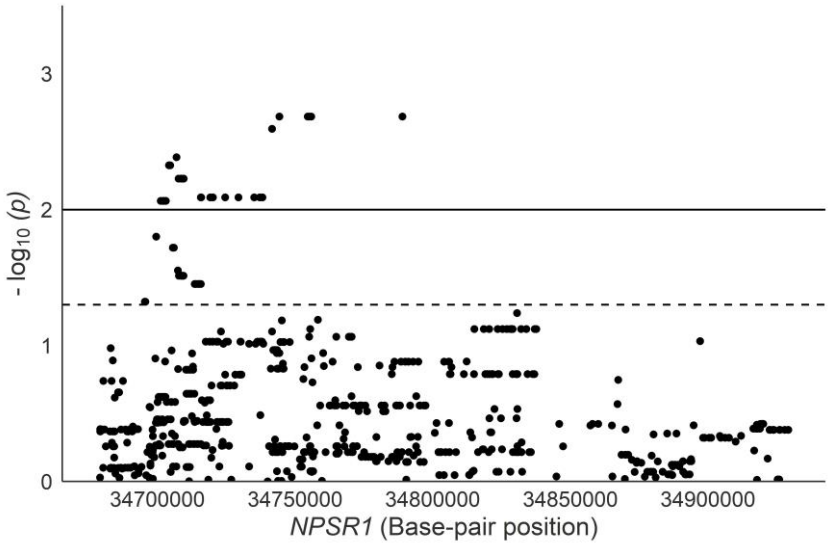


Figure 5. Plotted results of gene-wide single marker analysis for *NPSR1* and *AUCg delta* in both groups together. *Note.* On the x-axis genomic coordinates of the single nucleotide polymorphisms (SNPs) are depicted and, on the y-axis, the negative logarithm of the corresponding *p*-value is displayed for each SNP. The horizontal lines indicate association thresholds: solid line $p < .01$ and dashed line: $p < .05$.

The NPS/NPSR1 gene-set was not significantly associated with the change in perceived stress levels from t1 until the exam (GxE: $p = .136$; GSA: $p = .089$). Additional explorative analyses of the single markers in both groups and within the SG for *NPS* and *NPSR1* did not detect SNPs with particularly small *p*-values (supplementary Figure 12). The 'p.a.' SNPs that were inspected separately, showed no notable association with the *AA stress scale delta* (both groups: $ps \geq .429$; SG: $ps \geq .128$; CG: $ps \geq .233$). In *NPS* only few SNP showed an association with the *AA stress scale delta*.

We could not find any association of the NPS/NPSR1 gene-set with the change in anxiety symptoms over the observation period (GxE: $p = .593$; GSA: $p = .247$). The explorative gene-wide single marker analysis revealed only a small number of SNPs in *NPSR1* showing a weak association with *anxiety delta* (supplementary Figure 13). Visual inspection of the plots for the analysis of both groups and within the CG showed some SNPs, possibly weakly associated with *anxiety delta*. In *NPS* only one of the examined SNPs showed a nominal significant association with *anxiety delta*. None of the 'p.a.' SNPs showed an association with *anxiety delta* (both groups: $ps \geq .086$; SG: $ps \geq .417$; CG: $ps \geq .147$).

4.5 Discussion

The present analysis investigated the relation between chronic stress responses in everyday life and genetic variability in the NPS/NPSR1 system. We proposed that this system constitutes a promising target for our candidate gene study for at least two reasons. First, converging

evidence from animal models and studies in humans suggests its relevance for stress (Tobinski and Rappeneau, 2021). Second, sequence variation in only two genes needs to be assessed to capture the genetic variability in the NPS/NPSR1 system. Therefore, comprehensive evidence regarding the association between phenotypes of interest and genetic variability in a stress-related neuropeptide system can be obtained with a confined effort. Moreover, we assumed that our study design increased the *a priori* probability for reliable findings (see introduction). We are aware that the investigation of GxE interactions holds specific challenges, including the questions of how to assess the environment, how to avoid confounding due to gene-environment correlation, and the trade-off between the need for large sample sizes and precise assessment of (intermediate) phenotypes (Bakermans-Kranenburg and van IJzendoorn, 2015; Moffitt et al., 2005). These challenges have been proposed to be important reasons for inconsistent and non-replicated GxE findings. To partially overcome these difficulties, we conducted a (quasi-) experimental GxE study. GxE experiments with adequate control groups were suggested to increase the power of GxE analyses (Bakermans-Kranenburg and van IJzendoorn, 2015; van IJzendoorn et al., 2011). The experimental design reduces the measurement error and leads to more control of the E component. This is an important factor, as a better measurement of the environment may for certain research questions be more crucial than larger sample sizes (Wong et al., 2003). Furthermore, experimental approaches diminish uncontrollable correlations between genes and the environment. Previous experimental candidate gene studies focusing on single or only a few SNPs, already found genotype-intervention effects (Belsky and van IJzendoorn, 2015). For example, effects of an alcohol misuse intervention were found to be associated with a *GABRA2* SNP in the randomized PROSPER study (Russell et al., 2018). In another randomized controlled trial, intervention effects on behavioral problems of infants with attention deficit/hyperactivity disorder were reported to be linked to a dopamine transporter gene polymorphism (van den Hoofdakker et al., 2012). In addition to a (quasi-) experimental approach, we assessed stress-related phenotypes in everyday life on different psychobiological levels, including psychological variables measured via AA combined with a marker of cortisol regulation. AA measures psychological state at high frequency and proximal in time to the immediate experience, thus potentially reducing the measurement error of the outcome variable (Trull and Ebner-Priemer, 2014). As environment variable, we used (the preparation for) the first state examination for law students. Over the observation period we found significant increases in perceived stress levels in everyday life and anxiety symptoms in the stress group until the exam, whereas non-exam students stayed relatively stable. Furthermore, the SG showed, compared to the CG, a significantly blunted CAR at timepoint 4 during the examination days. Although both, an increased and an attenuated CAR, have been associated with chronic stress, it was suggested that studies with a

longitudinal design and rigorous methods are more likely to find a decreased CAR (Law and Clow, 2020). This could be confirmed by our results which were based on a longitudinal design, an appropriate baseline measurement and a control group. Further support comes from other studies investigating chronic examination stress with a longitudinal design (Duan et al., 2013; Koudela-Hamila et al., 2020). Moreover, one week after the exam we observed a quick recovery of the CAR. While the rapid regeneration of cortisol was unexpected and more research is needed to examine dynamic alterations in the CAR, it coincides with the trajectories of our psychological variables, namely perceived stress levels, anxiety, and depression. We assume that the blunted CAR at t4 can be interpreted as a temporary hypocortisolism in otherwise healthy young adults that might be of psychobiological relevance results are discussed in Giglberger et al. (2022).

Although in our GxE analysis we still focused on one candidate gene system, we conducted gene-set analyses to aggregate the genetic variation across 936 SNPs within the NPS/NPSR1 system and estimated their joint association with the phenotypes. The aggregation of single markers to genes reduces the number of tests, thereby improves power and enables to detect effects composed of multiple weaker SNP-phenotype-associations (de Leeuw et al., 2015). The analysis of the NPS/NPSR1 system and the two CAR parameters *AUCg* and *AUCi* revealed a GxE interaction. We found a significant association between genetic variability in the NPS/NPSR1 system and *AUCg* as well as a trend towards a significant association with *AUCi* in the GxE GSA. Consistent with our hypothesis, further *post hoc* investigation revealed a significant genotype-phenotype association for both parameters within the SG but not in the CG. This suggests that the association between genetic differences in the NPS/NPSR1 system and the CAR becomes visible under the environmental condition 'chronic stress exposure'. This is the most prominent finding of the present analysis, and it can be assumed that the heritability of the CAR is, to a certain extent, mediated by genetic variability in the NPS/NPSR1 system. As already stated, animal models demonstrated a close interaction of NPS and the hypothalamus-pituitary-adrenal (HPA) axis (Jüngling et al., 2012; Smith et al., 2006). However, direct evidence regarding this interplay in humans is scarce. Previously, we and others found that in male subjects the minor and more active T allele of the *NPSR1* rs324981 was associated with increased salivary cortisol responses to acute psychosocial stress induction (Kumsta et al., 2013). Similar results were found in a haplotype analysis covering three functional *NPSR1* SNPs (rs2530547, rs324981, rs727162; Streit et al., 2017). These findings and our results suggest that the NPS/NPSR1 system and the HPA axis also interact closely in humans and we assume that this interaction is more pronounced under stress. Moreover, it is known that the NPS/NPSR1 system is related to arousal, alertness as well as to the sleep–wake rhythm and that it plays a role in the regulation of morning awakening

(Kushikata et al., 2021). Therefore, as the CAR is supposed to be associated with the transition from sleep to wakefulness (Clow et al., 2010), it appears also possible that the NPS/NPSR1 system impacts the CAR via its interplay with these circuits in a stress-sensitive manner. Of course, this assumption is currently highly speculative.

Contrary to our assumptions, no association could be found between genetic variability in the NPS/NPSR1 system and changes in perceived stress levels and anxiety symptoms. A strong association between anxiety-related variables and the NPS/NPSR1 system was found in animal studies (Tobinski and Rappeneau, 2021). As already mentioned, evidence from research in humans mainly derived from studies on the association between *NPSR1* rs324981 and anxiety-related phenotypes. While medium-sized case-control studies found rs324981 to be related to panic disorder (Domschke et al., 2011; Donner et al., 2010) also healthy individuals have been investigated. For example, Klauke et al. (2011) found an association between anxiety sensitivity and rs324981 in healthy participants. Furthermore, they detected a significant GxE interaction of rs324981 and childhood maltreatment, but no consistent results regarding the impact of recent life events. In a recent study by Schiele et al. (2020), an interaction of rs324981 with retrospectively assessed childhood maltreatment and self-efficacy on trait anxiety was found but no genetic main effect on trait anxiety. The effect of childhood maltreatment on trait anxiety in A allele carriers was moderated by self-efficacy, whereas no effect on self-efficacy was found in TT homozygotes. Domschke et al. (2011) reported an association between anxiety sensitivity and the rs324981 in patients with panic disorder but not in healthy controls. It remains unclear why we failed to find an association with perceived stress levels and anxiety. In general, differences in sample structure, study design and outcome variables can certainly lead to inconsistent results. Moreover, in contrast to previous studies, the present investigation explored the association between overall genetic variability in the NPS/NPSR1 system and anxiety symptoms. As we particularly aimed at minimizing the risk of false positive findings, gene-set analyses have been applicated. This conservative strategy could possibly explain why we could not confirm each association which was found with single SNPs in previous studies.

The GSA approach is a promising, robust, and conservative alternative to multiple single candidate SNP analyses (Windhorst et al., 2016) and the usage of this method substantially contributes to our confidence in the reliability of the detected GxE interaction. However, the approach does not generate information about the type of association or the single variants. Therefore, we combined the GSA with explorative gene-wide single marker analysis. Though we are aware that the sample size of our study is too small to conduct valid gene-wide single marker analyses, we assume that it allows us to obtain some preliminary indications, which might be useful for further research. The gene-wide single marker analyses revealed some SNPs possibly associated

with *AUCi delta* and *AUCg delta*. Interestingly, in the analysis for the *AUCi delta* the 'p. a.' SNP rs324981 was among those SNPs. We have to emphasize again that the results should be interpreted with caution and need further replication. The depicted threshold lines in the figures only serve as rough orientation and illustration.

Our study has some limitations that need to be considered. First, compared to the general population, our study sample was relatively young and had presumably above-average intelligence and socioeconomic status. Hence, the generalizability of our findings is limited. Additionally, like any other study that investigates stress in real life, we cannot rule out a certain selection bias. Students who felt particularly stressed and who expected a particularly stressful exam preparation phase did possibly not participate as this was related to additional burden. An obvious limitation of our (quasi-) experimental design was the fact that a randomized assignment to the stress and the control group was not possible. To outweigh this issue, our CG consisted of individuals who were as similar as possible to our SG participants. This was a rather conservative strategy, since students in this group also had a substantial workload and experienced study-related stress which may have caused underestimation of group differences. Regarding the CAR, an additional assessment 60 minutes after awakening might have helped to broaden our understanding of dynamic changes in morning cortisol concentrations. Moreover, the sample size of our study has to be discussed. Although some features of our study design presumably increased the power and although a conservative analysis strategy has been applied to reduce the risk of false positive findings, our cohort was relatively small. Our findings need to be replicated in an independent sample. Moreover, assigning the SNPs to *NPS* and *NPSR1* we applied a commonly used window size of 20 kb up- and downstream the transcript region, but it should be noted that a different window might have altered the results.

To conclude, we would like to point out that the LawSTRESS project was conceptualized as a study which can be used to assess the association between responses to chronic stress exposure in everyday life and genetic variability. *A priori* neuropeptide S and its receptor have been selected as target system. Without any doubt, the present candidate gene analysis has certain weaknesses. Particularly, the sample size restricted the options for genetic statistical modeling. However, the study has also particular strengths including the prospective, longitudinal, and (quasi-) experimental design, the extensive phenotyping and the measuring of the overall genetic variability in the NPS/NPSR1 system. Therefore, we conclude that our study contributes to research on the interaction of the NPS/NPSR1 system and stress regulation in humans. Our analysis could not confirm previously reported associations of genetic variability in the NPS/NPSR1 system and changes in perceived stress levels and anxiety symptoms. However, with a conservative statistical method, we found a significant gene-environment interaction, suggesting that

changes in the cortisol awakening response in individuals exposed to chronic stress are associated with genetic variability in the NPS/NPSR1 system. This finding does not necessarily contradict the assumption that NPS influences cortisol regulation also under resting conditions but it suggests that the relative size of the assumed modulatory effect may increase when the system is under long-term challenge.

Chapter 5

5 Association of polygenic scores for depression and neuroticism with perceived stress in daily life during a long-lasting stress period

5.1 Abstract

Genetic factors contribute significantly to interindividual differences in the susceptibility to stress-related disorders. As stress can also be conceptualized as environmental exposure, controlled gene-environment interaction (GxE) studies with an in-depth phenotyping may help to unravel mechanisms underlying the interplay between genetic factors and stress.

In a prospective-longitudinal quasi-experimental study, we investigated whether polygenic scores (PGS) for depression (DEP-PGS) and neuroticism (NEU-PGS), respectively, were associated with responses to chronic stress in daily life. We examined law students (*n* = 432) over 13 months. Participants in the stress group experienced a long-lasting stress phase, namely the preparation for the first state examination for law students. The control group consisted of law students without particular stress exposure. In the present manuscript, we analyzed perceived stress levels assessed at high frequency and in an ecologically valid manner by ambulatory assessments as well as depression symptoms and two parameters of the cortisol awakening response. The latter was only assessed in a subsample (*n* = 196).

No associations between the DEP-PGS and stress-related variables were found. However, for the NEU-PGS we found a significant GxE effect. Only in individuals experiencing academic stress a higher PGS for neuroticism predicted stronger increases of perceived stress levels until the exam. At baseline, a higher NEU-PGS was associated with higher perceived stress levels in both groups. Despite the small sample size, we provide preliminary evidence that the genetic disposition for neuroticism is associated with stress level increases in daily life during a long-lasting stress period.

5.2 Introduction

Differences in the susceptibility to mental disorders can in part be explained by genetic factors (Caspi and Moffitt, 2006; Pluess, 2015). Regarding depression, twin studies have estimated the heritability to range between 30% and 40% (Kendler et al., 2006; Sullivan and Geschwind, 2019; Sullivan et al., 2000) and recent large-scale genome-wide association studies (GWAS) have identified several related genomic loci (Howard et al., 2019; Levey et al., 2020). Similarly, numerous loci have been found to be associated with neuroticism (Luciano et al., 2018; Nagel et al., 2018a), a personality trait which is also a risk factor for mental disorders (Kendler and Myers, 2010; Kotov et al., 2010; Prince et al., 2021). In twin studies the heritability of neuroticism was found to be around 40% (Jang et al., 1996; Vukasović and Bratko, 2015). Overall, recent GWAS confirmed the hypothesized polygenic nature of complex traits and common disorders, with each associated genetic variant being characterized by a very small effect size (Duncan and Keller, 2011; Howard et al., 2019; Nagel et al., 2018a; Visscher et al., 2017).

Identification of genetic main effects and of gene variants forming the molecular basis of these effects are important goals of genetic psychiatry. Moreover, gene-environment interaction (GxE) effects are of substantial interest as well (Assary et al., 2018; Musci et al., 2019; Uher and Zwicker, 2017). At this point, an interesting overlap emerges between genetics and stress research. Modern stress concepts define stress as a transactional relationship between individuals and their environment (Lazarus and Folkman, 1984). Nevertheless, stress - or in the narrow sense 'stressors' - can also be perceived as a significant environmental exposure, which is known for decades to increase the risk for several physical as well as mental disorders including depression (Chrousos, 2009; Galatzer-Levy et al., 2018; Kivimäki et al., 2015a; Kivimäki et al., 2015b; Madsen et al., 2017; Nillni et al., 2013). Furthermore, neuroticism is related to stress sensitivity. Individuals with high levels of neuroticism perceive life as more stressful and report a higher negative affect in response to stress (Lahey, 2009; McCrae, 1990; Schneider et al., 2012).

The majority of past GxE studies related to stress research investigated single candidate genes (Assary et al., 2018; Musci et al., 2019; Sharma et al., 2016; Smoller, 2016). However, in the last decade a growing number of first genome-wide by environment interaction studies (GWEIS; Arnau-Soler et al., 2018; Werme et al., 2021) as well as polygenic score (PGS) analyses studies emerged. PGS are estimates of the genetic disposition to a specific trait at the individual level. The effects of many common SNPs are aggregated to account for the polygenic nature of stress-related disorders and complex behavior (Choi and O'Reilly, 2019; Wray et al., 2014). PGS are estimated as the sum of effect alleles weighted by the corresponding estimated effect size of this allele derived from a respective GWAS on the examined trait (Choi and O'Reilly, 2019). This

approach enables to estimate the genetic disposition to a specific trait across the whole genome at the individual level. PGS analyses are an interesting approach to combine psychological and genetic research and to examine GxE effects with more predictive power than single candidate SNP analyses (Dudbridge, 2013; Harden, 2021; Iyegbe et al., 2014). Studies applying the PGS approach to investigate the interplay between genetic and different environmental factors, mainly examined childhood trauma and stressful life events (Fang et al., 2020; Mullins et al., 2016; Musliner et al., 2021). So far, results have been mixed (Arnau-Soler et al., 2019; Coleman et al., 2020; Domingue et al., 2017; Fang et al., 2020; Mullins et al., 2016; Musliner et al., 2021; Musliner et al., 2015; Peyrot et al., 2018). These inconsistent results probably arise from methodological differences and lack of power. Most of the studies focused on retrospective assessments of stressful life events or childhood maltreatment, an approach which is important but at the same time known to be susceptible to recall bias and cognitive errors (Colman et al., 2016; Monroe and Reid, 2008; Zammit and Owen, 2006). If feasible, prospective-longitudinal designs are preferable as they have the potential to uncover causal relationships between stress exposure and alterations in psychobiological systems or disease vulnerabilities. In our view, the combination of such a design with methods like ambulatory assessment (AA) which enables the ecologically valid recording of momentary experience and behavior (Trull and Ebner-Priemer, 2014) is a promising approach to overcome some difficulties of previous studies (Fox and Beevers, 2016). Moreover, AA offers a high reliability due to repeated real-time and real-life measurements and was proposed to provide higher sensitivity for examining the interplay between psychological and biological processes (Conner and Barrett, 2012; Trull and Ebner-Priemer, 2014). First studies investigating associations between PGS and carefully assessed phenotypes obtained promising results (Monninger et al., 2022; Pries et al., 2020; Schick et al., 2022). Schick et al. (2022) investigated 248 subjects and found that a PGS for schizophrenia (SCZ-PGS) was associated with psychotic experiences in response to minor daily stressors. Another study, investigating 70 subjects with AA, reported that a SCZ-PGS and the quantity of social contacts were associated with positive affect in daily life (Monninger et al., 2022). These studies document the usefulness of PGS analyses in studies with smaller sample sizes and highlight the importance to investigate the association between genetic factors and precisely assessed (intermediate) phenotypes to understand mechanisms involved in the etiology of psychiatric disorders and stress regulation. Conceptually, a thorough phenotyping may increase the size of the effect of interest. However, it should be noted that phenotyping quality can surely not fully compensate for the lack of power in studies with small samples.

Our prospective-longitudinal quasi-experimental LawSTRESS project aimed at identifying predictors of chronic stress responses in daily life to unravel molecular mechanism of stress

regulation and interindividual differences (Giglberger et al., 2022). Besides psychological and neural factors, the identification of genetic predictors was of special interest. The main objective of the genetic study arm was to perform gene-set analyses to examine the association between chronic stress responses and the overall genetic variability of the neuropeptide S (NPS) system, consisting of the genes for NPS and its receptor (NPSR1; Peter et al., 2022a). Our previous analyses did not confirm associations between genetic variability in the NPS/NPSR1 system and perceived stress levels or anxiety symptoms. However, we found a significant association with alterations of salivary cortisol regulation, in particular under the environmental condition 'chronic stress exposure'. The aim of the present analyses was to expand this candidate gene approach and to conduct secondary exploratory PGS analyses. We investigated the associations between a PGS for depression (DEP-PGS) and neuroticism (NEU-PGS), respectively, and three stress-related phenotypes, namely perceived stress levels, depression symptoms and cortisol regulation, assessed repeatedly over the 13 months observation period. Law students were examined while preparing for their first state examination which is considered one of the most stressful exam periods in the German university system. In Bavaria, this exam consists of six written exams of several hours each within eight days, it can be repeated only once, has a failure rate of about 24% to 30%, and the final mark is of crucial importance for the future career of the candidate. Additionally, we assessed an adequate control group, consisting of law students in earlier semesters experiencing usual study-related workload. Especially, perceived stress levels measured at high frequency with AA in 432 participants represent an interesting in-depth phenotype which complements previous studies using categorial phenotypes (Mullins et al., 2016; Musliner et al., 2021). The AA was combined with assessments of the cortisol awakening response (CAR). The CAR is characterized by a sharp increase of cortisol concentrations in the first 30 to 45 minutes after morning awakening (Pruessner et al., 2003a; Stalder et al., 2016). Regulatory mechanisms of the CAR partly differ from the basal diurnal secretion pattern as it is evoked by morning awakening (Wilhelm et al., 2007). A moderate heritability of the CAR was consistently found in twin studies (Kupper et al., 2005; Wüst et al., 2000a). Besides the repeated measurement of the stress related variables and the detailed phenotyping, the (quasi-) experimental design of our study holds further advantages for the investigation of GxE effects. To a certain degree, it reduces the measurement error in the environmental component and diminishes the uncontrollable influence of gene-environment correlations, which hinders the discovery of true GxE interactions (Bakermans-Kranenburg and van IJzendoorn, 2015; van IJzendoorn et al., 2011).

The investigation of the genetic disposition to depression and neuroticism seems promising for several reasons. Besides the high relevance of depression and neuroticism in stress research, large-scale GWAS for both phenotypes are available, enabling to compute PGS with substantial

power (Howard et al., 2019; Nagel et al., 2018a). Furthermore, although both phenotypes are highly correlated, genetically as well as phenotypically, they also have distinct genetic influences (Adams et al., 2020; Kendler et al., 2006; Luciano et al., 2018). Thus, we assume that they complement each other in the search for genetic predictors of stress responses during a long-lasting stress phase as the PGS for depression captures the genetic disposition to develop a clinical depression whereas the NEU-PGS probably has a broader range and stronger overlap with stress reactivity.

We were especially interested in the GxE effect of the DEP-PGS as well as the NEU-PGS in combination with the environmental variable 'chronic examination stress'. The main hypothesis was that the DEP-PGS and the NEU-PGS predict perceived stress levels which were assessed at high frequency with AA over the observation period. We expected this association particularly in the stress group, experiencing chronic academic stress. Furthermore, we investigated whether alterations in depression symptoms and different parameters of the cortisol awakening response were associated with genetic disposition for depression and neuroticism, respectively.

5.3 Materials and Methods

5.3.1 Sample

In the LawSTRESS project, we recruited 470 law students from Bavarian universities. Genetic data were analyzed for 451 participants who completed at least the first sampling timepoint. Another 19 participants were excluded during the quality control (QC) steps of the genetic data (see section 2.4) resulting in a final sample of 432 students for the following analyses. For a detailed sample description of the total sample, health and university study-related information, and trajectories of several psychological questionnaires, please see https://doi.org/10.5283/epub.51920 and Giglberger et al. (2022).

Two different cohorts were recruited, each consisting of a stress group (SG), experiencing a long-lasting and significant stress phase, namely the preparation for the first state examination for law students, and a control group (CG) experiencing usual study-related workload. Cohort A consisted of 196 students (SG: n = 95 and CG: n = 101) mainly from the University of Regensburg. Cohort B comprised 236 (SG: n = 123 and CG: n = 113) law students from other Bavarian universities who underwent a modified examination protocol (less extensive AA, no CAR data; see section 2.3.1).

Exclusion criteria were: (self-reported) current psychiatric, neurological, or endocrine disorders, treatment with psychotropic medications, any other medication affecting central nervous system or endocrine functions, or regular night-shift work. The study was approved by the local

ethics committee. All participants provided written informed consent and received monetary compensation and individual feedback.

5.3.2 General procedure

As reported elsewhere (Giglberger et al., 2022), the study comprised six sampling timepoints (t1 – t6) over 13 months (Figure 1). T1 for the SG took place one year before the exam; the remaining timepoints were scheduled three months (t2) and one week (t3) prior to the exam, on the weekend during the eight-days exam period (t4), as well as one week (t5) and one month (t6) after the exam. For the CG the same procedure applied, except that there was no exam at t4. Data collection lasted from March 2018 until April 2021. At t1, exclusion criteria were checked and written informed consent obtained. An online questionnaire battery to inquire baseline data, psychometrics, physical health, health behavior, and university studies-related variables was sent out. Furthermore, participants received the material and detailed description for the first AA and a buccal swab for DNA analysis was collected. At t2 - t6 the AA was conducted. Moreover, a trajectory questionnaire was assessed at all timepoints except for t4 (Figure 1), comprising health, health behavior and psychological variables.

5.3.3 Acquisition of behavioral and endocrine data

5.3.3.1 Ambulatory assessment

As previously reported (Giglberger et al., 2022; Peter et al., 2022a), the AA in cohort A encompassed the collection of saliva samples after awakening for later assessment of the CAR and an assessment of current perceived stress via the newly developed, five-item AA stress scale. A description of the generation of the AA stress scale consisting of the items ‘time pressure’, ‘relaxed’, ‘tense’, ‘overstrained’ and ‘I am disappointed with my performance’ with a seven-point Likert scale as response format (‘strongly disagree’ to ‘strongly agree’) can be found in Giglberger et al. (2022). For the AA, the combined smartphone app and web platform movisensXS (Version 1.3.2 to 1.5.13; movisens, Karlsruhe, Germany) was used. At t1, t2, t5, and t6, ten queries per day were presented on two consecutive working days. At the timepoints close to the exam (t3 and t4), AA was performed on one day only to limit the study-related burden. 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 was presented immediately at the individually chosen awakening time between 05:00 and 07:30 a.m. and the last one at 09:00 p.m. The remaining eight queries took place at pseudo-randomized times between 08:30 a.m. and 08:00 p.m. with a minimum interval of 60 minutes between two queries. Participants who did not have a compatible

smartphone received a device provided by the institute (Motorola G4, Motorola Play G4, Motorola Play G6).

The measurement of the CAR was based on three saliva samples, collected using cortisol Salivettes (Sarstedt, Nümbrecht, Germany) immediately after awakening as well as 30 and 45 minutes later. Saliva samples were collected on the first day of each AA phase; except for t1, when CAR was assessed on both sampling days. During this period, participants were briefed not to eat, drink (except from water), smoke or brush their teeth. To enhance compliance and sampling accuracy, in 51% to 72% (varying over sampling points) of the measurements, functional and non-functional ('sham') electronic monitoring devices to verify times of sample collection (MEMS caps, AARDEX Ltd., Zug, Switzerland) were used (Broderick et al., 2004; Kudielka et al., 2003). In addition, participants were instructed to transfer a random three-digit code to the sampling tube for each saliva sampling, which was displayed to them via smartphone. In our lab 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% and 7%; inter-assay coefficients of variation were between 7% and 9%.

In cohort B, the AA stress scale was assessed via SoSci Survey (alerts via SMS and e-mail; https://www.soscisurvey.de/; Leiner, 2014) in the morning at 07:30 a.m. and in the evening at 09:00 p.m. The query had to be answered within 90 minutes.

5.3.3.2 Questionnaires

Demographic variables (age, sex, etc.) and different psychological constructs were assessed online with SoSci Survey at t1. Depression symptoms were inquired with the depression subscale of the Hospital Anxiety and Depression Scale (HADS; Herrmann-Lingen et al., 2011) at t1, t2, t3, t5 and t6. Please see Giglberger et al. (2022) and https://doi.org/10.5283/epub.51920 for additionally assessed variables not included in the present report.

5.3.4 DNA sampling, genotyping, quality control and genotype imputation

As described previously (Peter et al., 2022a), we used a non-invasive DNA sampling via buccal swabs (Analytik Jena GmbH, Jena) and salting out procedure for DNA isolation (Miller et al., 1988). Genotyping was conducted using the Illumina Infinium™ Global Screening Array 3.0 with Multi-disease drop in (Illumina, San Diego, CA, USA) at the Life & Brain facilities, Bonn, Germany. Quality control of the data was conducted with PLINK 1.9 (see www.cog-genomics.org/plink/1.9/; Chang et al., 2015). SNPs with minor allele frequency (MAF) of < .01, deviating from Hardy-Weinberg equilibrium (HWE) with a *p*-value of < 10^{-6}, and with missing

data > .02 were removed. Participants were excluded in case of missingness > .02, sex-mismatch, and heterozygosity rate > |.20|. Filtering for relatedness and population structure was performed on a SNP set filtered for high quality (HWE p > .02, MAF > .20, missing rate = 0) and linkage disequilibrium pruning (pairwise r^2 = .10). In the case of relatedness (pi-hat > .20), one participant was excluded at random. To adjust for population stratification, principal components (PC) were computed. Outliers on any of the first 20 PCs (|z| > 4.5) were eliminated. In total, 19 participants were excluded. In a last step, data was checked for duplicate SNPs and one was retained at random. Thus, the final data set contained 432 subjects and 476,701 SNPs.

After quality control, genotype imputation was performed with Eagle v2.4.1 (Loh et al., 2016) and Minimac4 (Das et al., 2016). Data from 1,000 Genomes Phase3 v5 (Auton et al., 2015) was used as reference panel. For the analyses, we used the estimated most likely genotype and only SNPs with an info score ≥ .90. In a last step, data was again checked for MAF of > .01, for duplicate SNPs, retaining one at random, and SNP rs IDs were added resulting in a total of 5,278,541 SNPs used for PGS analysis. Detailed information on genotyping, quality control steps, and genotype imputation has been previously described in Peter et al. (2022a).

5.3.5 Polygenic Scores

DEP-PGS for each participant were calculated based on summary statistics of GWAS using data from the Psychiatric Genomics Consortium (PGC), the UK Biobank and 23andMe Inc. (containing 246,363 cases and 561,190 controls; Howard et al., 2019). The NEU-PGS were computed based on summary statistics of the meta-analysis of GWAS for neuroticism excluding data from 23andMe, only including data from the UK Biobank, and the Genetics of Personality Consortium (GPC; containing 390,278 subjects; Nagel et al., 2018a). Calculation of PGS was performed with PRSice 2.3.3 (Choi and O'Reilly, 2019). PGS were calculated as weighted sums of each participant's trait-associated alleles across SNPs retained after clumping (250 kb sliding window, linkage disequilibrium r^2 > 0.1) and after removal of variants within the major histocompatibility complex region (--x-range chr6 26000000 - 33000000). For the inclusion of SNPs, a p-value threshold (P_T) of ≤ .05 for DEP-PGS and P_T ≤ .10 for NEU-PGS, respectively, was applied since they explained the largest proportion of phenotypic variance in their original GWAS. Otherwise, default settings were used. The final DEP-PGS contained 29,523 SNPs and the NEU-PGS contained 49,816 SNPs.

As positive control, PGS for height were calculated with PRSice using summary statistics from Yengo et al. (2018) for the p-value thresholds $5*10^{-08}$, 10^{-06}, .0001, .001, .01, .05, .10, .2, .5, and 1. Height PGS were tested for association with measured height and with sex, age, and PC1 to 5 as covariates.

5.3.6 Statistical analysis

Because of the hierarchical and longitudinal structure of our data, the associations between the PGS and the stress-related variables were tested in two level linear mixed models (timepoints nested within participants) using R (version 4.0.3; R Core Team, 2020). Since we were interested in the association between the PGS and the trajectory of the investigated variables under chronic stress conditions, only the timepoints until the exam were included (t1-t4, for depression: t1-t3). In a first step, the final group models investigating group differences over the observation period are shortly presented. Some of these models have already been presented in Giglberger et al. (2022) and Peter et al. (2022a). However, sample sizes are slightly different, only timepoints until the exam were examined, and aggregated parameters of the CAR were used instead of single cortisol values (see section 2.6.1). The aggregation of the single cortisol values was necessary in order to facilitate interpretability of the final models. In a second step, we then added the PGS to these models to test our hypotheses. All models were estimated with Maximum Likelihood and the significance level was set at $\alpha = .05$.

5.3.6.1 Model structure of the group models to test for group differences

The trajectories of the AA stress scale (*n* = 432; observations = 12,230) and depression symptoms (*n* = 432; observations = 1,231) were calculated using generalized linear mixed models (package glmmTMB; Brooks et al., 2017). The final group models (group.model) contained the fixed effects *group* (0 = CG, 1 = SG), *timepoint* (centered at the first timepoint) as linear and quadratic time trend, their interactions with *group* (0 = CG; 1 = SG), and the covariates *sex* (0 = men; 1 = women) and *cohort* (0 = cohort A; 1 = cohort B), the latter only in the AA stress scale model. To account for dependencies in the data, random intercepts and slopes for *timepoint* by participant were estimated. To model the CAR (*n* = 196; observations = 919), we used the two parameters area under the curve with respect to the ground (*AUCg*), serving as measurement of the total hormonal output and the AUC with respect to the increase (*AUCi*), representing the time-dependent change of cortisol in the morning (Pruessner et al., 2003a). Raw cortisol values were used since the residuals of the final models displayed satisfactory approximation to normal distribution. Fourteen cortisol values were excluded because of participants' self-reported non-adherence to the study protocol and physiologically implausible values (e.g., only one extremely high value within one CAR assessment). Linear mixed models were computed with the package nlme (Pinheiro et al., 2021). The models contained similar fixed effects as presented above, except that *AUCg* was best represented by a linear time trend only and without a random slope for *timepoint*. As covariates, we added the person-mean centered variable *time of awakening*

(in minutes) and instead of *sex* the *hormonal status* was used (0 = women not using hormonal contraceptives, 1 = women using hormonal contraceptives and 2 = men).

5.3.6.2 Models containing PGS to test main hypotheses

To test our hypotheses that the PGS for depression and the PGS for neuroticism are associated with the trajectories of the stress-related variables, the following fixed effects were added simultaneously to the group.model: *PGS*, the interaction of *PGS* with *group* and the linear and quadratic time trend as well as the three-way interaction (*timepoint/timepoint*^{2} x *group* x *PGS;* PGS.model). The PGS were z-standardized; for models containing only the SG or the CG, the PGS were standardized within the group. Adding the *PGS*, we used the decrease in AIC and log-likelihood ratio test to evaluate improvement in model fit. In order to control for genetic ancestry, grand mean-centered PC1-5 were added to the PGS.model (PC.model). The covariates PC1-5 were only retained in the model if a significant improvement in model fit was observed (AIC and change in -2log-likelihood with χ^2-test) or if their addition led to changes in the results. In total, eight main models were tested, four for the DEP-PGS and four for the NEU-PGS, respectively. *Post-hoc*, two additional models were computed for the variable *AUCg*. In order to unravel the interaction between the NEU-PGS and the group, separate models for the SG and the CG were calculated. The explained variance of the fixed effects of the final models was calculated via marginal R squared (R^2; Nakagawa and Schielzeth, 2013). The predictive power of the PGS was then measured by the 'incremental R^{2}', defined as the increase of marginal R^2 when the PGS and its interactions were added to the model.

5.4 Results

5.4.1 Demographics

Demographic information of the sample can be taken from Table 6. As the control group consisted of students in earlier semesters, the significant age difference between SG and CG was not surprising ($t(430) = -11.45$, $p < .001$).

Table 6. Demographic characteristics of the total sample.

	Stress group	Control group
n	218	214
Age (Mean ± standard deviation)	22.96 (± 1.72)	21.04 (± 1.77)
Women	*n* = 160 (73 %)	*n* = 165 (77 %)
Women using hormonal contraception	*n* = 102	*n* = 103

Note. Recruiting was separated in two cohorts. Cohort A (*n* = 196) underwent the elaborate study protocol with laboratory visits in Regensburg and the assessment of the cortisol awakening response whereas cohort B (*n* = 236) consisted of law students from other Bavarian universities who completed a less detailed study protocol (see section 2.1).

5.4.2 Association of DEP-PGS and NEU-PGS with stress-related phenotypes

The focus of our analysis was to investigate the association of the DEP-PGS and the NEU-PGS with the rise in momentary perceived stress levels due to the examination stress. Furthermore, we assessed whether the PGS were associated with the alterations in depression symptoms as well as the CAR parameters *AUCg* and *AUCi* until the exam at t4. As recently reported, trajectories of perceived stress levels, depression symptoms, and the CAR were significantly different between SG and CG (Giglberger et al., 2022; Peter et al., 2022a). The present analyses, based in part on different sample sizes and aggregated variables for the CAR (see Section 2.6.1), yielded very similar results.

In none of the final PGS models the addition of the covariates PC1-5 did improve the model fit. Moreover, only minor effects on the beta values but no alterations of the overall results were observed. Therefore, always the less complex PGS.model without the covariates is presented in the following. Information on the PC.models that include PC1-5 can be found in supplementary Tables 18-27. The positive control, height PGS, showed a positive association with measured height (strongest association: $P_T \leq .10$, $R^2 = 12.26\%$).

5.4.2.1 AA Stress scale

The AA stress scale represented the most relevant self-report instrument used in the present study as it was assessed at high frequency as well as in real-time and real-life to capture the momentary perceived stress. Considering only t1 - t4, a compliance rate of 94% was reached. As already presented elsewhere (Peter et al., 2022a), we found significant differences between SG and CG in the trajectories of perceived stress levels until the exam at t4 (*timepoint x SG b* = .18, $p < .001$; *timepoint*^{2} *x SG b* = -.04, $p < .001$). Mean perceived stress levels in the SG increased, whereas perceived stress levels in the CG stayed relatively stable (see supplementary Table 18).

Additionally, the SG showed slightly higher perceived stress levels at the baseline measurement, compared to the CG, resulting in a significant difference at t1 (*SG b* = .10, *p* = .003).

Entering the DEP-PGS to the model did not lead to an improvement of the model (group.model vs. PGS.model $\chi^2(6) = 8.63$, $p = .196$, ΔAIC = -3.37). Therefore, our hypothesis that the increase of stress perception in the SG is predicted by the DEP-PGS could not be confirmed (Figure 6). Please see supplementary Table 18 for all model parameters, the explained variance and a detailed model comparison. However, adding the NEU-PGS resulted in an improved model fit (group.model vs. PGS.model $\chi^2(6) = 38.76$, $p < .001$, ΔAIC = 26.77), indicating a significant association between NEU-PGS and perceived stress levels. We found a significant effect of the NEU-PGS on the trajectory of perceived stress levels in the SG (*NEU-PGS x timepoint x SG b* = -.06, *p* = .001; *NEU-PGS x timepoint² x SG b* = .02, *p* < .001) but not in the CG (*NEU-PGS x timepoint b* = .02, *p* = .093). Only the quadratic trend in the CG seems to be associated slightly with the PGS *(NEU-PGS x timepoint² b* = -.01, *p* = .005). However, as there is nearly no change in perceived stress levels of the CG over the time period, results should be viewed with caution. Additionally, we found a significant effect of the NEU-PGS on the baseline measure of perceived stress levels at t1 (*NEU-PGS b* = .05, *p* = .034). The effect did not differ between the two groups (*NEU-PGS x SG b* = .02, *p* = .460). Thus, individuals with a low genetic disposition for neuroticism showed lower perceived stress levels at t1 in both groups as well as a lower increase of stress levels in the SG under chronic examination stress (see Figure 7, Table 7 & supplementary Table 19). In the PGS.model, 1.53% of the variance could be explained by the NEU-PGS parameters.

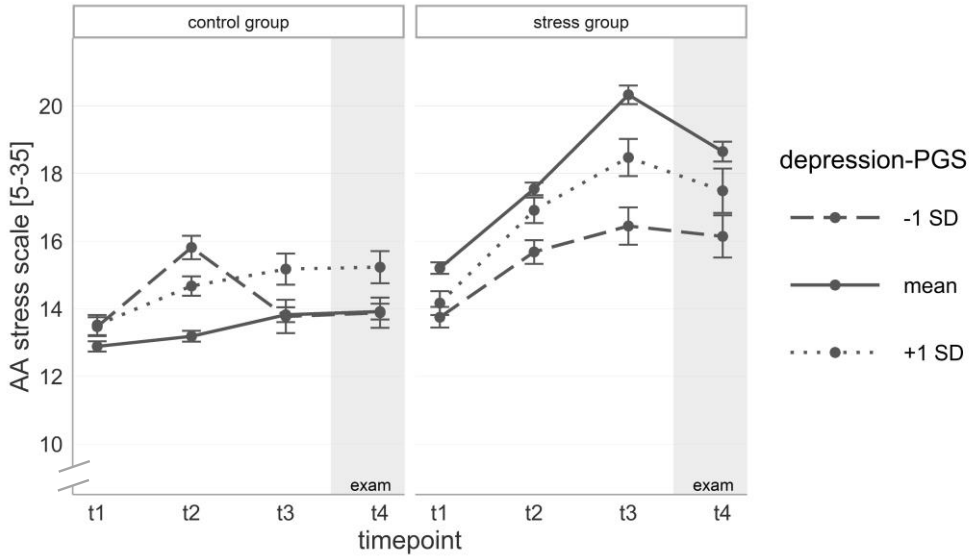


Figure 6. Time course of mean perceived stress levels (± SEM) in stress group (SG) and control group (CG) separated by polygenic score (PGS) for depression (grouping based on *SD* for illustrative purposes only). *Note*. SD = standard deviation.

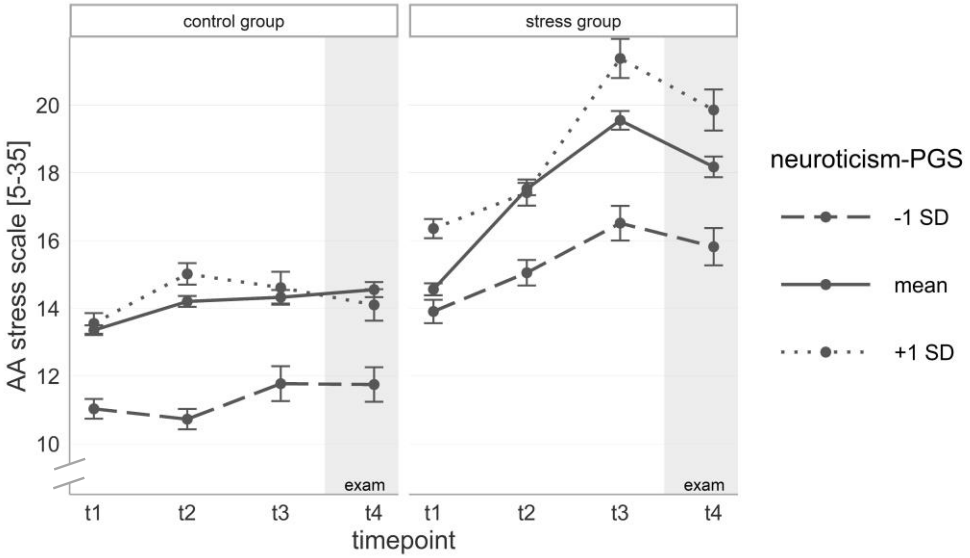


Figure 7. Time course of mean perceived stress levels (± SEM) in the stress group (SG) and control group (CG) separated by polygenic score (PGS) for neuroticism (grouping based on *SD* for illustrative purposes only). *Note. SD* = standard deviation.

Table 7. Parameter estimates for overall effects for the final PGS.model with perceived stress as dependent variable and the polygenic score for neuroticism as predictor.

Fixed Effects	Estimates	*SE*	*p*
Intercept	2.46	0.04	**< .001**
Timepoint	.07	0.01	**< .001**
Timepoint^2	-.02	0.00	**< .001**
Women (vs. men)	.07	0.03	**.049**
Cohort B (vs. cohort A)	.18	0.03	**< .001**
SG (vs. CG)	.09	0.03	**.004**
Timepoint x SG	.18	0.02	**< .001**
Timepoint^2 x SG	-.05	0.00	**< .001**
PGS	.05	0.02	**.034**
PGS x SG	.02	0.03	.460
PGS x Timepoint	.02	0.01	.093
PGS x Timepoint^2	-.01	0.00	**.005**
PGS x Timepoint x SG	-.06	0.02	**.001**
PGS x Timepoint^2 x SG	.02	0.00	**< .001**
Random Effects	*SD*	Correlation Intercept	
Participant (Intercept)	0.31		
Timepoint	0.11	-.27	

Note. CG = Control group; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error; SG = Stress group.

5.4.2.2 Depression symptoms

Regarding depression symptoms, we found a difference between the SG and the CG over time (*timepoint x SG b* = .50, *p* < .001; *timepoint^2 x SG b* = -.11, *p* = .044). No difference was found at the baseline measure (t1) between both groups (*SG b* = -.08, *p* = .285). The SG showed a steep

increase until the exam, whereas the CG stayed relatively stable (see supplementary Figure 14 & supplementary Table 20). As already presented in Giglberger et al. (2022) 18% of the students in the SG exceeded the clinically relevant score of 11 for depression symptoms at t3, compared to 2% at the baseline measurement and 3% to 5% of the CG. Neither including the DEP-PGS nor the NEU-PGS resulted in an improved model fit (DEP-PGS: group.model vs. PGS.model $\chi^2(6) = 7.07$, $p = .314$, ΔAIC = -4.92; NEU-PGS: group.model vs. PGS.model $\chi^2(6) = 8.89$, $p = .180$, ΔAIC = -3.11). Thus, no association between the DEP-PGS nor the NEU-PGS and depression symptoms could be assumed (see supplementary Figures 15 and 16 & Tables 20 and 21).

5.4.2.3 Cortisol awakening response: *AUCg* and *AUCi*

Regarding the CAR parameters *AUCg* and *AUCi,* we found significant differences between both groups over time (*AUCg*: *timepoint x SG b* = -19.15, $p = .016$; *AUCi*: *timepoint*^{2} *x SG b* = -19.81, $p = .015$). No differences were found at t1 (*AUCg*: *SG b* = 2.83, $p = .888$; *AUCi*: *SG b* = -10.60, $p = .511$). The SG showed a strong decline of *AUCg* and *AUCi* at t4 (see supplementary Figures 17 and 20 & Tables 22 and 26). Thus, our previously reported finding of a blunted CAR in the SG compared to the CG could be reproduced by the present analyses of the aggregated CAR parameters (Giglberger et al., 2022).

For the *AUCg* and the *AUCi*, addition of the DEP-PGS did not improve the global model fit (*AUCg*: group.model vs. PGS.model $\chi^2(6) = 2.64$, $p = .620$, ΔAIC = -5.36; *AUCi*: group.model vs. PGS.model $\chi^2(6) = 2.42$, $p = .877$, ΔAIC = -9.58). Hence, the DEP-PGS was not related to the CAR (see supplementary Figures 18 and 21 & Tables 22 and 26). Also, regarding the NEU-PGS and the CAR we could not confirm our GxE hypothesis. The NEU-PGS was not related to the alteration of the *AUCg* and *AUCi* over the time period (*AUCg*: *ps* ≥ .538; AUCi: *ps* ≥ .068; see supplementary Tables 23 and 27). We found an association between the NEU-PGS and the baseline measurement of the *AUCg* which differed significantly between the two groups (*PGS b* = -47.40, $p < .001$; *PGS* x SG *b* = 48.23, $p = .014$). Subsequently calculated separate models for the CG and SG confirmed this association solely for the CG (CG.model vs. PGS.model $\chi^2(2) = 12.33$, $p = .002$, ΔAIC = 8.33; *PGS b* = -48.42, $p < .001$; supplementary Table 24) but not for the SG (SG.model vs. PGS.model $\chi^2(2) = 0.21$, $p = .900$, ΔAIC = -3.79; *PGS b* = 1.49, $p = .911$; supplementary Table 25). The PGS parameters in the model containing only the CG explained 5.77% of the variance. For the *AUCi,* a tendency for this association within the CG could also be found (*PGS b* = -25.33, $p = .021$), though the model did not improve significantly (group.model vs. PGS.model $\chi^2(6) = 7.75$, $p = .257$, ΔAIC = 0.01; supplementary Table 27). Individuals in the CG with lower genetic disposition for neuroticism showed a higher *AUCg* (supplementary Figure 19) and, probably, also a stronger increase in cortisol upon awakening at t1 (supplementary Figure 22).

5.5 Discussion

In the present analyses, we studied if polygenic scores capturing the genetic disposition for depression and neuroticism, were associated with chronic stress responses in everyday life. The focus was to investigate GxE effects of both PGS and the environmental exposure 'chronic examination stress' in a quasi-experimental and prospective longitudinal design. The main outcome variable, the increase in perceived stress levels, was assessed at high frequency and in an ecological valid manner with AA in 432 subjects. As previously reported (Giglberger et al., 2022), significant differences between the SG and the CG over the 13 months period could be found: The SG showed increases in perceived stress levels and depression symptoms as well as decreases in the CAR parameters *AUCg* and *AUCi* until the exam compared to the CG. Hence, we can conclude that the chronic examination stress resulted in alterations in psychological well-being as well as in cortisol regulation.

To examine whether these alterations are associated with genetic factors, PGS for depression and neuroticism were investigated. Both phenotypes are related to stress (Dunn et al., 2015; Kendler et al., 1999; Kessler, 1997; Lahey, 2009). Although they are highly correlated, both phenotypically as well as genetically (Jylhä and Isometsä, 2006; Kendler et al., 2006; Luciano et al., 2018; Nagel et al., 2018a), they have also distinct genetic influences (Adams et al., 2020) complementing each other in the search for genetic factors influencing chronic stress responses. Contrary to our hypothesis, no relation was found between the DEP-PGS and perceived stress levels over the observation period. Thus, we did not observe any difference between individuals with elevated or low polygenic disposition for depression regarding their perceived stress levels in daily life over a long-lasting stress phase. Usually, phenotyping in large-scale GWAS is not very extensive and recent studies showed that this strategy can result in unspecific PGS capturing not only the risk to develop clinically relevant depression but also related constructs and comorbid disorders (Cai et al., 2020; Mitchell et al., 2021). However, it can be assumed that the DEP-PGS particularly represents the risk for clinical depression as the investigated GWAS sample was enriched for patients with diagnosed depression. This risk is probably not fully congruent with the risk to reach high perceived stress levels in the context of academic stress. Thus, we suppose that the DEP-PGS was not entirely suitable to uncover GxE effects in our study sample consisting of healthy students. Although academic stress was shown to be associated with increased depression symptoms (O'Flynn et al., 2018; Rotenstein et al., 2016), we expected that the majority of our participants would be rather stress resilient. This notion was supported by our findings that most of the students showed a fast recovery after the exam regarding perceived stress levels as well as other psychometric variables, including anxiety and depression symptoms,

reported sleep disturbances, and other facets of chronic stress (Giglberger et al., 2022). It appears plausible that a larger and more heterogenic sample regarding stress vulnerability would be needed to find meaningful associations between DEP-PGS and perceived stress levels. Regarding the NEU-PGS, we found support for our hypothesis as we observed a significant GxE effect. The higher the genetic disposition for neuroticism, the more pronounced was the increase of perceived stress until the exam at t4 in the SG. Additionally, we observed an effect at the baseline measurement in both groups. The higher the NEU-PGS, the higher were perceived stress levels. The PGS and the PGS x stress exposure effect explained 1.53% of the variance in perceived stress levels. The present findings are in accordance with previous studies reporting an association between neuroticism and stress sensitivity (McCrae, 1990; Rietschel et al., 2014; Schneider et al., 2012). Furthermore, it was shown in a twin study using AA that the phenotypic association between the variability in negative affect in daily life and neuroticism can be partly explained by genetic effects (Jacobs et al., 2011).

Taken together, our analyses using PGS on the individual level, reflecting the polygenicity of neuroticism, expand the current knowledge as they suggest a shared genetic basis of neuroticism and reported momentary stress levels under normal conditions as well as under chronic stress conditions. Furthermore, the findings support the notion that the NEU-PGS which was proposed to capture the genetic predisposition to subclinical symptoms of anxiety and depression (Thorp et al., 2021) has a stronger overlap with stress reactivity than the DEP-PGS. We assume that the NEU-PGS probably reflects partly the genetic disposition of how individuals react to stress whereas the DEP-PGS reflects to a higher extent the genetic susceptibility for the disorder itself.

Regarding the prediction of depression symptoms, no effect of neither the DEP-PGS nor the NEU-PGS was found. Thus, in our sample the genetic disposition to depression and neuroticism was not related to depression symptoms. Several reasons why we failed to find any association with depression symptoms are conceivable. First, the lack of power due to the small sample size has to be noted, especially since depression symptoms were not assessed with AA in contrast to perceived stress levels. This probably resulted in a lower validity due to the lower proximity to the momentary experience as well as in a lower reliability of the self-report as symptoms were only assessed once per measurement timepoint. Second, although we found an increase in depression symptoms in the SG, most participants reported only low to moderate depression symptoms, in particular compared to clinical cases. Thus, our variance in the outcome variable could have been too small. Other recent studies which have used DEP-PGS or NEU-PGS did find significant GxE effects on depression symptoms (de Moor et al., 2015; Fang et al., 2020; Li et al., 2019b; Rietschel et al., 2017). Fang et al. (2020) examined 5,227 training physicians under

chronic stress conditions, more precisely during their medical internship year. They found that depression symptoms under stress were predicted by DEP-PGS, and that this association was stronger than with depression symptoms at baseline. In another longitudinal study, Li et al. (2019b) found a significant association between a NEU-PGS and late life depression investigating a sample of 4,877 participants. This association was partly mediated by retrospectively assessed stressful life events. While the phenotyping in these investigations was less extensive, the samples were considerably larger than in our study, which probably explains why Fang et al. (2020) as well as Li et al. (2019b) could detect an effect of the PGS on depression symptoms. Two additional interesting longitudinal studies in this context with slightly smaller sample sizes also found significant associations between DEP-PGS and depression symptoms (Domingue et al., 2017; Lobo et al., 2021). However, results are not necessarily comparable to our study as the investigated trauma-like type of stressor (motor vehicle collision and death of spouse) differed substantially from chronic academic stress.

Investigating the two CAR parameters, *AUCg* and *AUCi*, we found no association with the DEP-PGS. Furthermore, no association between the NEU-PGS and alterations of the CAR parameters under chronic stress conditions was observed. In general, it is a well-known phenomenon that biological indicators of stress are often not or only moderately correlated with subjective stress-related variables (Campbell and Ehlert, 2012; Fahrenberg, 1979; Giglberger et al., 2022). As the PGS were primarily generated based on self-report data, they predominantly capture the genetic disposition for phenotypes assessed on a subjective psychological level. Thus, the genes influencing these phenotypes may show only limited overlap with the genes modulating alterations of the CAR under chronic examination stress. The baseline effect of the NEU-PGS on the *AUCg* only in the CG was somewhat unexpected, as we would assume that any effect at the baseline should be visible in both groups. Therefore, and due to the fact that the power of the CAR analyses was substantially lower compared to analyses of perceived stress levels and depression symptoms (n = 196, less frequent assessment), results should be interpreted with caution.

Our study has some limitations that need to be considered. Our sample consisted of young students who probably have better overall health and a higher socioeconomic status compared to the general population. Furthermore, only students with European ancestry were investigated. Thus, the generalizability of the present results is limited. Furthermore, like any other study examining real-life stress, a certain selection bias cannot be ruled out. Students who already felt stressed by their regular study program and who anticipated an exceedingly stressful exam (preparation 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 stress group to a certain extent. An obvious limitation of our quasi-experimental design was the missing randomization regarding the assignment to the stress and the control group. To compensate for this issue, our CG contained individuals who were as similar as possible to our SG participants. This rather conservative strategy might have caused an underestimation of group differences as students in the CG as well had substantial study-related stress. Additionally, as mentioned earlier, the sample size has to be discussed. Although some features of our study design, as for example the quasi-experimental design as well as the repeated measurements, likely increased the power, our sample size was quite small, particularly for the analysis of depression symptoms and the CAR. Hence, the statistical power to reveal three-way interaction effects was limited, and the findings need to be replicated in an independent sample. Additionally, the number of covariates within the model could have caused overfitting with too small variance left for the PGS to explain, possibly further reducing the power of our analyses. However, we assume that overfitting due to the number of covariates did not impact results as no alterations of the reported results were observed recomputing the models without covariates. Due to the risk of overfitting, no additional covariates were tested. Although, it would be desirable to control not only for covariates but also for their interactions with the PGS and the environmental variable, to account for potential confounders on the interaction term (Keller, 2014). It further has to be noted that the variance currently explained by PGS represents only a marginal proportion of genetic contribution and therefore is still very small (Wray et al., 2021).
In summary, we conclude that the present PGS analysis in a cohort that has been thoroughly phenotyped in a longitudinal study including a meaningful, long-lasting and real-life stress exposure, provided relevant information on the association between genetic disposition and chronic stress responses in daily life. In particular, we found that individuals with a higher NEU-PGS were more stress sensitive, as they generally reported higher perceived stress levels and showed stronger increases over the stressful period. Assumed associations between genetic disposition for depression and stress-related phenotypes could not be confirmed. Due the small sample further replication is needed. Future studies could combine polygenic scores with additional factors, such as brain activation changes in response to acute stress, functional connectivity, or other physiological stress markers to predict chronic stress responses in daily life. Such a combination of PGS and other relevant factors was already shown to be useful for disease risk stratification and for the prediction of medication treatment outcomes (Torkamani et al., 2018; Wang et al., 2022).

Chapter 6

6 General Discussion

The present dissertation aimed at investigating chronic stress responses in daily life and analyzing GxE effects. To examine stress regulation under chronic stress conditions we conducted the LawSTRESS project, a study with a prospective-longitudinal (quasi-) experimental design. Law students in preparation for the first state examination were investigated over 13 months. In *Study I* (Chapter 3) it was examined whether the long-term preparation phase for and the exposure to the first state examination resulted in alterations in perceived stress levels, the CAR, and other stress-related variables. Additionally, the association between repeatedly assessed psychological stress ratings and the CAR was examined, and the predictive value of several psychometric variables assessed at the first timepoint was analyzed. To investigate associations between genetic factors and stress regulation new genetic methods capturing the variability of a whole gene system and the whole genome, respectively, were applied to overcome well-known limitations of single candidate gene studies. In *Study II* (Chapter 4) GSA were computed to investigate the genetic variability within the NPS/NPSR1 system and its association with the examined stress-related phenotypes. In *Study III* (Chapter 5) exploratory PGS analyses were conducted.

In the following chapter the findings of the three studies are shortly summarized and results are discussed within the framework of the current state of research (Chapter 2). Moreover, strengths and future challenges are outlined, and a final conclusion is presented.

6.1 Summary of main findings

6.1.1 Effects of chronic exam stress

Overall, we observed a significant impact of the exam period on the students' health and well-being. Regarding the most relevant self-report instrument used in the present study, the AA stress scale, we found significant increases of perceived stress levels in the stress group until the exam, whereas students of the control group stayed relatively stable. Furthermore, we observed increases in self-reported anxiety and depression symptoms, sleep disturbances as well as regarding several facets of perceived chronic stress. Although most of the students within the SG showed no concerning increases in anxiety and depression symptoms, a considerable number of participants could be identified who temporarily exceeded the cut-off levels (Giglberger et al., 2022). These results are in line with previous findings and confirm that academic stress depicts a great burden for many students and comes along with biopsychological alterations (González-Cabrera et al., 2014; Grützmacher, 2018; Koudela-Hamila et al., 2020; Sheldon et al., 2021; Weber et al., 2020). Four weeks after the exam, mean values of the stress-related variables returned to baseline levels indicating a fast recovery.

Regarding the CAR, we observed that chronic exam stress was associated with blunted CAR followed by a fast recovery as cortisol concentrations in the morning increased to initial levels one week after the exam. This is in line with the trajectories of the psychological variables. To date, studies investigating the impact of academic stress on the CAR yielded mixed results (Duan et al., 2013; Hewig et al., 2008; Koudela-Hamila et al., 2020; O'Flynn et al., 2018; Weik and Deinzer, 2010). It is assumed that methodological differences, e.g., heterogenous samples or varying durations and intensities of the exam period are probably an explanation for these partly contradictory results. Law and Clow (2020) summarized that in longitudinal studies with thorough methods chronic stress was often related to a blunted CAR. Previous longitudinal studies (Duan et al., 2013; Koudela-Hamila et al., 2020) as well as our results which are based on a longitudinal design, a baseline measurement and a control group confirmed these findings. Although more research is needed to investigate dynamic alterations in the CAR, we assumed that the attenuated CAR might be of psychobiological relevance and represents a temporary hypocortisolism (see section 3.5). As already discussed in Chapter 3, no evidence was found to support the theory of a preceding hyperactivity of the HPA axis in the development of a hypocortisolism (Fries et al., 2005; Miller et al., 2007). However, evidence for this theory is scarce and it should be emphasized that besides the timing and duration of the stressor, several features of the stressor and characteristics of the person experiencing it influence the impact of chronic stress on the HPA axis (Boggero et al., 2017; Fries et al., 2009; Miller et al., 2007). Further research is needed to examine possible effects of this short-term reduction in morning cortisol.

In a subsequent step, we examined predictors of the alterations of the CAR. First, we were interested in the association between the alterations in the CAR and the repeatedly measured perceived stress levels. As already summarized (section 3.5), the missing covariation of subjective stress experience and markers of cortisol regulation is a well-known phenomenon (Campbell and Ehlert, 2012; Fahrenberg, 1979). Previous studies already failed to find such association or yielded inconsistent results (Anand et al., 2022; Pruessner et al., 2003b; Weekes et al., 2008). Nevertheless, we assumed that several features of our study design which presumably enhanced the validity and reliability of the measured perceived stress levels (extensive AA) would facilitate to find an association between perceived stress and the CAR, theoretically representing indicators of the same construct ‘stress’. However, within participants perceived stress and the CAR were not significantly associated. Second, we investigated the predictive value of psychometric variables assessed at the first timepoint, namely anxiety and depression symptoms, test anxiety and perceived chronic stress, for the alterations of the CAR. The observed decrease of the CAR was not associated with any of the variables.

To conclude, our findings support the view, that exam stress constitutes a robust stressor. In our sample chronic exam stress was associated with alterations in psychological and biological stress parameters. Although the homogenous sample of only law students reduces the generalizability, we assume that the overall psychosocial profile of this stressful period is generally comparable to stress individuals frequently experience in schools, universities or at the workplace. Additionally, the number of participants temporally exceeding clinically relevant cut-off scores of depression and anxiety symptoms seem alarming. However, in young and healthy students, these changes appear to be temporary and return to initial levels after the exam. We assume that the changes are probably necessary alterations to cope successfully with the stress triggered by the exam and represent a healthy reaction to the demand. Further research is needed to investigate whether such a life phase leads to improved coping strategies and capacity or has consequential effects in some individuals, in particular when individuals are confronted with additional stressful events later in life.

6.1.2 Chronic stress responses and genetic variability within the NPS/NPSR1 system

To examine genetic factors of the stress response, we analyzed the association between the genetic variability within the *a priori* selected NPS/NPSR1 system and stress-related alterations during chronic stress. The relevance of the NPS/NPSR1 system for stress was shown repeatedly in animal models and studies in humans (Tobinski and Rappeneau, 2021). Furthermore, since genetic variability within the neuropeptide system can be covered by assessing genetic variants in only two genes (*NPS* and *NPSR1*), its association with stress-related variables can be examined with comparatively low effort. To capture the genetic variability within the system adequately, we computed gene-set analyses aggregating the genetic variation across 936 SNPs within the NPS/NPSR1 system and examining their joint association with the phenotypes.

Our analysis could not confirm previously reported associations of genetic variability in the NPS/NPSR1 system and changes in perceived stress levels and anxiety symptoms. As summarized in section 4.5, animal models as well as single candidate studies in humans, in particular investigating the *NPSR1* rs324981, showed a strong association between anxiety-related variables and the NPS/NPSR1 system (Domschke et al., 2011; Donner et al., 2010; Klauke et al., 2011; Schiele et al., 2020; Tobinski and Rappeneau, 2021). In contrast to previous studies which focused on one SNP of *NPSR1*, the present analyses investigated the association between overall genetic variability in the NPS/NPSR1 system and anxiety symptoms. The application of GSA aimed at minimizing the risk of false positive findings and providing adequate coverage of the entire system. We assume that this rather conservative strategy possibly explains why previously found associations of the single SNP studies could not be confirmed. Additionally,

differences in sample structure, study design and outcome variables can certainly lead to inconsistent results.

However, we revealed a gene-environment interaction between the NPS/NPSR1 system and the two CAR parameters *AUCg* and *AUCi*. Our findings suggest that genetic variability in the NPS/NPSR1 system is associated with changes in the CAR in individuals exposed to chronic stress. It seems that the association between genetic variability in the NPS/NPSR1 system and the CAR only becomes relevant under the environmental condition 'chronic stress exposure'. As already summarized (section 4.5), evidence regarding the interplay between the NPS/NPSR1 system and the HPA axis in humans is scarce. In response to acute psychosocial stress, it was shown that the T allele of *NPSR1* rs324981 was associated with increased salivary cortisol (Kumsta et al., 2013; Streit et al., 2017). Although these results are not necessarily transferable to our findings, as the mechanisms regulating the increase of cortisol in the morning differ from mechanisms regulating the secretion of cortisol in response to acute stress (Wilhelm et al., 2007), it seems that the NPS/NPSR1 system and the HPA axis interact closely in humans. Moreover, there is substantial evidence that the NPS/NPSR1 system is associated with the sleep–wake rhythm and that it is involved in the regulation of morning awakening (Kushikata et al., 2021). Thus, as the CAR is supposed to be associated with the transition from sleep to wakefulness (Clow et al., 2010), the NPS/NPSR1 system might be related to the CAR via its interplay with these circuits in a stress-sensitive manner. It should be noted that this assumption is purely speculative.

Although the exact interplay is still unknown, we found suggestive evidence that alterations of the CAR during a long-lasting stress phase seem to be related to the genetic variability within the NPS/NPSR1 system. It thus can be assumed that the heritability of the CAR is, to a certain extent, mediated by genetic variability in the NPS/NPSR1 system. The conducted GSA approach is a promising, robust, and conservative alternative to single candidate SNP analyses (Windhorst et al., 2016) and might complement large-scale genome-wide analyses in the uncovering of stress-related disease mechanisms. The joint association of 936 SNPs within the NPS/NPSR1 system with the phenotypes of interest reduced the number of tests needed and ensured an adequate coverage of the genetic variability of the NPS/NPSR1 system.

6.1.3 Chronic stress responses and the genetic disposition to depression or neuroticism

In a subsequent explorative step, we conducted PGS analyses investigating GxE effects of the genetic disposition to depression and neuroticism, respectively, and the environmental exposure 'chronic exam stress'. As presented in section 5.2, depression and neuroticism are moderately heritable phenotypes (Jang et al., 1996; Kendler et al., 2006; Sullivan and Geschwind, 2019; Vukasović and Bratko, 2015), are both of high relevance in stress research (Dunn et al., 2015;

Kendler et al., 1999; Kessler, 1997; Lahey, 2009) and probably complement each other in the search for genetic factors influencing chronic stress responses (Adams et al., 2020).

Contrary to our hypothesis, no association between the PGS for depression and the increase in perceived stress levels was found. We assumed that the PGS for depression, reflecting to a high extent the genetic disposition to develop a clinical depression was not entirely suitable to uncover GxE effects in our study sample consisting of healthy students. As stated in section 5.5, the risk to develop a clinical depression is probably not fully congruent with the risk to reach high perceived stress levels in the context of academic stress. Moreover, we expected that the majority of our participants would be rather stress resilient which was supported by the findings of a fast recovery after the exam in most of the students (Giglberger et al., 2022). It appears plausible that a larger and more heterogenic sample would be needed to find meaningful associations between DEP-PGS and perceived stress levels. Regarding the NEU-PGS, we observed a significant GxE effect. Individuals with a higher NEU-PGS seem to be more stress sensitive. They generally reported higher perceived stress levels and showed a more pronounced increase of perceived stress over the stressful period. These findings are in line with current research consistently showing associations between neuroticism and stress sensitivity (Jacobs et al., 2011; McCrae, 1990; Rietschel et al., 2014; Schneider et al., 2012). Considering the polygenicity of neuroticism, our analyses expand the current knowledge and suggest a shared genetic basis of neuroticism and reported momentary stress levels under normal conditions as well as under chronic stress conditions. Furthermore, they support the notion that the NEU-PGS has a stronger overlap with stress sensitivity than the DEP-PGS. Due to its high relevance for stress-related disorders, especially depression and anxiety, a lot of research has been conducted regarding the genetic basis of neuroticism (Adams et al., 2020; Belonogova et al., 2021; Lahey, 2009; Luciano et al., 2018; Thorp et al., 2021). Nagel et al. (2020, 2018b) investigated neuroticism at item-level and found three genetically specific item clusters, representing 'depressed affect', 'worrying' and 'sensitivity to environmental stress and adversity'. The last cluster indicates that the NEU-PGS reflects partly a sensitivity to stress which fits our conclusion that the NEU-PGS seems to be a good approximation to uncover the genetic basis of stress sensitivity during chronic stress conditions in a healthy sample. Given the high genetic heterogeneity of neuroticism, analyses using PGS that separately capture the three clusters would be promising to unravel genetic architecture of perceived stress levels.

No association was found between either DEP-PGS or NEU-PGS and depression symptoms. It seems conceivable that we fail to find associations due to a lack of power, especially since depression symptoms were not assessed at high frequency with AA. Similar longitudinal studies observing associations between DEP-PGS and depression symptoms under chronic stress

investigated 5,227 and 4,877 participants, respectively (Fang et al., 2020; Li et al., 2019b). Additionally, although we observed increases in depression symptoms, mean values were still low in particular compared to clinical cases. Also, regarding the CAR parameters, *AUCi* and *AUCg* no GxE effects were found with neither the DEP-PGS nor the NEU-PGS. As already discussed (section 5.5), results should be interpreted with caution, especially because of the small sample size (*n* = 196).

To sum up, the PGS analyses provided interesting insights into the interplay between genetic disposition to depression and neuroticism and chronic stress responses in daily life. Individuals with a high NEU-PGS appeared to be more stress sensitive as they reported higher perceived stress levels and larger increases under chronic stress conditions could be observed. No associations were found between the genetic disposition to depression and stress-related phenotypes. However, replication of the findings is necessary to further uncover the genetic architecture of perceived stress levels and other stress-related phenotypes.

6.2 Gene-set analyses and polygenic scores in future stress research

Many studies have been conducted to unravel the genetic architecture of the stress response and to uncover GxE effects in the etiology of stress-related disorders. As outlined in the introduction most studies investigated single candidate SNPs. These candidate gene studies and cGxE studies have often been criticized (see section 2.3.3). At the same time GWAS became increasingly successful in uncovering genetic variants associated with complex traits and psychiatric disorders (Visscher et al., 2017; Yengo et al., 2018). Furthermore, first GWEIS were conducted investigating GxE effects on genome-wide level (Arnau-Soler et al., 2018; Coleman et al., 2020; Dunn et al., 2016; Ikeda et al., 2016; Otowa et al., 2016; Werme et al., 2021). However, the usage of deep phenotyping approaches and high-quality assessment of the environment in GWAS or GWEIS is often not feasible due to the large sample size required.

These challenges illustrate that close collaborations between genetics and social sciences are long overdue (Duncan and Keller, 2011; Harden and Koellinger, 2020). Psychologist and social scientists have rich phenotypic, multimodal, and longitudinal data as well as experience to assess environmental factors (Dick et al., 2018; Duncan et al., 2014). With increasing affordability of genotyping, the next step is to add high-quality measurement of the genome. Furthermore, to move beyond single SNP analyses the application of new biostatistical tools, investigating many SNPs simultaneously, is needed.

Therefore, we conducted GSA and PGS analyses assuming that these approaches are possibly suitable to complement previous findings from cGxE as well as large-scale GWAS and GWEIS. GSA enable to detect effects composed of multiple weaker SNP-phenotype-associations

because the aggregation of single markers to genes and gene sets reduces the number of tests and thereby improves power (de Leeuw et al., 2015; Fridley and Biernacka, 2011). Furthermore, they are biologically informative as gene-sets are composed of genes with shared functional or biological characteristics (de Leeuw et al., 2015). Also, in PGS analyses the effects of many SNPs are investigated simultaneously. PGS analyses are a 'hypothesis-free' genome-wide approach (Kullo et al., 2022). They were proposed to be especially useful in smaller sample sizes (Harden, 2021). Furthermore, they can be added in complex statistical models like linear mixed models. This enables a thorough analysis of longitudinal data and of the intra-individual variability regarding stress sensitivity (Schick et al., 2022). Additionally, as PGS summarize the effect of many genetic factors in one value at the individual level they can easily be combined with other susceptibility factors, such as brain activation changes in response to acute stress, functional connectivity, or other physiological stress markers to predict chronic stress responses in daily life and may serve to identify individuals at risk. Such a combination of PGS and other relevant factors was already shown to be useful for disease risk stratification and for the prediction of medication treatment outcomes (Torkamani et al., 2018; Wang et al., 2022). Another useful application would be the implementation of PGS in experimental designs and longitudinal studies with deep-phenotyping approaches. To save resources, only individuals with low and high PGS values could be included and compared (Choi et al., 2020; Wray and Maier, 2014).

Although more research and replication are needed to determine whether PGS and GSA analyses are suitable to explore GxE effects in small, well-characterized study samples, we conclude that they offer a promising alternative to single candidate gene studies. Overall modern cGxE studies that are based on findings from large-scale GWAS and use methods capturing the variability within the gene system adequately seem to offer great potential to reveal stress-related disease mechanism in humans. Furthermore, our as well as other findings (Domingue et al., 2017; Fang et al., 2020; Li et al., 2019b; Lobo et al., 2021) illustrate the usefulness of PGS and their implementation in well-designed studies with quasi-experimental designs or fine-grained data. Nevertheless, sufficiently large sample should be ensured for reliable statements.

6.3 Ambulatory assessment in GxE studies

Our findings revealed interesting insights into the relationship between genetic factors and stress sensitivity in daily life. The usage of the AA method to measure perceived stress levels as well as the CAR over the time period depicts one major advantage of the present project. Multimodal approaches and data collection in form of AA became much more affordable and feasible with the growing usage of smartphones as well as wearable devices during the last decades (Insel, 2017). These devices offer a novel tool to collect data including GPS, mobility patterns, as

well as physiological data as for instance the pulse in daily life from many individuals (Torous et al., 2017). Due to this development, more studies will have the possibility to investigate the association between genetic factors and dynamic psychological processes throughout the day and different biological and physiological parameters assessed at high frequency in daily life. This can complement previous studies mainly investigating categorial and heterogenous phenotypes and help to elucidate underlying mechanisms of stress-related disorders. It was for example shown that a heightened stress sensitivity in daily life to minor stressors was associated with the development of psychiatric disorders, above all depression and psychosis (Reininghaus et al., 2016b; Wichers et al., 2009). Stress sensitivity seems to represent an interesting intermediate phenotype for psychopathology (Rauschenberg et al., 2022; Wichers et al., 2009; Wichers et al., 2007). Thus, the investigation of genetic factors associated with stress sensitivity under chronic stress conditions probably enables a deeper understanding of the stress-disease-link to identify individuals at risk and provide individualized treatment (Reichert et al., 2021; van Winkel et al., 2014). In a subsequent step it has been proposed to develop ecological momentary interventions targeting specific behavioral patterns in daily life of patients as well as prevention programs applied to individuals at high risk for psychopathology (Myin-Germeys et al., 2016; Reininghaus et al., 2016a; Schick et al., 2021). In this context it would be of special interest to investigate whether individuals with a high genetic disposition to a specific phenotype differ regarding the benefit from an intervention compared to individuals with a low genetic disposition (Choi and O'Reilly, 2019; Wray et al., 2014).

Measurements of momentary experiences as well as mental states with AA represent interesting (intermediate) phenotypes enabling to examine underlying behavioral patterns and mechanisms as well as offering new targets for prevention and intervention programs. We conclude that the measurement of dynamic mental states as well as other biological parameters in daily life with AA in genetic studies is a promising approach to broaden the knowledge of the interplay between genetic factors and chronic stress and the impact on different systems of stress response. In particular the combination of the PGS with AA enables to conduct interesting analyses investigating the association between genetic factors and different trajectories as well as within-person daily variation.

6.4 Future challenges investigating GxE effects

The implementation of genetic factors into psychological studies and the investigation of GxE effects are essential to reveal psychopathology of stress-related disorders (Rees and Owen, 2020). However, investigating GxE effects several aspects have to be considered. Due to limited resources as well as the complexity of human stress responses and GxE effects, it is nearly

impossible to consider all aspects in the analyses and study planning. However, in addition to the problems and challenges already addressed, the following issues should also be considered in the design and interpretation of future studies.

The main conceptual frameworks within which the majority of GxE studies have be embedded are the diathesis-stress model (Monroe and Simons, 1991) and the differential susceptibility theory (DST; Belsky and Pluess, 2009). The diathesis-stress models states that some individuals are more vulnerable to detrimental environmental factors than others because of their genetic disposition. According to the model, psychopathology develops in individuals with inherent vulnerability who are exposed to adverse environmental factors (Assary et al., 2018). The DST on the other hand assumes that individuals differ regarding their susceptibility not only to negative but also to positive environments. Proposing that individuals which are affected most by adverse environmental factors are also the ones who would benefit the most of positive environmental events (Belsky and van IJzendoorn, 2017). The focus of most current research on the investigation of negative environmental factors only, was proposed to be an explanation for the inconclusive findings in GxE studies (Homberg and Jagiellowicz, 2022). Neglecting positive or protective factors may hinder the discovery of GxE effects as positive events may counteract the effects of environmental stress (Homberg and Jagiellowicz, 2022). There is some evidence that social support, higher self-esteem, low rumination tendencies and positive parenting can buffer the effects of stressful live events (Askeland et al., 2020; Fritz et al., 2018). Regarding genetic variability, a candidate gene study investigating the *5-HTTLPR* found that boys with at least one S allele reported more depression symptoms compared to boys homozygous for the L allele. However, when they experienced high family support, they reported the fewest depression symptoms (Li et al., 2013). Another example is *GABRA2* as individuals with the susceptibility allele seem to be more affected by parents and peers in a positive as well as in a negative way (Burmeister and Sen, 2021; Trucco et al., 2017; Trucco et al., 2016). There are also first attempts to pursue polygenic approaches examining a polygenic sensitivity score capturing a phenotype associated with a higher sensitivity to environmental events (Davidson et al., 2021; Keers et al., 2016). Although research mainly was conducted in children and mainly single SNPs have been investigated, specific positive characteristics of a person, as for example coping strategies or self-efficacy as well as protective environmental factors, e.g., social support may have an impact on the interaction between genetic factors and environmental stress (Homberg and Jagiellowicz, 2022; Schiele et al., 2020). Therefore, to investigate how genetic and environmental factors interact to shape the stress response as well as psychopathology, future research should consider including positive factors of the environment or individual coping abilities which possibly influence GxE effects.

In addition, interpreting PGS GxE effects, researchers should be aware that the interaction between stressful life events and psychopathology could be moderated by genetic variants that are not captured in case-control GWAS. These GWAS do not capture environmentally reactive SNPs (Halldorsdottir and Binder, 2017). It is conceivable that the SNPs included in our PGS are not susceptible to environmental stressors and are associated with depression or neuroticism only in the sense of a genetic main effect. Therefore, large-scale GWEIS are needed to identify variants that are associated with the phenotype of interest in interaction with the experience of stressful life events or positive events (Halldorsdottir and Binder, 2017; Lehto et al., 2018; Werme et al., 2021).

Another challenge investigating genetic factors of stress responses is the fact that mainly information about individuals with European ancestry is available (Fatumo et al., 2022). It was for example shown that PGS deriving from GWAS including primarily individuals with European ancestry do not perform equivalently well in individuals from other ancestries (Kullo et al., 2022; Martin et al., 2017; Martin et al., 2019b). This reinforces the already existing focus of psychological studies on the so-called WEIRD (western, educated, industrialized, rich and democratic) populations (Ghai, 2021; Henrich et al., 2010; Martin et al., 2017; Martin et al., 2019b; Shattuck, 2019). Thus, one challenge investigating GxE effects as well as unraveling the stress-disease link will be to examine mechanisms across population groups. Focusing on existing knowledge about the WEIRD population and neglecting other populations will broaden disparities (Martin et al., 2019b). Much research remains to be done to understand the genetic architecture of the stress response, to identify individuals at risk for stress-related psychopathology, and to develop treatments for stress-related disorders based on precise knowledge about mechanisms. Nevertheless, research should focus on strategies to integrate different population groups.

6.5 Final conclusion

The stress response is a highly complex phenomenon that involves the interplay between multiple systems and many different genes (Joëls and Baram, 2009; Smoller, 2016). Despite considerable effort, the identification of genetic factors modulating the stress response as well as the investigation of the interplay between environmental stressors and genetic factors remains a major challenge. The need for new study designs, novel statistical approaches, and close cooperations across disciplines became evident to unravel GxE effects in psychopathology of stress-related disorders (Dick et al., 2018; Harden and Koellinger, 2020).

With the LawSTRESS project we presented a study design which was conceptualized to investigate GxE effects. The results of the current dissertation showed that academic stress constitutes a good model to examine chronic stress as we found significant differences between the SG and

the CG in perceived stress levels, the CAR, and other stress-related variables. Although we are aware of some limitations, our study design had several advantages such as the quasi-experimental design, the multimodal data collection, and the prospective-longitudinal approach. Additionally, we collected highly differentiated phenotypes including hormonal markers, thorough psychometric characterization, and valid acquisition of current stress perception with AA, which can reduce measurement error in the outcome variable. Thus, we conclude that our design may be useful to investigate associations between chronic stress, HPA axis alterations, psychological well-being, and genetic factors.

To investigate GxE effects, we used two approaches that analyze the effect of many SNPs simultaneously to overcome some of the challenges of previous GxE studies. Examining the association between genetic variability within the NPS/NPSR1 system and stress-related alterations with GSA, we found suggestive evidence for an association between the 936 SNPs within the NPS/NPSR1 system and the CAR parameters under chronic stress conditions. Regarding the genetic disposition to depression and neuroticism, respectively, we found that NEU-PGS was associated with an increased stress sensitivity under normal and chronic stress conditions. Besides the relatively small sample size, we conclude that our study contributed to the research on the interaction between genetic factors and stress regulation in humans. However, replication of results is needed. We assume that the combination of in-depth phenotyping, a quasi-experimental design and the application of GSA and PGS analyses represent a promising approach to complement GWAS and GWEIS and offer great potential to reveal stress-related disease mechanisms in humans. A better understanding of GxE effects, as well as a better understanding of the underlying mechanisms of the relationship between stress and disease, will allow for the identification of individuals at risk and the development of improved and individualized prevention and intervention programs.

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8 Appendix

8.1 Supplementary Methods

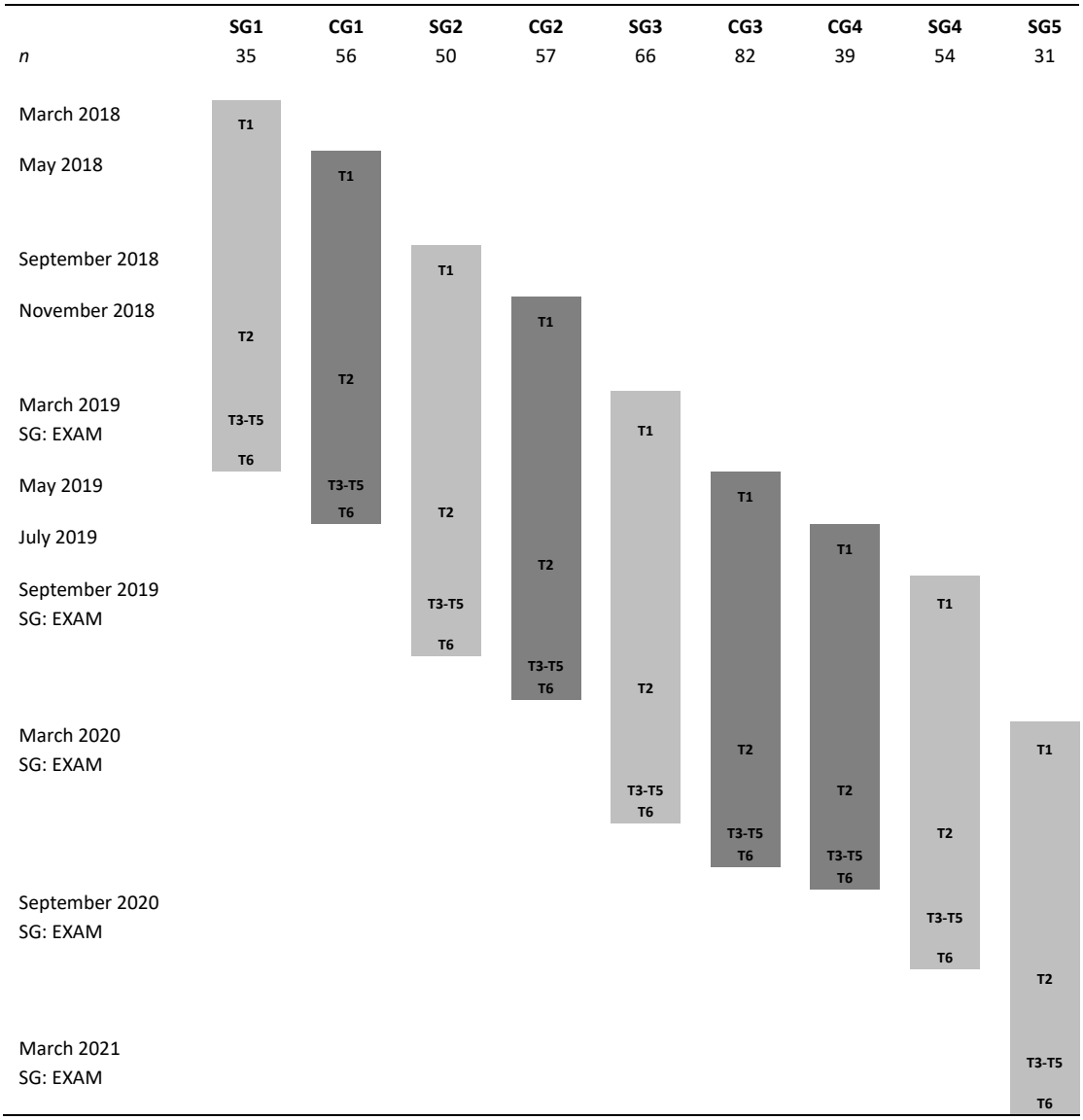

Figure 8. Nested data collection of the LawSTRESS project *Note.* CG = Control group; SG = Stress group.

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 8). Building upon this, confirmatory factor analyses of all surveys were conducted and all showed good fit indices (all CFI > .99; all RMSEA < .15).

Table 8. 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 Statistical analysis

To analyze the impact of the exam preparation on the CAR, perceived stress levels and anxiety symptoms, we computed hierarchical models using R (version 4.0.3, R Core Team, 2020). The models were estimated with Maximum Likelihood and the significance level was set at $\alpha = .05$. The analysis was described in Giglberger et al. (2022). The CAR was only assessed in cohort A, resulting in a sample of $n = 182$ for the present report.

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 et al., 2021) and MuMIn (Bartoń, 2013) were used for the analysis. Random intercepts for participants and timepoints as well as random slopes for minutes were estimated. The final model contained the categorial variables *timepoint* (t1 – t6) and *minutes* (0, 30 and 45 minutes after awakening), *group* (0 = CG, 1 = SG) and the covariates *hormonal status* (0 = women not using

hormonal contraceptives, 1 = women using hormonal contraceptives and 2 = men) and *awakening time* (person-mean centered). Cortisol data was log-transformed to base 10. For the analysis of the AA stress scale and anxiety symptoms, the whole sample of *n* = 403 was included and two level generalized linear mixed models with the package glmmTMB (Brooks et al., 2017) were computed. For the AA stress scale, the time trend was modelled with the variable *timepoint* as linear, quadratic, and cubic trend. As for the variable *AA stress scale delta* for cohort A only the first query after awakening and the last query at 09:00 p.m. were used for the present analysis to increase the homogeneity of AA stress scale measurements in cohorts A and B. Trajectories of anxiety symptoms were modelled with linear and quadratic time trend. As fixed effects the variable *group*, and the interactions between the time trends and *group* were included. Additionally, random intercepts and slopes for the participants were added. As covariate the variable *sex* was tested as main effect and in interaction with *group*. The effects are only reported in the case of model improvement. As reported in Giglberger et al. (2022) perceived stress levels in cohort B were somewhat higher in both the stress and control group over the entire study period, but the trajectories in SG and CG were very similar in both cohorts.

8.1.3 Imputation

In a first step, we used the Genotype Harmonizer v1.4.23 (Deelen et al., 2014) to detect strand flips or unresolvable ambiguous variants. Detected variants were either corrected or removed using the CEU population (Utah Residents (CEPH) with Northern and Western European ancestry) out of the 1000 Genomes Phase3 v5 (Auton et al., 2015) to provide the expected linkage disequilibrium relationships. We activated the flag --mafAlign (.30), otherwise we used the default settings.

After strand alignment, we performed phasing using Eagle v2.4.1 (Loh et al., 2016). For Eagle, we changed one parameter to improve accuracy (flags = --Kpbwt = 40000; Loh et al., 2016) and otherwise applied default settings. Genotype imputation was carried out with Minimac4 (Das et al., 2016). 1000 Genomes Phase3 v5 (Auton et al., 2015) was used as reference panel and default settings were applied.

For further analysis, only SNPs of high imputation quality (info score >= .90) were included and as output format the estimated most likely genotype was used. The final data was again checked to remove duplicate SNPs and SNPs with minor allele frequency < .01.

8.2 Supplementary Results

Table 9. Parameter estimates for overall effects for the final model with perceived stress as dependent variable.

Fixed Effects	Estimate	*SE*	*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; *SE* = Standard error.

Table 10. 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	*SE*	*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; *SE* = Standard error.

Table 11. 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	*SE*	*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; *SE* = Standard error.

Table 12. Parameter estimates for overall effects for the model of the stress group (SG.model) with the cortisol awakening response as dependent variable.

Parameter	Estimate	*SE*	*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; *SE* = Standard error.

Table 13. 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 14. Parameter estimates for overall effects of the final model with perceived stress levels as de-pendent variable.

Fixed Effects	Estimate	*SE*	*p*
Intercept	2.59	0.03	**<.001**
Timepoint	.00	0.02	.988
Timepoint^2	.01	0.01	.220
Timepoint^3	.00	0.00	**.050**
SG	.02	0.04	.507
Timepoint x SG	.45	0.03	**<.001**
Timepoint^2 x SG	-.20	0.02	**<.001**
Timepoint^3 x SG	.02	0.00	**<.001**
Random Effects	*SD*	Correlation	
		(Intercept)	
Subject (Intercept)	0.33		
Timepoint	0.06	-.26	
Marginal R^2	.09		
Conditional R^2	.67		

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

Table 15. Fit indices of the models with perceived stress levels as dependent variable.

	Model 1	Model 2	Model 3	Model 4	Model 5
AIC	37600.48	36725.17	36224.47	36159.22	35683.71
BIC	37613.90	36758.73	36264.73	36206.20	35757.53
Log Likelihood	-18798.22	-18357.59	-18106.23	-18072.61	-17830.85
Num. obs.	6069	6069	6069	6069	6069

Note. Model 1 only consists of a random intercept for participant; in model 2 a fixed effect and a random slope for the linear time trend are added; in model 3 and 4 quadratic and cubic time trends are added; model 5 (full model) includes the fixed effect for *group* and its interaction with the linear, quadratic and cubic time trend.

Table 16. Parameter estimates for overall effects of the final model with anxiety symptoms as dependent variable.

Fixed Effects	Estimate	*SE*	*p*
Intercept	1.89	0.04	**< .001**
Timepoint	.02	0.03	.383
Timepoint^2	-.01	0.00	.219
SG	-.03	0.05	.627
Timepoint x SG	.30	0.04	**< .001**
Timepoint^2 x SG	-.06	0.01	**< .001**
Random Effects	*SD*	Correlation (Intercept)	
Subject (Intercept)	0.38		
Timepoint	0.04	-.88	
Marginal R^2	.05		
Conditional R^2	.65		

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

Table 17. Fit indices of the models with anxiety symptoms as dependent variable.

	Model 1	Model 2	Model 3	Model 4
AIC	10338.94	10308.81	10186.52	10089.72
BIC	10350.13	10336.79	10220.10	10140.09
Log Likelihood	-5167.47	-5149.40	-5087.26	-5035.86
Num. obs.	1991	1991	1991	1991

Note. Model 1 only consists of a random intercept for participant; in model 2 a fixed effect and a random slope for the linear time trend are added; in model 3 the quadratic time trend is added; model 4 (full model) includes the fixed effect for *group* and its interaction with the linear and quadratic time trend.

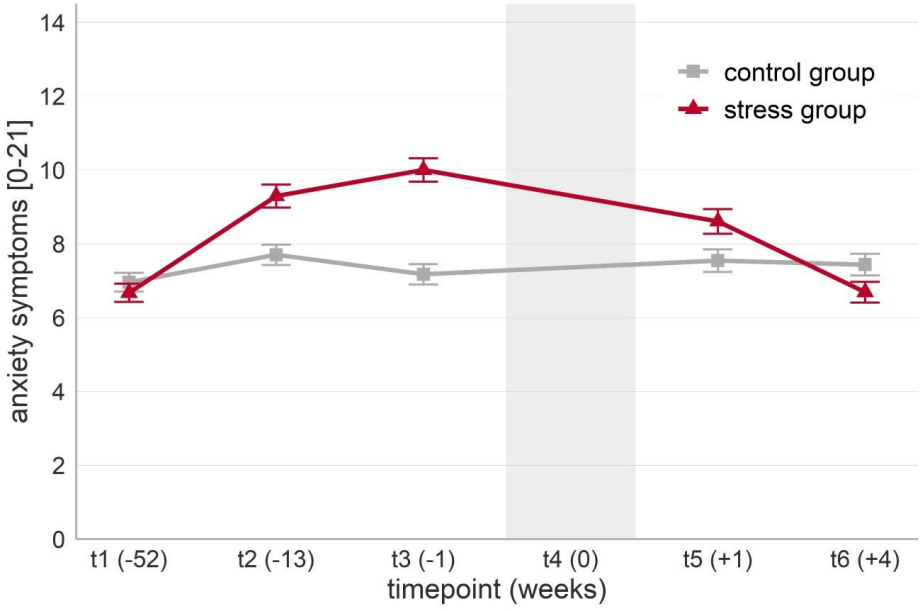

Figure 9. Mean anxiety levels (± SEM) for stress group (SG) and control group (CG) over the study period. *Note*. Anxiety symptoms were measured with the anxiety subscale of the Hospital Anxiety and Depression Scale.

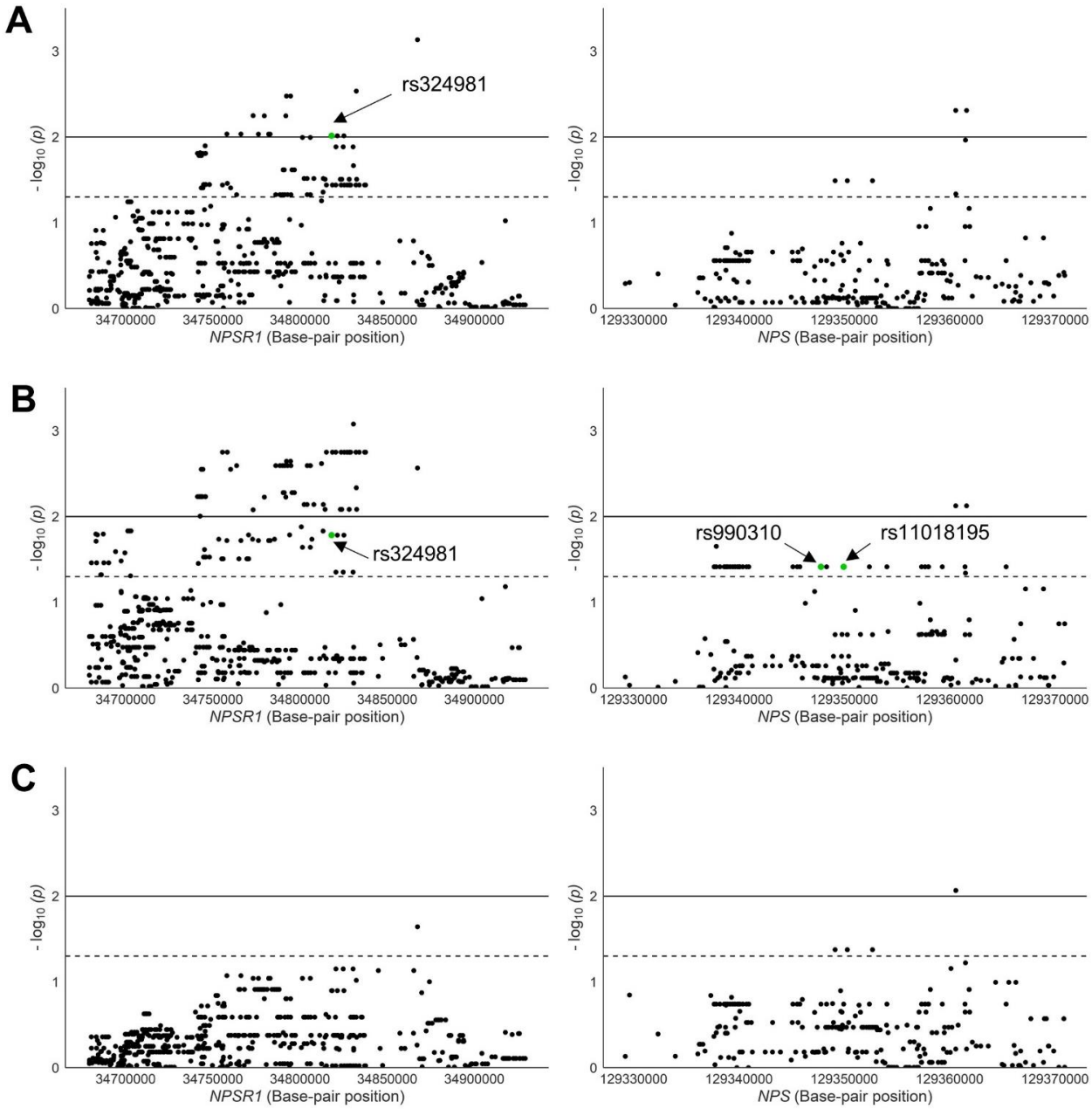

Figure 10. Plotted results of gene-wide single marker analysis for the NPS/NPSR1 system and *AUCi delta* in both groups (A), in the stress (B) and control group (C). *Note.* On the x-axis, genomic coordinates of the single nucleotide polymorphisms (SNPs) are depicted and, on the y-axis, the negative logarithm of the corresponding *p*-value is displayed for each SNP. The horizontal lines indicate association thresholds = solid line $p < .01$ and dashed line = $p < .05$. The SNPs rs324981, rs990310 and rs11018195 are marked in green.

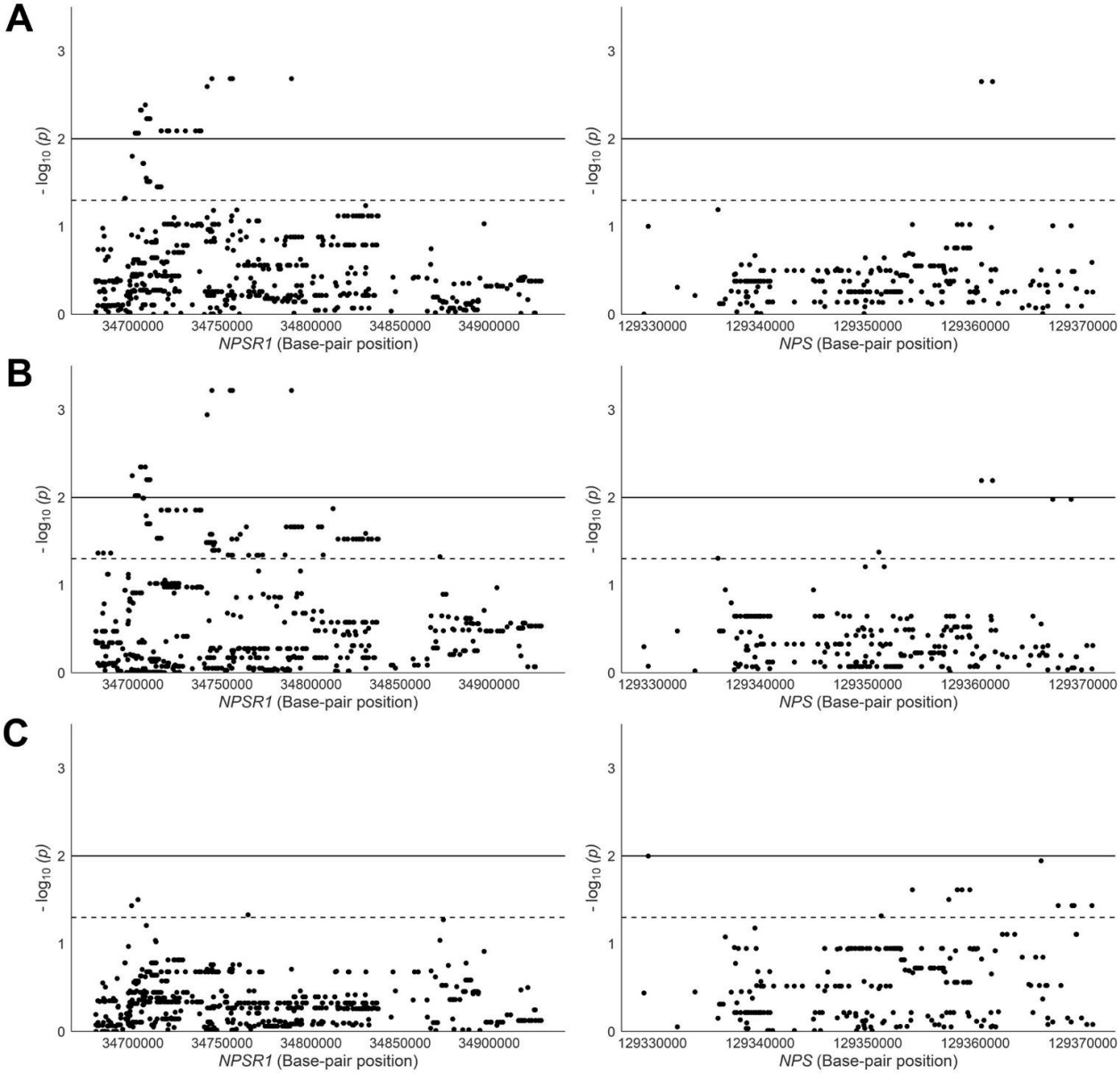

Figure 11. Plotted results of gene-wide single marker analysis for the NPS/NPSR1 system and *AUCg delta* in both groups (A), in the stress (B) and control group (C). *Note.* On the x-axis, genomic coordinates of the single nucleotide polymorphisms (SNPs) are depicted and, on the y-axis, the negative logarithm of the corresponding *p*-value is displayed for each SNP. The horizontal lines indicate association thresholds = solid line = solid line $p < .01$ and dashed line = $p < .05$.

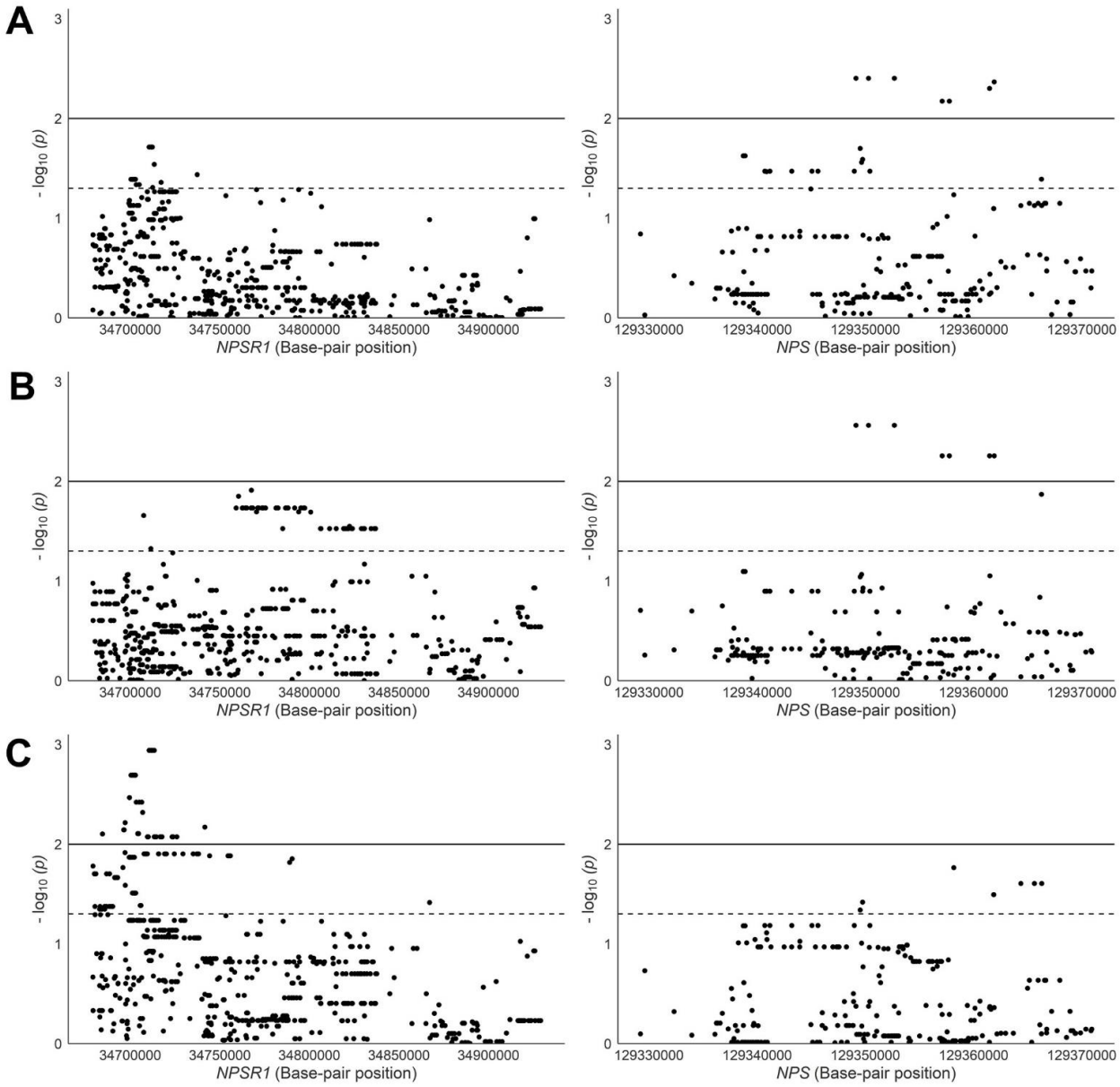

Figure 12. Plotted results of gene-wide single marker analysis for the NPS/NPSR1 system and *AA stress scale delta* in both groups (A), in the stress (B) and control group (C). *Note.* On the x-axis, genomic coordinates of the single nucleotide polymorphisms (SNPs) are depicted and, on the y-axis, the negative logarithm of the corresponding *p*-value is displayed for each SNP. The horizontal lines indicate association thresholds = solid line $p < .01$ and dashed line = $p < .05$.

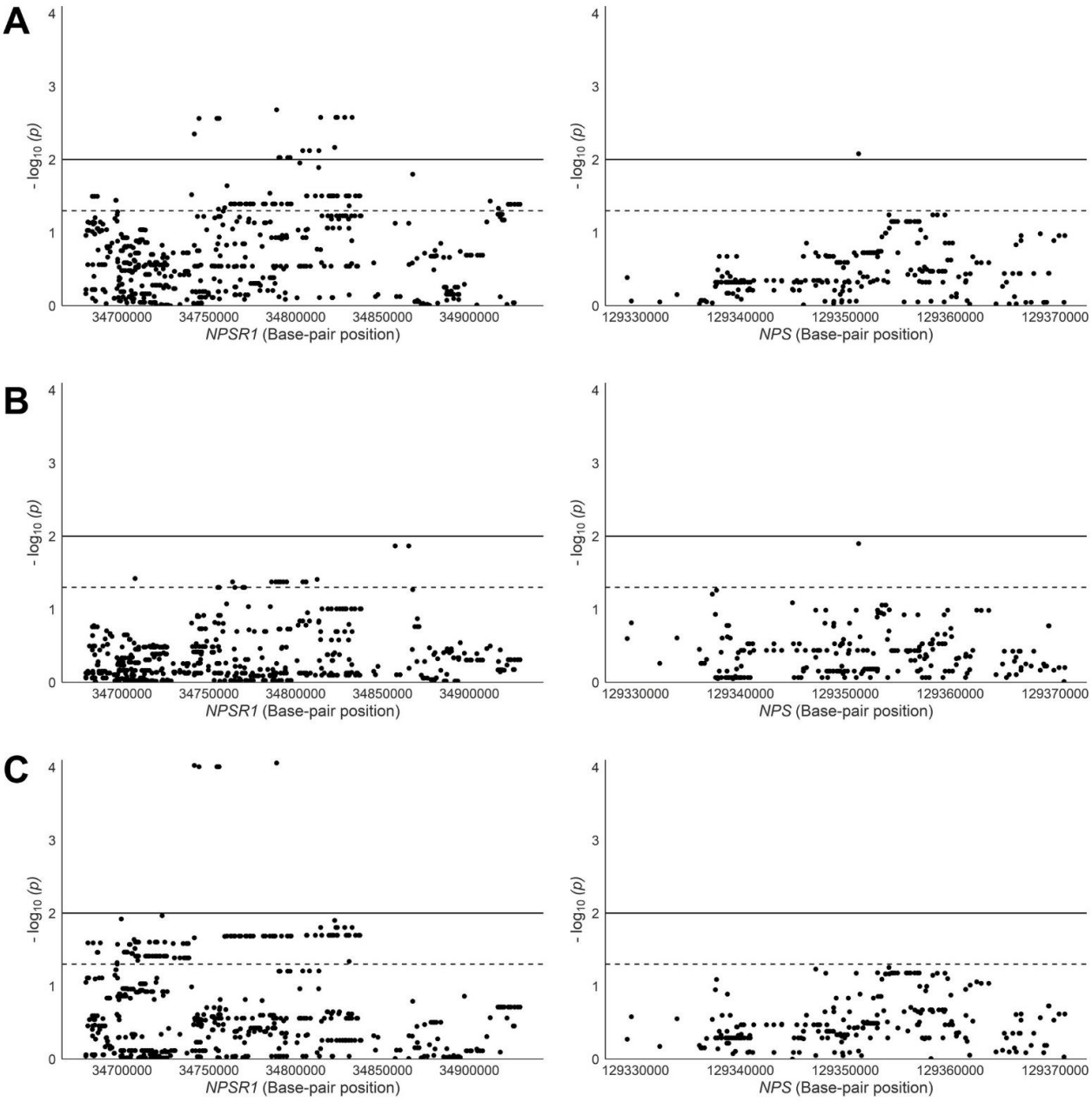

Figure 13. Plotted results of gene-wide single marker analysis for the NPS/NPSR1 system and *anxiety delta* in both groups (A), in the stress (B) and control group (C). *Note.* On the x-axis, genomic coordinates of the single nucleotide polymorphisms (SNPs) are depicted and, on the y-axis, the negative logarithm of the corresponding *p*-value is displayed for each SNP. The horizontal lines indicate association thresholds = solid line $p < .01$ and dashed line = $p < .05$

Table 18. Parameter estimates for overall effects of the models with perceived stress as dependent variable and the polygenic score for depression as predictor.

	group.model			PGS.model			PC.model		
	Estimates	*SE*	*p*	Estimates	*SE*	*p*	Estimates	*SE*	*p*
Intercept	2.46	0.04	**< .001**	2.46	0.04	**< .001**	2.46	0.04	**< .001**
Timepoint	.07	0.01	**.001**	.07	0.01	**.001**	.07	0.01	**.001**
Timepoint^2	-.02	0.00	**< .001**	-.02	0.00	**< .001**	-.02	0.00	**< .001**
Women (vs. men)	.07	0.03	.054	.07	0.03	.062	.07	0.04	**.040**
Cohort B (vs. cohort A)	.17	0.03	**< .001**	.17	0.03	**< .001**	.17	0.03	**< .001**
SG (vs. CG)	.10	0.03	**.003**	.09	0.03	**.003**	.10	0.03	**.003**
Timepoint x SG	.18	0.02	**< .001**	.18	0.02	**< .001**	.18	0.02	**< .001**
Timepoint^2 x SG	-.04	0.00	**< .001**	-.04	0.00	**< .001**	-.04	0.00	**< .001**
PGS				-.01	0.02	.586	-.01	0.02	.539
PGS x SG				.04	0.03	.201	.04	0.03	.180
PGS x timepoint				-.02	0.01	.171	-.02	0.01	.173
PGS x timepoint^2				.01	0.00	**.018**	.01	0.00	**.018**
PGS x timepoint x SG				.02	0.02	.248	.02	0.02	.249
PGS x timepoint^2 x SG				-.01	0.00	**.047**	.01	0.00	**.047**
PC1							-.23	0.33	.497
PC2							-.64	0.34	.063
PC3							.06	0.34	.872
PC4							-.22	0.34	.521
PC5							-.02	0.34	.950
AIC			69670.06			69673.43			69678.97
BIC			69751.59			69799.43			69842.03
Log-likelihood			-34824.03			-34819.71			-34817.49
Observations			12230			12230			12230
n			432			432			432
SD: participant (Intercept)			0.31			0.31			0.31
SD: timepoint			0.11			0.11			0.11
Correlation: participant x timepoint			-.27			-.27			-.27
Marginal R^2			0.1005			0.1032			0.1071
Conditional R^2			0.6612			0.6612			0.6610

Note. The group.model consists of a random intercept for participant, a random slope for the linear time trend and the fixed effects for the linear and quadratic time trend, group and its interactions with the time trends as well as the covariates sex and cohort; The PGS.model includes the additional fixed effects PGS, PGS in interaction with group as well as with the linear and quadratic time trend, and the three way interaction of the PGS, group and the time trends; In the PC.model the covariates PC1 to 5 are added; CG = Control group; PC = Principal component; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error; SG = Stress group.

Table 19. Parameter estimates for overall effects of the models with perceived stress as dependent variable and the polygenic score for neuroticism as predictor.

	group.model			PGS.model			PC.model		
	Estimates	*SE*	*p*	Estimates	*SE*	*p*	Estimates	*SE*	*p*
Intercept	2.46	0.04	**< .001**	2.46	0.04	**< .001**	2.45	0.04	**< .001**
Timepoint	.07	0.01	**< .001**	.07	0.01	**< .001**	.07	0.01	**< .001**
Timepoint^2	-.02	0.00	**.003**	-.02	0.00	**< .001**	-.02	0.00	**< .001**
Women (vs. men)	.07	0.03	.054	.07	0.03	**.049**	.08	0.03	**.027**
Cohort B (vs. cohort A)	.17	0.03	**< .001**	.18	0.03	**< .001**	.18	0.03	**< .001**
SG (vs. CG)	.10	0.03	**.003**	.09	0.03	**.004**	.09	0.03	**.003**
Timepoint x SG	.18	0.02	**< .001**	.18	0.02	**< .001**	.18	0.02	**< .001**
Timepoint^2 x SG	-.04	0.00	**< .001**	-.05	0.00	**< .001**	-.05	0.00	**< .001**
PGS				.05	0.02	**.034**	.05	0.02	**.025**
PGS x SG				.02	0.03	.460	.02	0.03	.443
PGS x timepoint				.02	0.01	.093	.02	0.01	.092
PGS x timepoint^2				-.01	0.00	**.005**	-.01	0.00	**.005**
PGS x timepoint x SG				-.06	0.02	**.001**	-.06	0.02	**.001**
PGS x timepoint^2 x SG				.02	0.00	**< .001**	.02	0.00	**< .001**
PC1							-.39	0.33	.242
PC2							-.63	0.34	.061
PC3							.05	0.34	.892
PC4							-.28	0.34	.409
PC5							.00	0.33	.993
AIC			69670.06			69643.29			69647.62
BIC			69751.59			69769.29			69810.67
Log-likelihood			-34824.03			-34804.65			-34801.81
Observations			12230			12230			12230
n			432			432			432
SD: participant (Intercept)			0.31			0.31			0.30
SD: timepoint			0.11			0.11			0.11
Correlation: participant x timepoint			-.27			-.27			-.27
Marginal R^2			0.1006			0.1159			0.1198
Conditional R^2			0.6612			0.6609			0.6602

Note. The group.model consists of a random intercept for participant, a random slope for the linear time trend and the fixed effects for the linear and quadratic time trend, group and its interactions with the time trends as well as the covariates sex and cohort; The PGS.model includes the additional fixed effects PGS, PGS in interaction with group as well as with the linear and quadratic time trend, and the three way interaction of the PGS, group and the time trends; In the PC.model the covariates PC1 to 5 are added; CG = Control group; PC = Principal component; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error; SG = Stress group.

Table 20. Parameter estimates for overall effects of the models with depression symptoms as dependent variable and the polygenic score for depression as predictor.

	group.model			PGS.model			PC.model		
	Estimates	*SE*	*p*	Estimates	*SE*	*p*	Estimates	*SE*	*p*
Intercept	1.31	0.08	**< .001**	1.31	0.08	**< .001**	1.31	0.08	**< .001**
Timepoint	.17	0.09	.059	.17	0.09	.058	.17	0.09	.062
Timepoint^2	-.06	0.04	.123	-.06	0.04	.122	-.06	0.04	.122
Women (vs. men)	-.14	0.07	**.043**	-.14	0.07	**.045**	-.14	0.07	.050
SG (vs. CG)	-.08	0.08	.285	-.08	0.08	.282	.50	0.12	**< .001**
Timepoint x SG	.50	0.12	**< .001**	.50	0.12	**< .001**	-.11	0.06	**.047**
Timepoint^2 x SG	-.11	0.06	**.043**	-.11	0.06	**.047**	-.08	0.08	.270
PGS				.07	0.06	.241	.06	0.06	.299
PGS x SG				-.05	0.08	.520	-.04	0.08	.585
PGS x timepoint				-.06	0.09	.527	-.06	0.09	.532
PGS x timepoint^2				.01	0.04	.865	.01	0.04	.868
PGS x timepoint x SG				.20	0.12	.105	.20	0.12	.108
PGS x timepoint^2 x SG				-.07	0.06	.198	-.07	0.06	.202
PC1							-.13	0.68	.847
PC2							-.50	0.71	.479
PC3							.76	0.69	.272
PC4							.82	0.71	.252
PC5							.47	0.70	.497
AIC			5835.56			5840.48			5846.94
BIC			5886.71			5922.33			5954.37
Log-likelihood			-2907.78			-2904.24			-2902.47
Observations			1231			1231			1231
n			432			432			432
SD: participant (Intercept)			0.58			0.58			0.57
SD: timepoint			0.18			0.18			0.18
Correlation: participant x timepoint			-.31			-.30			-.28
Marginal R^2			0.0848			0.0878			0.0923
Conditional R^2			0.6076			0.6085			0.6078

Note. The group.model consists of a random intercept for participant, a random slope for the linear time trend and the fixed effects for the linear and quadratic time trend, group and its interactions with the time trends as well as the covariates sex and cohort; The PGS.model includes the additional fixed effects PGS, PGS in interaction with group as well as with the linear and quadratic time trend, and the three way interaction of the PGS, group and the time trends; In the PC.model the covariates PC1 to 5 are added; CG = Control group; PC = Principal component; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error; SG = Stress group.

Table 21. Parameter estimates for overall effects of the models with depression symptoms as dependent variable and the polygenic score for neuroticism as predictor.

	group.model			PGS.model			PC.model		
	Estimates	*SE*	*p*	Estimates	*SE*	*p*	Estimates	*SE*	*p*
Intercept	1.31	0.08	**< .001**	1.32	0.08	**< .001**	1.32	0.08	**< .001**
Timepoint	.17	0.09	.059	.17	0.09	.057	.17	0.09	.061
Timepoint^2	-.06	0.04	.123	-.06	0.04	.121	-.06	0.04	.122
Women (vs. men)	-.14	0.07	**.043**	-.15	0.07	**.037**	-.14	0.07	**.043**
SG (vs. CG)	-.08	0.08	.285	-.08	0.08	.273	-.09	0.08	.260
Timepoint x SG	.50	0.12	**< .001**	.50	0.12	**< .001**	.50	0.12	**< .001**
Timepoint^2 x SG	-.11	0.06	**.043**	-.11	0.06	**.046**	-.11	0.06	**.046**
PGS				-.03	0.05	.527	-.03	0.05	.555
PGS x SG				.13	0.08	.089	.13	0.08	.095
PGS x timepoint				.09	0.09	.351	.09	0.09	.344
PGS x timepoint^2				-.05	0.04	.297	-.05	0.04	.294
PGS x timepoint x SG				-.04	0.13	.762	-.04	0.13	.759
PGS x timepoint^2 x SG				.03	0.06	.638	.03	0.06	.636
PC1							-.19	0.68	.774
PC2							-.54	0.70	.435
PC3							.81	0.69	.240
PC4							.70	0.71	.321
PC5							.49	0.69	.474
AIC			5835.56			5838.66			5845.05
BIC			5886.71			5920.51			5952.47
Log-likelihood			-2907.78			-2903.33			-2901.52
Observations			1231			1231			1231
n			432			432			432
SD: participant (Intercept)			0.58			0.58			0.57
SD: timepoint			0.18			0.18			0.18
Correlation: participant x timepoint			-.31			-.31			-.29
Marginal R^2			0.0848			0.0946			0.0991
Conditional R^2			0.6076			0.6075			0.6068

Note. The group.model consists of a random intercept for participant, a random slope for the linear time trend and the fixed effects for the linear and quadratic time trend, group and its interactions with the time trends as well as the covariates sex and cohort; The PGS.model includes the additional fixed effects PGS, PGS in interaction with group as well as with the linear and quadratic time trend, and the three way interaction of the PGS, group and the time trends; In the PC.model the covariates PC1 to 5 are added; CG = Control group; PC = Principal component; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error; SG = Stress group.

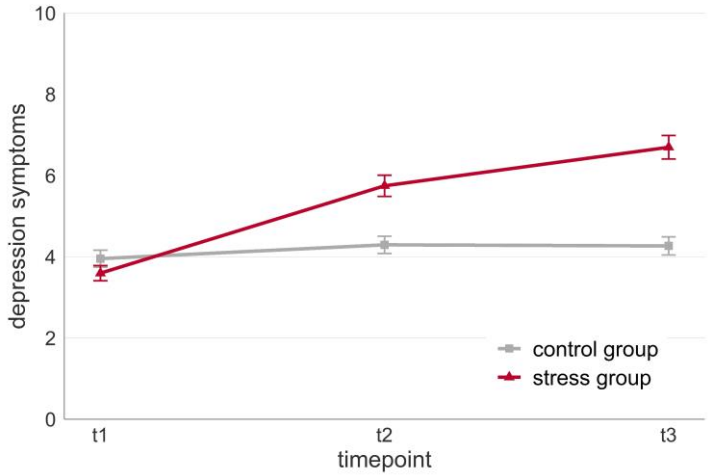

Figure 14. Time course of mean depression symptoms (± SEM) in the stress group (SG) and control group (CG) until one week prior exam.

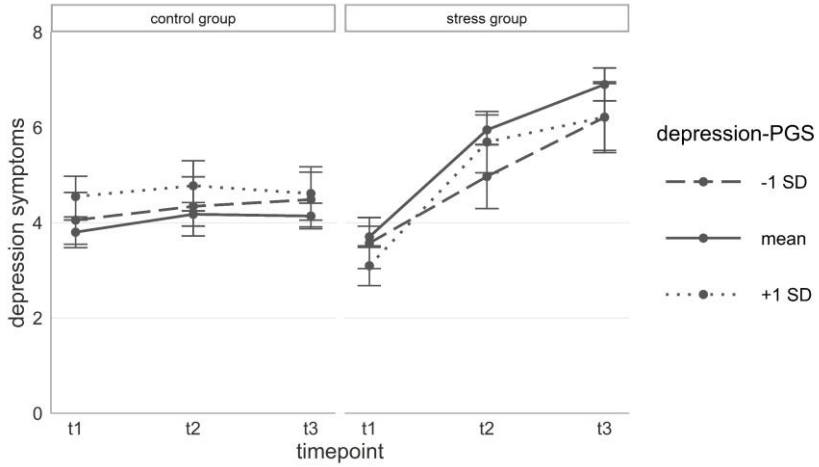

Figure 15. Time course of mean depression symptoms (± SEM) in the stress group (SG) and control group (CG) separated by polygenic score (PGS) for depression (grouping based on SD for illustrative purposes only). *Note. SD* = standard deviation.

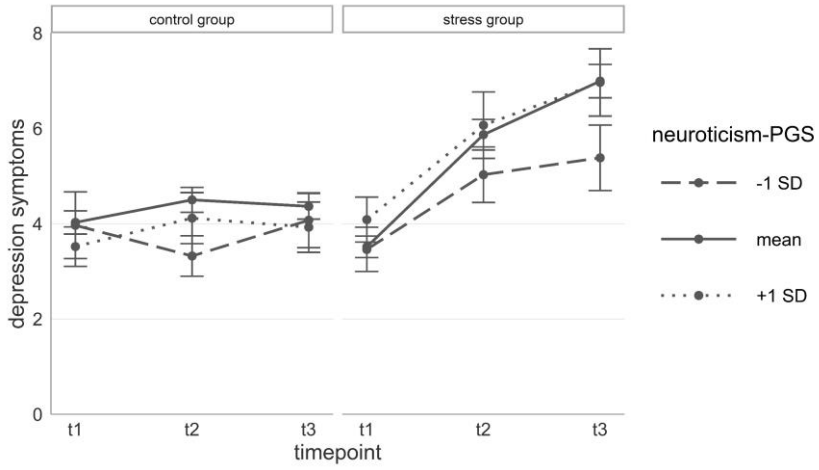

Figure 16. Time course of mean depression symptoms (± SEM) in the stress group (SG) and control group (CG) separated by polygenic score (PGS) for neuroticism (grouping based on SD for illustrative purposes only). *Note. SD* = standard deviation.

Table 22. Parameter estimates for overall effects of the models with area under the curve with respect to the ground (*AUCg*) as dependent variable and the polygenic score for depression as predictor.

	group.model			PGS.model			PC.model		
	Estimates	*SE*	*p*	Estimates	*SE*	*p*	Estimates	*SE*	*p*
Intercept	549.93	19.66	**< .001**	548.32	19.64	**< .001**	546.06	19.71	**< .001**
Timepoint	-7.62	5.40	.158	-7.67	5.41	.157	-7.69	5.42	.157
Women using HC (vs. other groups)	-77.64	21.73	**< .001**	-76.61	21.68	**.001**	-73.31	21.82	**.001**
Men (vs. other groups)	-64.31	25.22	**.012**	-60.03	25.31	**.019**	-60.35	25.54	**.019**
Awakening time	27.02	12.81	**.035**	26.84	12.83	**.037**	26.83	12.87	**.037**
SG (vs. CG)	2.83	20.00	.888	2.39	19.96	.905	3.39	19.90	.865
Timepoint x SG	-19.15	7.89	**.016**	-18.99	7.91	**.017**	-18.96	7.93	**.017**
PGS				-13.55	14.02	.335	-13.44	14.00	.339
PGS x SG				21.59	19.88	.279	24.48	19.87	.220
PGS x timepoint				-3.24	5.41	.550	-3.30	5.43	.543
PGS x timepoint x SG				2.43	7.83	.757	2.47	7.85	.753
PC1							-325.34	199.88	.105
PC2							72.69	205.90	.725
PC3							-45.71	208.08	.826
PC4							-115.45	215.26	.592
PC5							143.68	20.91	.475
AIC			11879.83			11885.19			11891.58
BIC			11923.21			11947.85			11978.34
Log-likelihood			-5930.91			-5929.59			-5927.79
Observations			196			196			196
n			916			916			916
SD: participant (Intercept)			105.40			104.48			103.14
Marginal R^2			0.0553			0.0615			0.0700
Conditional R^2			0.4083			0.4090			0.4088

Note. The group.model consists of a random intercept for participant and the fixed effects for the linear time trend, group and its interactions with the time trend as well as the covariates hormonal status and awakening time; The PGS.model includes the additional fixed effects PGS, PGS in interaction with group as well as with the linear time trend, and the three way interaction of the PGS, group and the linear time trend; In the PC.model the covariates PC1 to 5 are added; CG = Control group; HC = Hormonal contraception; PC = Principal component; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error; SG = Stress group.

Table 23. Parameter estimates for overall effects of the models with area under the curve with respect to the ground (*AUCg*) as dependent variable and the polygenic score for neuroticism as predictor.

	group.model			PGS.model			PC.model		
	Estimates	*SE*	*p*	Estimates	*SE*	*p*	Estimates	*SE*	*p*
Intercept	549.93	19.66	**< .001**	547.89	19.08	**< .001**	544.98	19.19	**< .001**
Timepoint	-7.62	5.40	.158	-7.66	5.41	.157	-7.67	5.42	.158
Women using HC (vs. other groups)	-77.64	21.73	**< .001**	-75.86	21.01	**< .001**	-71.86	21.21	**.001**
Men (vs. other groups)	-64.31	25.22	**.012**	-59.06	24.42	**.017**	-57.57	24.74	**.021**
Awakening time	27.02	12.81	**.035**	27.47	12.89	**.033**	27.46	12.92	**.034**
SG (vs. CG)	2.83	20.00	.888	2.39	19.47	.903	3.33	19.45	.864
Timepoint x SG	-19.15	7.89	**.016**	-19.25	7.91	**.015**	-19.28	7.93	**.015**
PGS				-47.40	13.24	**< .001**	-46.66	13.31	**.001**
PGS x SG				48.23	19.36	**.014**	48.99	19.42	**.013**
PGS x timepoint				2.67	5.28	.614	2.66	5.29	.615
PGS x timepoint x SG				-4.91	7.96	.538	-5.03	7.98	.529
PC1							-216.52	194.26	.267
PC2							121.61	20.22	.544
PC3							-77.85	202.28	.701
PC4							-137.73	21.26	.513
PC5							164.52	194.88	.400
AIC			11879.83			11873.43			11880.74
BIC			11923.21			11936.09			11967.50
Log-likelihood			-5930.91			-5923.72			-5922.37
Observations			196			196			196
n			916			916			916
SD: participant (Intercept)			105.40			100.13			99.11
Marginal R^2			0.0553			0.0897			0.0956
Conditional R^2			0.4083			0.4089			0.4085

Note. The group.model consists of a random intercept for participant and the fixed effects for the linear time trend, group and its interactions with the time trend as well as the covariates hormonal status and awakening time; The PGS.model includes the additional fixed effects PGS, PGS in interaction with group as well as with the linear time trend, and the three way interaction of the PGS, group and the linear time trend; In the PC.model the covariates PC1 to 5 are added; CG = Control group; HC = Hormonal contraception; PC = Principal component; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error; SG = Stress group.

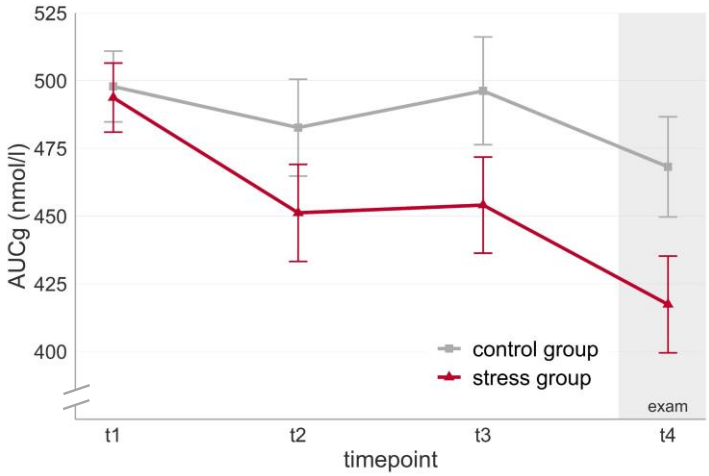

Figure 17. Time course of mean area under the curve with respect to the ground (*AUCg* ± SEM) in the stress group (SG) and control group (CG) until the exam at timepoint 4.

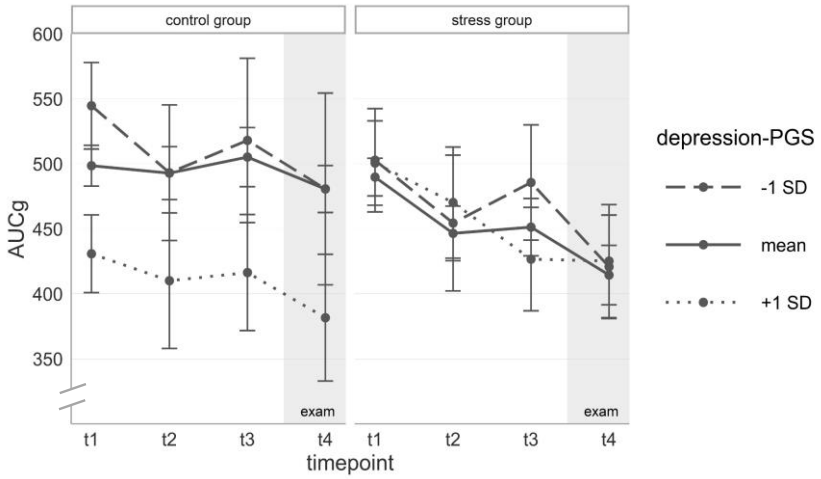

Figure 18. Time course of mean area under the curve with respect to the ground (*AUCg* ± SEM) in the stress group (SG) and control group (CG) separated by polygenic score (PGS) for depression (grouping based on SD for illustrative purposes only). *Note. SD* = standard deviation.

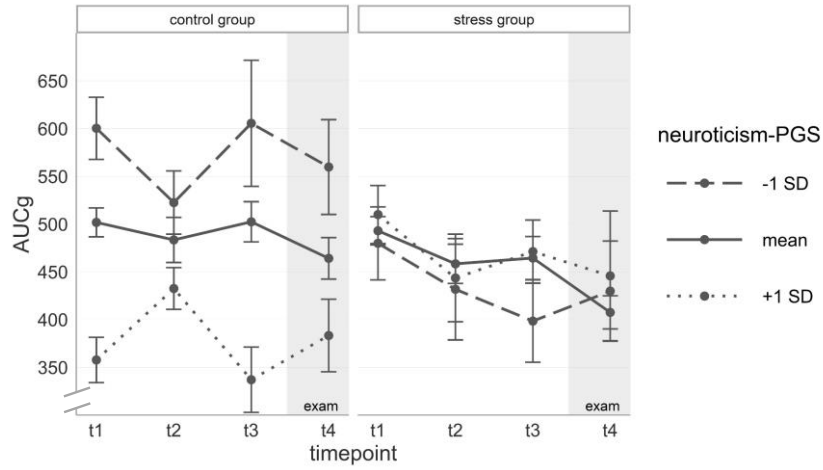

Figure 19. Time course of mean area under the curve with respect to the ground (*AUCg* ± SEM) in the stress group (SG) and control group (CG) separated by polygenic score (PGS) for neuroticism (grouping based on SD for illustrative purposes only). *Note. SD* = standard deviation.

Table 24. Parameter estimates for overall effects of the models only containing the stress group (SG) with area under the curve with respect to the ground (*AUCg*) as dependent variable and the polygenic score for neuroticism as predictor.

	SG.model			PGS.model			PC.model		
	Estimates	*SE*	*p*	Estimates	*SE*	*p*	Estimates	*SE*	*p*
Intercept	534.47	27.39	**< .001**	534.45	27.45	**< .001**	532.94	27.67	**< .001**
Timepoint	-24.28	5.95	**< .001**	-24.43	5.97	**< .001**	-24.49	6.01	**< .001**
Women using HC (vs. other groups)	-64.75	3.99	**.040**	-64.69	31.08	**.040**	-61.47	31.62	.055
Men (vs. other groups)	-34.11	34.05	.319	-34.09	34.13	.321	-35.44	34.45	.307
Awakening time	-12.77	21.39	.551	-13.09	21.45	.542	-12.85	21.59	.552
PGS				1.49	13.29	.911	1.52	13.55	.911
PGS x timepoint				-2.61	5.93	.660	-2.72	5.97	.649
PC1							-8.63	264.33	.761
PC2							-92.67	271.67	.734
PC3							-271.02	274.99	.327
PC4							15.64	318.23	.961
PC5							256.08	265.92	.338
AIC			5527.23			5531.02			5538.89
BIC			5555.63			5567.53			5595.68
Log-likelihood			-2756.62			-2756.51			-2755.44
Observations			95			95			95
n			427			427			427
SD: participant (Intercept)			89.86			89.87			88.15
Marginal R^2			0.0530			0.0534			0.0633
Conditional R^2			0.3384			0.3388			0.3380

Note. The SG.model consists of a random intercept for participant and the fixed effects for the linear time trend and the covariates hormonal status and awakening time; The PGS.model includes the additional fixed effects PGS and PGS in interaction with the linear time trend; In the PC.model the covariates PC1 to 5 are added; HC = Hormonal contraception; PC = Principal component; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error; SG = Stress group.

Table 25. Parameter estimates for overall effects of the models only containing the control group (CG) with area under the curve with respect to the ground (*AUCg*) as dependent variable and the polygenic score for neuroticism as predictor.

	CG.model			PGS.model			PC.model		
	Estimates	*SE*	*p*	Estimates	*SE*	*p*	Estimates	*SE*	*p*
Intercept	559.91	24.31	**< .001**	556.84	23.09	**< .001**	557.02	23.01	**< .001**
Timepoint	-8.74	5.38	.105	-8.86	5.39	.101	-8.84	5.42	.103
Women using HC (vs. other groups)	-83.66	3.30	**.007**	-81.17	28.65	**.006**	-82.98	28.62	**.005**
Men (vs. other groups)	-91.68	37.28	**.016**	-81.52	35.35	**.023**	-79.39	35.65	**.028**
Awakening time	49.60	15.91	**.002**	50.78	16.03	**.002**	50.77	16.11	**.002**
PGS				-48.42	14.02	**.001**	-46.55	14.07	**.001**
PGS x timepoint				3.59	5.32	.501	3.55	5.35	.508
PC1							-436.21	285.16	.130
PC2							395.85	294.46	.182
PC3							127.72	295.02	.666
PC4							-272.26	285.04	.342
PC5							102.57	284.72	.720
AIC			6352.37			6344.04			6349.39
BIC			6381.72			6381.78			6408.09
Log-likelihood			-3169.19			-3163.02			-3160.70
Observations			101			101			101
n			489			489			489
SD: participant (Intercept)			116.41			107.88			104.57
Marginal R^2			0.0599			0.1176			0.1382
Conditional R^2			0.4609			0.4618			0.4616

Note. The CG.model consists of a random intercept for participant and the fixed effects for the linear time trend and the covariates hormonal status and awakening time; The PGS.model includes the additional fixed effects PGS and PGS in interaction with the linear time trend; In the PC.model the covariates PC1 to 5 are added; CG = Control group; HC = Hormonal contraception; PC = Principal component; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error.

Table 26. Parameter estimates for overall effects of the models with area under the curve with respect to the increase (*AUCi*) as dependent variable and the polygenic score for depression as predictor.

	group.model			PGS.model			PC.model		
	Estimates	*SE*	*p*	Estimates	*SE*	*p*	Estimates	*SE*	*p*
Intercept	228.30	14.50	**< .001**	227.94	14.55	**< .001**	224.85	14.62	**< .001**
Timepoint	-35.13	16.11	**.030**	-34.97	16.14	**.031**	-34.88	16.19	**.032**
Timepoint^2	9.09	5.36	.091	9.06	5.38	.092	9.03	5.39	.094
Women using HC (vs. other groups)	-78.03	14.37	**< .001**	-77.96	14.43	**< .001**	-74.34	14.53	**< .001**
Men (vs. other groups)	-75.75	16.77	**< .001**	-75.25	16.94	**< .001**	-72.68	17.09	**< .001**
Awakening time	-38.44	11.76	**.001**	-38.04	11.79	**.001**	-37.99	11.82	**.001**
SG (vs. CG)	-10.60	16.11	.511	-10.34	16.13	.523	-9.12	16.15	.573
Timepoint x SG	38.46	24.14	.112	38.31	24.20	.114	38.21	24.27	.116
Timepoint^2 x SG	-19.81	8.12	**.015**	-19.80	8.14	**.015**	-19.81	8.16	**.016**
PGS				-13.12	11.33	.249	-13.22	11.35	.246
PGS x SG				10.46	16.08	.516	11.14	16.11	.490
PGS x timepoint				20.60	16.28	.206	20.31	16.33	.214
PGS x timepoint^2				-4.89	5.42	.368	-4.84	5.44	.374
PGS x timepoint x SG				-19.18	23.59	.416	-18.77	23.66	.428
PGS x timepoint^2 x SG				4.87	7.87	.536	4.78	7.90	.545
PC1							-55.59	133.48	.678
PC2							86.14	137.37	.531
PC3							-24.82	139.19	.859
PC4							-172.11	144.34	.235
PC5							216.73	134.13	.108
AIC			11527.59			11537.17			11543.19
BIC			11590.25			11628.75			11658.87
Log-likelihood			-5750.80			-5749.58			-5747.59
Observations			196			196			196
n			916			916			916
SD: participant (Intercept)			78.30			77.99			77.50
SD: timepoint			34.39			34.08			33.95
Correlation: participant x timepoint			-.61			-.61			-.62
Marginal R^2			0.1118			0.1143			0.1207
Conditional R^2			0.3843			0.3853			0.3851

Note. The group.model consists of a random intercept for participant, a random slope for the linear time trend and the fixed effects for the linear and quadratic time trend, group and its interactions with the time trends as well as the covariates hormonal status and awakening time; The PGS.model includes the additional fixed effects PGS, PGS in interaction with group as well as with the linear and quadratic time trend, and the three way interaction of the PGS, group and the time trends; In the PC.model the covariates PC1 to 5 are added; CG = Control group; HC = Hormonal contraception; PC = Principal component; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error; SG = Stress group.

Table 27. Parameter estimates for overall effects of the models with area under the curve with respect to the increase (*AUCi*) as dependent variable and the polygenic score for neuroticism as predictor.

	group.model			PGS.model			PC.model		
	Estimates	*SE*	*p*	Estimates	*SE*	*p*	Estimates	*SE*	*p*
Intercept	228.30	14.50	**< .001**	227.68	14.41	**< .001**	224.17	14.48	**< .001**
Timepoint	-35.13	16.11	**.030**	-35.13	16.12	**.030**	-34.98	16.16	**.031**
Timepoint^2	9.09	5.36	.091	9.08	5.37	.091	9.03	5.38	.094
Women using HC (vs. other groups)	-78.03	14.37	**< .001**	-77.61	14.27	**< .001**	-73.50	14.35	**< .001**
Men (vs. other groups)	-75.75	16.77	**< .001**	-73.98	16.68	**< .001**	-70.61	16.83	**< .001**
Awakening time	-38.44	11.76	**.001**	-38.04	11.81	**.001**	-38.04	11.84	**.001**
SG (vs. CG)	-10.60	16.11	.511	-10.75	16.01	.503	-9.54	16.04	.553
Timepoint x SG	38.46	24.14	.112	38.23	24.18	.114	37.99	24.25	.118
Timepoint^2 x SG	-19.81	8.12	**.015**	-19.65	8.13	**.016**	-19.62	8.15	**.016**
PGS				-25.33	1.91	**.021**	-25.99	1.98	**.019**
PGS x SG				28.93	15.94	.071	30.61	16.02	.058
PGS x timepoint				28.63	15.69	.068	28.72	15.73	.068
PGS x timepoint^2				-8.82	5.23	.092	-8.86	5.24	.091
PGS x timepoint x SG				-36.28	23.72	.127	-36.55	23.78	.125
PGS x timepoint^2 x SG				12.09	7.95	.129	12.12	7.98	.129
PC1							-23.52	131.97	.859
PC2							109.82	135.87	.420
PC3							-39.00	137.61	.777
PC4							-189.28	143.26	.188
PC5							227.49	132.25	.087
AIC			11527.59			11531.84			11537.37
BIC			11590.25			11623.42			11653.05
Log-likelihood			-5750.80			-5746.92			-5744.69
Observations			196			196			196
n			916			916			916
SD: participant (Intercept)			78.30			77.07			76.72
SD: timepoint			34.39			34.44			34.32
Correlation: participant x timepoint			-.61			-.61			-.63
Marginal R^2			0.1118			0.1217			0.1287
Conditional R^2			0.3843			0.3881			0.3878

Note. The group.model consists of a random intercept for participant, a random slope for the linear time trend and the fixed effects for the linear and quadratic time trend, group and its interactions with the time trends as well as the covariates hormonal status and awakening time; The PGS.model includes the additional fixed effects PGS, PGS in interaction with group as well as with the linear and quadratic time trend, and the three way interaction of the PGS, group and the time trends; In the PC.model the covariates PC1 to 5 are added; CG = Control group; HC = Hormonal contraception; PC = Principal component; PGS = Polygenic Score; *SD* = Standard deviation; *SE* = Standard error; SG = Stress group.

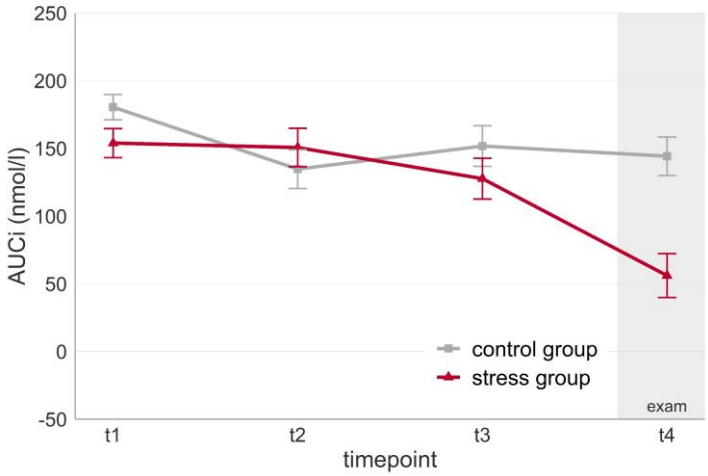

Figure 20. Time course of mean area under the curve with respect to the increase (*AUCi* ± SEM) in the stress group (SG) and control group (CG) until the exam at timepoint 4.

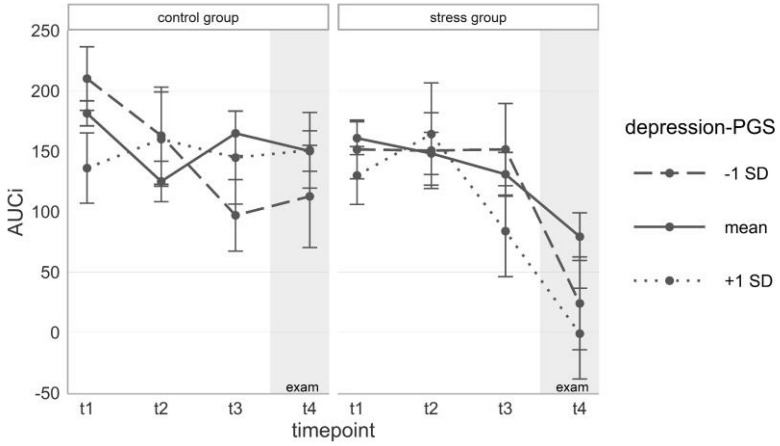

Figure 21. Time course of mean area under the curve with respect to the increase (*AUCi* ± SEM) in the stress group (SG) and control group (CG) separated by polygenic score (PGS) for depression (grouping based on SD for illustrative purposes only). *Note. SD* = standard deviation.

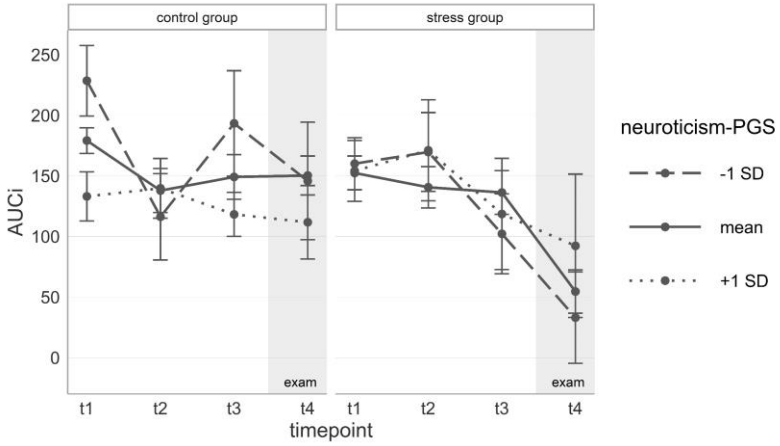

Figure 22. Time course of mean area under the curve with respect to the increase (*AUCi* ± SEM) in the stress group (SG) and control group (CG) separated by polygenic score (PGS) for neuroticism (grouping based on SD for illustrative purposes only). *Note. SD* = standard deviation.