From Caloric Load to the Measurement of Fear and Anxiety

Contributions to the Improvement of Methods in Biopsychological Stress Research

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Foreword

The present work comprises three manuscripts exploring the methodology of two biopsychological paradigms with the aim to provide improvements for future applications. The chapters were composed specifically for this thesis, with three chapters based on manuscripts that have already been published as review article (**Chapter 3**), original research paper (**Chapter 4**) or are currently under review as original research paper (**Chapter 5**). They are listed below in order of appearance within the thesis, and are currently not used or designated for use in other dissertations. For improved readability, contents, tables, and figures were numbered continuously and designed consistently, and journal-specific reference styles were standardised and integrated in one combined reference section.

The studies were conducted at the Department for "Medizinische Psychologie, Psychologische Diagnostik und Methodenlehre" (Medical Psychology, Psychological Diagnostics and Research Methodology) of the University of Regensburg.

Chapter 3 **Zänkert, S.**, Bellingrath, S., Wüst, S. & Kudielka, B. M. (2019). HPA axis responses to psychological challenge linking stress and disease: What do we know on sources of intra- and interindividual variability? *Psychoneurendocrinology, 105*, 86-97. `https://doi.org/10.1016/j.psyneuen.2018.10.027`

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Sandra Zänkert and Stefan Wüst developed the study concept and study design. Sandra Zänkert supervised the data collection. Sandra Zänkert performed data analysis and drafted the manuscript. Stefan Wüst and Brigitte M. Kudielka provided critical revisions.

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Contents

List of Abbreviations

5-HTTLPR	5HT transporter-linked polymorphic region
17-OHCS	17-hydroxycorticosteroids
AA	ambulatory assessment
ACTH	adrenocorticotropin
ANOVA	analysis of variances
AS	anxiety sensitivity
ASI-3	Anxiety Sensitivity Index - 3
BNST	bed nucleus of the stria terminalis
BOLD	blood oxygenation level dependent
CAR	cortisol awakening response
CAVE	Cave Automatic Virtual Environment
CeA	central nucleus of the amygdala
CBG	corticosteroid-binding-globulin
CPT	Cold Pressor Test
CRH	corticotropin-releasing hormone
CRH1	CRH receptor subtype 1
CRHR1	CRH receptor gene
CTRL	control group
CUE	presentation of cue during NPU-threat test
DE	dextrose equivalent
DNA	deoxyribonucleic acid
EEG	electroencephalogram
EMG	electromyogram
ERP	event related potential
f-TSST	Trier Social Stress Test, friendly version
FKBP5	FK506 binding protein
fMRI	functional magnetic resonance imaging
GxE	gene-environment interaction
GLMM	generalized-linear mixed models
HPA axis	hypothalamus-pituitary-adrenal axis
GAD	generalized anxiety disorder
GI	glycaemic index
GLUCO	glucose drink condition
GR	glucocorticoid receptor
GRAPE	grape juice condition
HSD	honestly significant difference
ICC	intraclass correlation
ITI	inter-trial interval during NPU-threat test
IU	intolerance of uncertainty
IU-18	Intolerance of Uncertainty Scale
LMM	linear mixed models
MALTO	maltodextrin drink condition
MAST	Maastricht Acute Stress Task
MIST	Montreal Imaging Stress Task
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging

N100	large negative-going evoked potential
NCUE	neutral condition of the NPU-threat test, cue
NITI	neutral condition of the NPU-threat test, inter-trial interval
NPU-threat test	threat of the predictable and unpredictable aversive events test
OC	oral contraception
OT	oxytocin
P-TSST	Trier Social Stress Test, placebo version
P300	event-related potential wave
PCUE	predictable condition of the NPU-threat test, cue
PITI	predictable condition of the NPU-threat test, inter-trial interval
PAF	Test Anxiety Questionnaire
PET	positron emission tomography
PD	panic disorder
POMC	pro-opiomelanocortin
PTSD	posttraumatic stress disorder
PVN	paraventricula nucleus of the hypothalamus
RDoC	Research Domain Criteria
RM	repeated measures
SAD	social anxiety disorder
SECPT	Socially Evaluated Cold Pressor Test
SPT	sensitivity to predictable threat
ssVEP	steady-state visual evoked potential
STADI	State-Trait Anxiety-Depression Inventory
STAI	State-Trait Anxiety Inventory
SUT	sensitivity to unpredictable threat
TSST	Trier Social Stress Test
TSST-C	Trier Social Stress Test for children
TSST-G	Trier Social Stress Test for groups
UCUE	unpredictable condition of the NPU-threat test, cue
UITI	unpredictable condition of the NPU-threat test, inter-trial interval
varb	between-person variance
varw	within-person variance
VR	virtual reality
VR-TSST	Trier Social Stress Test in virtual reality
WEIRD	western, educated, industrialised, rich and democratic
WHODAS	World Health Organization Disability Assessment Schedule
YIPS	Yale Interpersonal Stressor

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0 Summary

Reliable and valid standardised laboratory paradigms are a key element of modern empirical research. At the same time, the use of reliably assessable biomarkers for mental disorders has increased during the last decades and is presumably becoming even more important in the future. Biological psychology is an intersectional field of research, in-between basic and clinical disciplines, with the capability and expertise to develop and improve laboratory paradigms to help assess biological markers in the context of cognitive, emotional or socially challenging tasks.

Such standardised laboratory tasks are both, essential to uncover mechanisms mediating between health and disease and can cover different kinds of stressors and stimuli. Two widely renown examples are the Trier Social Stress Test (TSST, Kirschbaum et al., 1993), primarily used for the induction of psychosocial stress leading to a reliable activation of the HPA axis, and the NPU-threat test (Schmitz & Grillon, 2012), a psychophysiological paradigm to assess fear- and anxiety-potentiated startle reactions. Challenges, like the TSST or the NPU-threat test, require processing at higher brain levels. Apart from pharmacological or physical stimuli, these psychological challenges are a complementary necessity when investigating biological pathways leading to pathology, and especially psychopathology. Both challenges, the TSST, as well as the NPU-threat test, have proven useful tools for experimental research. However, there has been an ongoing debate on the lack of progress in the search for improved or novel treatment strategies in biological psychiatry. This is in part attributed to the challenging translation of results derived from preclinical animal studies to successful outcomes in clinical populations. It has been suggested that progression in this endeavour needs improved meta-methodological knowledge and more rigour when implementing biopsychological laboratory paradigms.

The first part of the thesis introduces stress research with the Trier Social Stress Test (TSST). An overview on the development of modern stress research is provided, which started with the assumption of a general nonspecific reaction of an organism to stress and progressed to the realisation that there is a high intra- and interindividual variability of the stress response. That necessary conclusions had mainly been drawn from field studies, observing individuals in different real-life situations or contexts. Derived from these observations, it became apparent that differences in processing of psychosocial

challenges play an important role for individual variability of the stress response.

The search for a reliable and valid laboratory research paradigm, however, lasted until the introduction of the TSST in 1993, which was found to reliably activate the HPA axis compared to other existing paradigms in a comprehensive meta-analysis approximately a decade later. Since then, the TSST has become the gold standard for induction of psychological stress in the laboratory. The present work depicts how methodological developments in the assessment of biological compounds accompanied the development of the TSST, with a special emphasis on the assessment of the hormone cortisol in saliva.

The success of the TSST led to accumulation of research on intra- and interindividual variability of the stress response. More recently, there is even a growing interest for research on the stress protocol of TSST itself, reflected in meta-methodological articles. This critical systematic reflection revealed significant variability in application of the protocol across different laboratories. Research with the TSST, without a doubt, improved our current understanding of the stress system. However, there still remains a wide range of inconsistent results. A better understanding on intra- and interindividual differences, also due to methodological variation, is one approach to put more rigour in the design of future TSST studies, their execution and the interpretation of results.

This thesis contributes an overview on sources of intra- and interindividual variability of the response to psychosocial stress in form of a narrative review in chapter 3. Starting with a short general introduction and summary of laboratory paradigms for the induction of psychosocial stress. The following paragraphs provide an overview on sources of intra- and interindividual variability from different domains like demographic and lifestyle behavioural factors, physiological and personality factors, but also effects of chronic stress and psychopathology. Knowledge on biological factors include updates in genetics and especially results from epigenetic research in humans. When appropriate, recommendations are provided on how to address possible moderators when conducting experimental studies on acute stress. The review concludes with a short summary on general methodological aspects that should be considered, such as time of day, effects of anticipation, stress appraisal and habituation to repeated testing, as well as protocol variation that can be seen across studies and laboratories.

A reflection on and further discussion of the narrative review is presented in chapter 6, extending on aspects that were not included in the review, but are suggested to become more important in future research. Research often proclaims stress as a global major health burden, yet most related work still primarily includes so-called WEIRD populations (western, educated, industrialised, rich and democratic). In this regard, initial results emphasise that future research on acute stress should concern to account for intersectional effects of ethnicity, culture, nationality and migration on possible moderators.

A further challenge, that emerged from recent meta-methodological research on the TSST, is the already mentioned protocol variation of the TSST. That the TSST is easily applicable for a variety of research questions and populations can, of course, be seen as an advantage of the protocol. However, unspecific protocol variation can impair comparability between studies and laboratories, and lack of standardised reporting on deviations from the original protocol limits replicability of studies. Unwanted variability of the stress response due to protocol variation might become even more important with the increasing use of advanced statistical approaches that allow to better model the full dynamic of changing cortisol levels.

The second part of chapter 2 elaborates on one of the first moderating effects that have been investigated in regard to the TSST, the association between energy load and the cortisol stress reaction. Early studies showed that the cortisol response to acute psychosocial stress is dependent on blood glucose levels, with impaired cortisol reactivity after hours of fasting and reinstatement of the stress response after consumption of a high dose of glucose. Recommendations to control for energy availability, when conducting experiments with psychosocial stress tests, have been around since then. However, these recommendations are implemented very heterogeneously, and are also not reported consistently. The work presented in chapter 4 was motivated by that observation. It further addresses a methodological shortcoming of the original studies, by including female participants. The aim of the empirical investigation was to further elucidate the association between glucose availability and the cortisol response to acute psychosocial stress and, if possible, derive advanced recommendations on how to best control for energy availability.

Therefore, the TSST was applied in a mixed-sex sample that was randomly assigned

to one of three conditions, receiving either a drink containing (1) grape juice, (2) a high dose of glucose or (3) a corresponding dose of maltodextrin, and were compared to (4) a control group that did not receive a drink but was instructed to fast for the same time of 3h prior to testing. Results showed elevated cortisol levels and higher responder rates after consumption of a caloric drink compared to no drink. However, 3h of fasting did not result in a depleted cortisol reaction to the TSST. Post-hoc explorative analysis indicated a possible interaction of sex that should be considered in future research.

An in-depth discussion on the results of this empirical work is provided in chapter 6, extending the discussion to include more recent publications. Updated recommendations on the control of energy availability in experimental stress research are provided. To sum up, glucose availability is positively associated with cortisol stress response in most studies and energy availability should be controlled for. Short fasting periods of around 3h might be sufficient to ensure euglycemic levels across participants without depletion of the cortisol stress response. With longer fasting times, exceeding 8 hours, glucose substitution can prevent a blunted cortisol response to acute stress. Possible mechanisms, that have been previously discussed, are shortly summarised, including the Selfish-Brain-Theory, proclaiming control over energy availability contrary to passive supply and the effect of cerebral insulin suppression due to the activation of the HPA axis. A final note emphasises the importance of a standard reporting whether or not fasting periods were implemented and/or if caloric supplements were administered at the beginning of a stress experiment.

Since introduction of the original TSST protocol, numerous original articles and a growing number of meta-analyses, have been published and illustrate the accumulation of knowledge on moderating factors of HPA axis functioning during stress, as well as its effects on emotion, cognition and behaviour. The TSST can be easily applied by scientists across disciplines and has been adapted to different research questions. Among other possible future developments and applications, increasing digitalisation will advance research in virtual environments or remote versions of the TSST, blending laboratory and field studies and allowing for innovative research questions. But as has been emphasised before, in order to take full advantage of these developments, more rigour must be put on an improved implementation of the TSST protocol and the reporting of its methodology.

This will help to improve, among others, the integration of results for meta-analyses and might increase replicability of results.

The NPU-threat test is investigated from a different perspective. The introduction provided in chapter 2 raises awareness to the question of external or ecological validity of biomarkers derived from laboratory protocols. Combined lab-field studies can be used to investigate this translation of laboratory measures to real-world emotional, cognitive or behavioural experiences. The NPU-threat test modulates predictability of threat of shock to elicit either fear- or anxiety-potentiation. Here, fear is seen as a phasic response to imminent threat, operationalised by predictable threat of shock (P) compared to no shock (N), whereas anxiety is a more sustained aversive state to uncertain future threat, operationalised by unpredictable threat of shock (U) compared to no shock. Especially anxiety-potentiated startle is considered an important biomarker in the translation between basic and clinical research.

To determine what is known of the ecological validity of the NPU-threat test, a summary on the differentiation of ecological validity, external validity and generalisability is provided. A systematic approach to ecological validity of experimental settings is introduced, where dimensions like the nature of stimuli, task and behaviour can be differentiated. Although some aspects of the NPU-threat test might thus be considered ecological valid, like the nature of the stimulus "threat of shock", there exist no evidence on external validity and generalisability to real life.

Following, Ambulatory Assessment (AA) is introduced as a tool set of research practices to study individuals in their natural environment. Although AA has been applied in anxiety research before, those studies almost exclusively applied a dichotomous approach to anxious pathology, treating healthy participants as control group for clinical populations. To measure variability within and between non-clinical participants, the use of validated psychometric scales proven to detect changes across contexts, is imparative.

In order to investigate the association between laboratory physiological markers for fear and anxiety with everyday anxious experiencing in healthy individuals, a lab-field study is presented in chapter 5. The NPU-threat test and a two-day AA were applied in three differently characterised mixed sex study samples. A first sample comprised of

non-preselected healthy individuals, a second sample was preselected for low vs. high trait anxiety and a third sample included participants in preparation to an important examination in the near future. The AA comprised items of the subscales state worry and emotionality of the State-Trait Anxiety-Depression-Inventory (STADI) which were delivered pseudo-randomised eight times a day. The AA proved to be sensitive to dispositional (second sample) and context variation (third sample), as an alteration in variability within- and between participants across the three samples could be observed. However, there were no differences in anxiety- or fear-potentiated startle between any of the three samples. Most importantly, there was no significant association between NPU-threat test measures and everyday worry and emotionality.

However, it was not concluded that the NPU-threat test lacks ecological validity in general. Due to underlying biological mechanisms, an effect might only be detectable in clinical populations with, for example, exaggerated anxiety- and fear-potentiated startle compared to healthy participants. An extended discussion is provided in chapter 6, introducing additional alternative approaches to assess ecological validity in the sense of external validity and generalisability of the NPU-threat test. Suggestions range from alternative outcome measures, including electroencephalography or functional magnetic resonance imaging, to the assessment of higher cognitive functioning, or alterations in AA study design, like longer assessments or the use of different assessment modalities. A concluding thought addresses variation of between-person characteristics associated with a higher risk to develop an anxiety pathology, like traits or even past experiences like having been bullied.

The final paragraphs provide a general outlook on the importance of meta-methodological research on biomarkers, as more personalised approaches to pathology and treatment strategies are becoming more relevant in clinical research. It is suggested that high degrees of standardisation and proper understanding of moderators are prerequisites to guarantee that advancements, due to increasing digitalisation, or progression in statistics or biotechnology, can live up to their full potential in future research. The importance of this conclusion is emphasised by an expected increasing demand to assess biomarkers, derived from biopsychological paradigms, in clinical settings.

Part I

Fundamentals and Background

1 Outline

Biological psychology, as part of behavioural neurosciences, is a rather young discipline, that has been steadily growing in outreach and attention over the last decades. It is situated at the interdisciplinary intersection between behavioural biology, clinical psychology, genetics, neuroscience, health sciences and similar disciplines. Biopsychologists are interested in understanding connections between biological mechanisms in the body (neuronal, endocrine, biochemical processes) and behaviour of animals and humans (Birbaumer & Schmidt, 2018). During the last decades, it has also emerged that especially mental health plays a key role in modern healthy societies. The prevalence for psychological disorders seems to be rising (Rehm & Shield, 2019) and as these disorders are responsible for an increasing number of sick days, they are even becoming a widely recognised economic factor (Trautmann et al., 2016).

Psychological stressors, like daily hassles, increasing demands in the workplace, uncertainty of the individual financial future or even worries about the ecological future, are perceived as major burdens in most modern societies (Bellingrath & Kudielka, 2016; Charles et al., 2013; Clayton, 2020; Cunsolo et al., 2020; Sayal et al., 2002; van Eck et al., 1996). And increased levels of stress are associated with most clinically relevant psychiatric diseases and can also affect physiological health (Chrousos, 2009; Kariuki-Nyuthe & Stein, 2014). In general, the stress response is a way for the body to react to acute challenges and prepare the body against potential harm. But these evolutionary advantageous mechanisms have shown opposite effects when an individuum is confronted with long lasting non-physiological challenges or stressors (Joëls & Baram, 2009).

At the psychological level, there is a complex interplay between perception, cognition and appraisal of acute situations (Gaab et al., 2005), incorporating the ability to manage future challenges, cope with living circumstances or activate resources (DeLongis et al., 1988; S. E. Taylor et al., 2008). On the physiological level, the body tries to adapt to experiences and environmental changes and maintain homeostasis via alterations in a network comprised of the nervous system, the immune system, metabolic hormones and mediators in the brain and throughout the body (McEwen, 2019). Chronic activation of the bodily threat or stress response systems can lead to altered functioning as well as either hypo- or hyperreactivity of bodily response systems and has thus been associated

with the development of affective disorders (Chrousos, 2009; Davis et al., 2010).

Stress and anxiety, for example, have intertwined behavioural responses and neural circuits (Daviu et al., 2019). Perception of psychological stressors, like situations that can potentially induce a homeostatic challenge, can be regarded to as anticipation to a perceived threat. Also, the physiological stress response is accompanied by an emotional response that is, in part, determined by the perception of the imminence of the threat (Daviu et al., 2019). Fear, for instance, is defined as "emotional response to a real or perceived imminent threat" and anxiety as "anticipation of future events". Also, the biological underpinnings that distinguish between stress, fear and anxiety are very complex (Daviu et al., 2019). Unraveling these non-linear networks of bodily systems and interconnections to psychological factors of risk and resilience for each, stress or anxiety, seems of major importance for successful prevention and treatment of clinically relevant affective disorders.

Over the last years, there has been an increasing recognition of the importance of biological markers for clinical research, including improved diagnostics and approaches to more individualised therapies (Grillon & Ernst, 2020; Moriarity & Alloy, 2021; Wright & Woods, 2020). Biological psychology can provide reliable and valid tools, like laboratory paradigms, that combine aspects of physiological, endocrine or other biological activities and cognitive processes to investigate associations between psychology and biology, advancing knowledge how mind and body affect each other.

At first glance, the assessment of biological or physiological processes seems relatively straight forward, such as the assessment of heart beat via an electrocardiogram or hormone levels in bodily fluids like blood or saliva. On the other hand, cognitive and psychological processes are often latent and demand more deductive approaches, for example, via observation of behaviour or self-report. The combination of both constitutes a completely new challenge as it opens a new corridor for possible mediating or confounding factors. A proper understanding and systematic documentation of mediating factors as well as investigations on psychometric criteria like reliability and validity of laboratory paradigms seems crucial for an advanced usage of derived biomarkers (Moriarity & Alloy, 2021). Additionally, due to accumulation of research, systematic documentation should be updated regularly. Furthermore, progression in methodology may facilitate the

investigation of new perspectives in regard to established biomarkers.

The work presented here aims to contribute new perspectives on two laboratory paradigms that are commonly used in biological psychology of stress and anxiety, namely the Trier Social Stress Test (TSST) and the NPU-threat test. Both laboratory paradigms come with their own empirical applications, as well as advantages and disadvantages. What both paradigms have in common, is that biomarkers derived specifically from them, play an increasingly important role in translational research, such as biological psychiatry. Therefore, it can be assumed that the use of these paradigms, by laboratories from different fields of research in clinical and basic (translational) science, will increase in the future.

The TSST (Kirschbaum et al., 1993) is considered the most widely used psychosocial stress task and has already been adopted by many laboratories to investigate the human stress system. The protocol comprises a free speech and an arithmetic task in front of an audience and has been found to elicit a reliable activation of the HPA axis resulting in an increase in cortisol levels (Dickerson & Kemeny, 2004). The glucocorticoid cortisol affects a wide variety of bodily tissues and is considered a major mediator of allostasis, which is defined as the active process of adaptation to maintain homeostasis in response to environmental changes and (stressful) experiences (McEwen, 2019). Measuring cortisol in response to an acute challenge like the TSST can provide insight on the efficiency of individual allostatic regulation (McEwen, 2019). As standardised laboratory psychosocial challenge, the TSST is the perfect tool to investigate factors of variability of the acute biological stress response and effects of increased cortisol levels on cognition, emotion and other psychological or physiological domains (Allen et al., 2017). A dysregulated HPA axis, with either hypo- or hyper-reactivity, has been associated with many psychiatric disorders but also physical diseases (Chrousos, 2009; Kudielka et al., 2009). Therefore, the HPA axis and its end product cortisol have become a research target by many scientific disciplines concerned with factors of health and disease.

The NPU-threat test is a startle response paradigm to elicit fear and anxiety in the laboratory, under controlled conditions, using threat of shock (Schmitz & Grillon, 2012). In short, it comprises of a neutral (N) condition in which no shock is applied, a predictable (P) condition where a shock is only applied when a cue is present and an unpredictable

(U) condition where a shock can be applied at any time, independent of cue presentation. Due to the resulting modulation of the startle response, the NPU-threat test allows for the assessment of fear- and anxiety-potentiated startle reactions. Despite their overlapping nature, this allows to distinguish between fear as phasic response to imminent threat and anxiety as a more sustained state of apprehension about future threat (Schmitz & Grillon, 2012). The NPU-threat test has proven to distinguish between anxiety disorders with different anxiety-related symptomatology (Grillon et al., 2017). Especially the biomarker anxiety-potentiated startle has been acknowledged in the novel framework of the Research Domain Criteria (RDoC) initiative to assess the construct Potential Threat ("Anxiety") (Lang et al., 2016). Further, the NPU-threat test is increasingly applied as experimental model of anxiety to investigate the nature of psychopathology, treatment mechanisms and treatment targets in healthy individuals, bridging the gap between basic and clinical science (Grillon et al., 2019).

The present thesis begins with this short introduction (chapter 1), which is followed by a more comprehensive elaboration on methodological developments and shortcomings regarding both laboratory paradigms (chapter 2). It covers the emergence of the TSST as the most widely used stress task today and discusses the effect of glucose on stress in more detail. The concepts ecological validity and Ambulatory Assessment will be elaborated, especially in regard to the NPU-threat task. The following part presents the contributions of this thesis with a narrative review on intra- and interindividual factors of the stress response to acute psychosocial stress (chapter 3), an empirical study on glucose load and the cortisol stress response to acute psychosocial stress (chapter 4) and an empirical investigation of the ecological validity of the NPU-threat test (chapter 5). The thesis ends with a closing discussion on the preceding chapters (chapter 6) and an integrated conclusion in light of the overall scope of the present work (chapter 7).

2 Theoretical Background

2.1 Introduction to Research on Stress

Stress is one of the key factors in the development of diseases in modern western societies and is seen as a major public health burden. The stress reaction to chronic and acute stress of an individuum is determined by a wide variety of factors, external and internal. Investigating modulating factors of the biological stress response has become an interdisciplinary endeavour as a better understanding of the stress system in health and disease seems crucial for better prevention of potential health problems and for developing new treatment strategies. The gain in scientific insights is strongly related to the development and advancement of methodology. In the following paragraph, the development of psychoneuroendocrine stress research during the last decades will be briefly outlined, identifying some key methodological developments.

The first to popularise the term stress in sense of a challenge for biological systems was Hans Selye, who is also referred to as the father of modern stress research (Agorastos & Chrousos, 2021). He stated the theory of a non-specific stress response of the body to biological, chemical, physical or social challenge via secretion of glucocorticoids (Selye, 1976) also known as the General Adaptation Syndrome. Although Selye was originally trying to discover a new sex hormone, his experiments not only initiated his stress concept, but also stimulated research in many areas of medicine and biology (Mason, 1975b). One integral part was the investigation of hormonal regulation including, but not limited to, the pituitary-adrenal cortical system (Mason, 1975b). Progress in this field was further driven by the availability of reliable biochemical methods of hormonal assays in the 1950s. Studies before 1955 mostly relied on a great variety of indirect and nonspecific indices of adrenal cortical activity, whereas later, e.g. measurement of 17-hydroxycorticosteroids (17-OHCS) as degradation products of cortisol in blood or urine, provided a more specific and more reliable method (Mason, 1968).

During an initial period of about 15 years, stress research was mainly focused on physiological stressors upon the organism, like noxious stimuli including radiation, surgical injury or extreme cold, until a growing interest was taken on effects of psychological stress. And especially observations of the effects of psychosocial stimuli in animals and

humans demonstrated a wide variety of intra- and interindividual responses of the HPA axis (Bovard, 1959; Mason, 1975a). The most crucial difference between the effect of physiological and psychosocial stimuli is, that the latter do not affect body cells directly. Thus, it was concluded that their effect on the hormonal regulation must be mediated by the central nervous system (Bovard, 1959). These observations and conclusions were accompanied by a paradigm shift from the concept of non-specificity of the stress response, popularised by Selye, to the realisation that specific situational and personal characteristics are crucial elements in the individual stress process (Mason, 1975a).

An array of, mainly, field studies led to the conclusion, that situations are perceived as stressful when factors like novelty, unpredictability, ambiguity or anticipation of potential negative consequences are involved (Mason, 1968). This led to the understanding, that different autonomic and neuroendocrine stress responses vary according to the quality of a challenge and the availability of a person's ability to cope (Lazarus & Folkman, 1984; Mason, 1968). In regard to these theoretical developments, exploring sources of variance and a general controversy in the field of stress research, was already considered crucial to update and revise stress concepts and to develop new research strategies (Mason, 1975b).

At that time, biological stress reactions were mainly assessed in field studies, via measurement of responses to acute (aircraft flights or final examinations in college students) or chronic stressful life situations (long-term studies of parents of leukemic children or in military staff). But there were also attempts to elicit "emotional disturbance" in the laboratory, with the aim to develop a standardised reliable protocol for investigation of stress across test sessions. Among others, stressful interview techniques, motion pictures or hypnotic induction of emotional distress were applied to induce emotional and biological responses. However, these techniques only evoked small changes in 17-OHCS, with a substantial range of individual differences (Mason, 1968).

This wide range of neuroendocrine reactions to different stress situations, especially in healthy populations, has often been explained by interindividual differences like personality traits, coping styles or cognitive appraisal of a stressful situation alone (Berger et al., 1987). Investigating intra-individual susceptibility of the HPA system had long been neglected, until a first systematic within-subject comparison of individual reactivity to

different stressors demonstrated the broad range of intra-individual cortisol responses (Berger et al., 1987). This further demonstrated, if stress research was interested in investigating factors contributing to variability in HPA function, it needed a standard laboratory protocol, that elicits a valid and reliable HPA axis activation.

Another constraint for psychoneuroendocrinological research at that time, was seen in the measurement of cortisol in plasma or serum (Kirschbaum & Hellhammer, 1989). Sampling blood via venepuncture in stress research, is associated with numerous issues ranging from reactivity of the method (Weckesser et al., 2014), but also ethical and practical constraints as well as economic factors (Kirschbaum & Hellhammer, 1989). These limitations were put aside with the development of new assays that allowed to measure corticosteroids and especially cortisol in saliva (Inder et al., 2012). Salivary cortisol provides a measure of active free cortisol with a slightly delayed peak compared to plasma cortisol (Kirschbaum & Hellhammer, 1994). With this method, samples can be obtained in any desired frequency, the non-invasiveness allows a collection independent of medical personnel and it is also applicable at home or anywhere outside the laboratory (Inder et al., 2012; Kirschbaum & Hellhammer, 1989).

However, in the beginning researchers remained uncertain about the validity of the measure and it took some time, and thorough reviewing of the available literature, for the method to gain broad acceptance as a valuable alternative in psychoneuroendocrine research (Kirschbaum & Hellhammer, 1994, 1989). From today's perspective, it can be said that collection of saliva for the assessment of cortisol levels probably lowered the initial entry barrier for other fields of research to study the HPA axis system in humans.

In retrospect, it is even tempting to speculate that the broader acceptance on the validity of salivary cortisol in stress research was accompanied by the publication of a newly developed laboratory stress protocol, the Trier Social Stress Test, and following empirical work (TSST, Kirschbaum et al., 1993; Kirschbaum & Hellhammer, 1994). This impression is confirmed by Citation Report graphics derived from Clarivate Web of Science (see Appendix D, Copyright Clarivate 202_. All rights reserved). Figure 4 shows the number of citations and publications referencing the term "salivary cortisol" limited to research areas in psychology, psychiatry and sociology (for complete search

documentation see Appendix D). As pictured, the number of publications including the term “salivary cortisol” in the respective fields of research stayed on a low level before 1995, whereas a steeper increase can be seen after 1995, two years after the publication of the TSST protocol (Appendix D, Figure 4). Of course, this is a very rough estimate and a more extensive bibliographic analysis, which is out of the scope of this thesis, would be needed to clarify this assumption.

Today, the TSST protocol is known to provide a highly standardised method to induce psychological stress in a laboratory environment with a reliable activation of the HPA axis (Dickerson & Kemeny, 2004; Narvaez Linares et al., 2020). In brief, it consists of an anticipation period followed by a test period comprised of a free speech and a mental arithmetic task in front of an audience. In early studies, it has been applied to investigate genetic factors, sex differences and smoking as possible sources of variance on interindividual variation in salivary cortisol responses (Kirschbaum & Hellhammer, 1994). However, at that time, still a wide variety of laboratory stressors was used, including mental arithmetic and or speech tasks, but also playing video games or watching movies (Biondi & Picardi, 1999).

The popularity of the TSST gained momentum after the protocol was put into focus following the publication of the today widely renowned meta-analysis by Dickerson and Kemeny (2004). They reviewed 208 laboratory studies using different acute psychological stressors and set out to test a theoretical model to characterise conditions capable of eliciting cortisol responses. It was concluded that tasks containing uncontrollable and social-evaluative elements were associated with the largest cortisol changes. Results of the meta-analysis suggested that a short-duration task combining public speaking and a cognitive task, like the TSST, is a clear and reliable way to elicit an activation of the HPA axis (Dickerson & Kemeny, 2004). It seems that this recommendation led to a considerable increase in use of the TSST, as could also be derived from Figure 4. Here, the number of citations of the original protocol (Kirschbaum et al., 1993) shows a steeper increase since 2005.

Currently, the TSST is long been considered the gold standard for the induction of psychosocial stress and is used by a wide variety of research groups from different scientific fields (Goodman et al., 2017; Narvaez Linares et al., 2020). Only few years

after publication of the meta-analysis, a first narrative review on "Ten Years of Research with the TSST" (Kudielka et al., 2007) provided an overview on various studies applying the TSST and summarised what could be derived on sources of intra- and interindividual differences of stress reactivity. It also included a more detailed description of the protocol including the suggestion that the instruction can be changed slightly to employ the TSST to test children or older adults (Kudielka et al., 2007).

And indeed, the scientific success of the TSST did inspire the development of versions of the original protocol, so it could be applied to a broader variety of study designs or target populations. These versions include a protocol for group settings (TSST-G, von Dawans et al., 2011), a version for children (TSST-C, Buske-Kirschbaum et al., 1997) and of course "no stress" control conditions in form of a placebo (P-TSST, Het et al., 2009) as well as a friendly (f-TSST, Wiemers et al., 2013) version. Additionally, there have been adaptations and developments of laboratory stress protocols inspired by the theoretical concept of the TSST, like the Socially Evaluated Cold Pressor Test (SECPT, Schwabe et al., 2008), combining a physiological stressor with social stress or the ScanSTRESS (Lederbogen et al., 2011; Streit et al., 2017), to elicit a reliable HPA axis activation in an MRI (magnetic resonance imaging) environment. With the development of the TSST, and its direct and indirect variations, biopsychology provided a toolset of standardised psychological stimuli, that elicit a reliable activation of the HPA axis.

Of course, a reliable activation of the HPA axis can also be achieved with pharmacological challenges, or after intense physical exercise (Kudielka et al., 2009). Both options are useful in their own way, and especially pharmacological challenges are applied to assess functionality of the HPA axis in medical conditions, for example sensitivity or capacity of the adrenal cortex, or dysregulation of the HPA axis in depression.

However, evidence from field studies on natural occurring stress suggested that situational and personal characteristics do also contribute to variation of the individual stress response, and are capable to significantly activate the HPA axis. Laboratory psychological stimuli, that require processing at higher brain levels of the HPA axis and are standardised across test sessions, are therefore crucial to uncover mechanisms in stress biology leading to disease (Kudielka et al., 2009). This allows not only for the investigation of moderating factors that affect the HPA axis response on different levels,

it also enables investigation of the effect of an activated HPA axis in response to a more naturalistic psychological stimuli, on emotion, cognition and behaviour.

In retrospect, it can be concluded that the combination of the easy to measure cortisol in saliva and the introduction of a standardised and reliable laboratory paradigm boosted stress research and advanced understanding on intra- and inter-individual sources of HPA axis functioning in health and disease.

2.2 Research On and With the TSST

As mentioned earlier, currently, the TSST is considered the gold standard for experimental stress research in humans and has been applied in laboratories worldwide. It has been utilised to study stress effects on learning, memory, mental and physical health (Goodman et al., 2017). Also, the literature today provides an extensive overview on various environmental, psychological and physiological factors that can affect the stress response to the TSST and other protocols for induction of acute psychosocial stress in the laboratory (Allen et al., 2017; Foley & Kirschbaum, 2010; Kudielka et al., 2009; Kudielka et al., 2007; Kudielka & Wüst, 2010). Advanced knowledge on these factors does not only help to better understand HPA axis functioning, but also enhances the controllability of possible confounding factors and allows for more complex designs with measurement of multiple biological and psychological outcomes prior to, during and after the acute stress response (Allen et al., 2017; Strahler et al., 2017).

However, the popularity of the TSST leads to a fast accumulation of results and keeping track on the latest literature can be a challenge for experts, as well as for novices in the field of stress research. Integrative and often narrative reviews, providing a general overview on factors of variability in HPA axis reactivity, can therefore be a helpful resource. A first summary of studies using the TSST protocol by the original authors reported on the factors genetics, sex and smoking (Kirschbaum & Hellhammer, 1994). Only about ten years later, this list could be extended immensely (Kudielka et al., 2007), combining, amongst others, investigations on sex steroids, use of oral contraception or breast feeding and lactation, but also age, life style behavioural and social factors as well as results on genotypes and heritability of the stress response. This review further provided recommendations on if and how to control for certain factors when planning

to investigate effects of or on acute stress like for example energy availability prior test sessions or consideration of menstrual cycle phase in women (Kudielka et al., 2007).

Since then, there have been similar narrative reviews focussing on the TSST and sources of variability in general (Allen et al., 2017; Foley & Kirschbaum, 2010), extended approaches including broader concepts of psychological challenge and pharmacological stimulation (Kudielka et al., 2009) or biomarkers of the TSST (Allen et al., 2014). Due to accumulating research with the TSST there is also a growing number of systematic reviews and meta-analysis on the acute cortisol stress response and for example factors like age, sex differences and gender (Kudielka et al., 2004a) or psychiatric disorders (Burke et al., 2005; Lopez-Duran et al., 2009). Despite the growing body of research, narrative but also systematic analysis often reveals a certain heterogeneity of results and interpretationsoften remains inconclusive (e.g. Liu et al., 2017). Of course, this can in part be attributed to the complexity of the stress response and numerous inconsistent findings still need to be addressed systematically in future studies (Goodman et al., 2017; Narvaez Linares et al., 2020).

2.3 Glucose and Stress

Investigating the association between energy availability and HPA axis activation has been of interest already in the early days of research with the TSST. The rationale included the believe that cortisol is required for energy mobilisation in times of increased metabolic demands, for example due to stress, to prepare a fight or flight reaction (Kirschbaum et al., 1997). It was therefore assumed that energy availability might be an important mediator of the cortisol response to acute stress. And indeed, a first study showed that fasting prior stress exposure resulted in a blunted cortisol response and that intake of glucose was able to restore HPA axis reactivity to acute stress (Kirschbaum et al., 1997).

A later study suggested that this association was an effect of glucose load rather than energy availability alone as neither fat or protein load restored HPA axis activity after a period of fasting (Gonzalez-Bono et al., 2002). The association between glucose and HPA axis activity was found to be in line with animal research, however the underlying mechanism remained poorly understood (Gonzalez-Bono et al., 2002; Kirschbaum et al.,

1997).

A review on effects of nutrition on HPA axis response integrated research on orexigenic and anorexigenic peptides. The authors aimed to elucidate the phenomenon of a blunted HPA axis reactivity after a period of fasting but concluded that current data was insufficient to explain this modulation (Rohleder & Kirschbaum, 2007). However, several reviews on modulating factors of the cortisol responses to acute stress, specifically the TSST, recommend to control for energy availability or standardise the nutritional state before onset of an experiment (Foley & Kirschbaum, 2010; Kudielka et al., 2007). Recommended strategies included control for fasting periods prior testing, consumption of standardised meals or administration of a glucose drink at the beginning of a stress experiment.

However, there seemed to be a mixed reception of these recommendations, as they have been applied by some, but not all studies. Whereas some laboratories might have included administration of a glucose drink (e.g., 250 ml grape juice in Steudte-Schmiedgen et al., 2017) and maybe even to their standard stress test protocol used across studies, there are also studies that do not report either fasting periods or standardisation of energy availability at all.

Mixed implementation of fasting and energy restoration is also illustrated by the following example. A recent meta-analysis, interested in TSST methodology, only included TSST studies that applied the original version of the TSST and also reported on several methodological aspects like testing time window, number of judges or cortisol collection (Narvaez Linares et al., 2020). The authors identified 35 eligible studies and provide an extensive supplemental matrix of their extracted information. Although they did not investigate or discuss fasting periods, nor glucose or energy availability, the supplemental material includes information on restricted activities prior to TSST administration. Out of 35 studies, only 24 report on eating restriction (fasting period) prior testing, that range between 60 till 270 minutes with a mean of about 100 minutes.

A post-hoc screening for information on energy restoration after fasting, or further control of nutritional state, in this pre-selected and non-representative subset of available TSST studies, revealed that only one study reported on precise caloric intake (fixed 500 cal meal, no information on fasting period, Wand et al., 2007), one reported that

subjects where allowed a light meal during the stay in the laboratory (1 h fasting prior testing, Fries et al., 2006) and two studies only further specified instructions what to eat prior fasting Reinelt et al. (2019). The remaining 30 studies did not provide additional information on general energy availability or restoration after fasting (Narvaez Linares et al., 2020).

The association between glucose and HPA axis activation seems to receive little attention as a reliable source of cortisol variability in most studies, probably also due to still unknown mechanisms. Also, the original studies did have some methodological shortcomings, particularly only investigating men. But so far, there has been no empirical follow up to extend the study design or simply replicate the assumed association. To further elucidate the association between energy availability and HPA axis reactivity to acute challenge, extending the research question to also include women and investigate the effect of dose dependency seems long overdue.

2.4 Laboratory Biomarkers in Anxiety Research

Standardised laboratory paradigms, like the TSST, are considered a gold standard in psychological science to determine causational relationships between variables. They allow for an improved controllability of the environment, a better control of confounding factors, which also often comes with a high internal validity, and promise a precise assessment of the dependent variables of interest (Fahrenberg et al., 2007; Trull & Ebner-Priemer, 2013; Wilhelm et al., 2012). However, real-world relevance of these laboratory findings and biomarkers has been questioned and demonstration of ecological validity is growing in importance (Trull & Ebner-Priemer, 2013). Studies who assure whether findings from the laboratory translate into real-world emotional, cognitive or behavioural experiences are still sparse (Trull & Ebner-Priemer, 2013; Wilhelm et al., 2012).

There has also been an ongoing discussion about a gap between scientific groundwork on the aetiology of fear- and anxiety-like behaviour studied in animal models and the successful translation into new and advanced therapy strategies in humans, for example for promising pharmacological agents (Grillon et al., 2019).

One approach to bridge this translational gap, is to investigate new hypotheses about biomarkers in non-clinical populations using experimental models of anxiety, like the

NPU-threat test. The premise of this approach is a dimensional conceptualisation of psychopathology, with a continuum ranging from normal to pathological anxiety. An equivalent continuity would be assumed for underlying cognitive or biological mechanisms (Grillon et al., 2019; Grillon & Ernst, 2020).

In general, fear as a phasic response to imminent threat and anxiety as a more sustained aversive state to uncertain future threat are considered separable features of anxiety disorders with distinct neurobiological mechanisms (Davis et al., 2010; Grillon et al., 2019). In humans, fear and anxiety can be evoked experimentally by symbolic representation of an aversive experience as the knowledge of future aversive events is sufficient to create a threatening context (Davis et al., 2010). The NPU-threat test is a laboratory paradigm that allows to assess fear- and anxiety-potentiated startle reactions.

The so-called startle is a very short latency reflex that is mediated by a simple pathway between brainstem and spinal cord. This reflex is found across species and can therefore be measured in humans and rodents alike (Fendt & Koch, 2013). The startle reflex can be triggered by different modalities, like sudden loud acoustic noises or air puff blasts. The startle reaction serves to protect vulnerable parts of the body and therefore also triggers an eyeblink (Fendt & Koch, 2013). Contraction of the involved musculature can be measured via electromyography. Additionally, the reflex is subject to a variety of modulations, like increases or decreases of its magnitude (i.e., muscle contraction and thus EMG signal).

The enhancement of the startle reflex by contexts that elicit fear or anxiety, have been recognised as valuable biomarkers in anxiety research (Grillon et al., 2019; Lang et al., 2016). The NPU threat test combines a no shock condition (N) as well as conditions of predictable (P) and unpredictable (U) threat of shock to elicit both, fear- and anxiety-potentiation (Schmitz & Grillon, 2012).

In brief, the participant is presented with a cue (geometric shape) or a blank inter-trial interval and is instructed about the probability of an electrical shock, applied to the forearm via an electrode. Startle probes are sampled across all conditions. In the N-condition, no shock is applied, in the P-condition a shock is only applied during cue presentation and in the U-condition a shock is possible at any time during cue

presentation as well as during inter-trial intervals. Fear-potentiation, or cued fear, is then calculated as difference between P_{CUE} – P_{ITI} and anxiety-potentiation, or contextual anxiety, is calculated as difference between U_{ITI} – N_{ITI}. The original protocol of the NPU-threat test has been described in great detail by Schmitz and Grillon (2012).

Fear- and anxiety-potentiated startle have been found to differentiate among anxiety disorders and are sensitive to pharmacological manipulation (Davis et al., 2010). Especially anxiety-potentiated startle has been proposed as intermediate biomarker in the novel framework of the Research Domain Criteria (RDoC) initiative to assess the construct Potential Threat ("Anxiety") (Lang et al., 2016) and might be relevant for research in healthy individuals to advance the search for novel anxiolytic treatments (Grillon & Ernst, 2020). However, it is not clear how psychophysiological measures from the NPU-threat test paradigm translate into everyday anxious experiencing in non-clinical populations. This question can be addressed with a combined study design incorporating the laboratory NPU-threat test with AA.

2.5 Ecological Validity

The term ecological validity seems to be employed more frequently in study designs, not only due to the growing popularity and use of AA. Yet it has been discussed that the term is often used in a relative vague way (Fahrenberg et al., 2007). This discussion also reflects in an ongoing debate on the definition and concept of ecological validity in regard to laboratory paradigms (Holleman et al., 2020; Shamay-Tsoory & Mendelsohn, 2019).

Currently, the concept is often used interchangeably with external validity, while in its original sense it was only referred to the nature of a stimuli or context (Holleman et al., 2020; Kihlstrom, 2021). To avoid confusion and arbitrariness, a more systematic approach for investigating ecological validity of experimental settings has been proposed (Holleman et al., 2020; Kieffer et al., 2015; Labonte-LeMoyne et al., 2018; Schmuckler, 2001). Accordingly, dimensions like nature of the stimuli, nature of the task, behaviour or response and the nature of the research context, should be differentiated. These dimensions could then be rated in regard to artificiality-naturality and simplicity-complexity (Schmuckler, 2001). Though this approach of course poses new challenges and raises questions on

objectivity, reliability and validity in itself (Schmuckler, 2001), a more careful and conscientious use of the terminology seems indispensable (Holleman et al., 2020).

On that note, AA is a method that is highly ecologically valid in regard to a naturalistic research context, whereas stimuli and task can vary depending on the research question (Hermans et al., 2019; Kihlstrom, 2021). In regard to lab-field studies, AA can be used to evaluate the generalisability of laboratory paradigms in the sense of 'how well do outcomes measures from the laboratory associate with behaviour or functioning in real life'. Therefore, in this regard, ecological validity refers mainly refers to the concept of external validity (Fahrenberg et al., 2007; Hermans et al., 2019).

To emphasise the importance of this terminological distinction, the TSST shall be used as a quick example. The TSST is often introduced as a mock job interview, which is a context taken from the real-world. However, the frequency of exposure to job interviews, and therefore experience with this specific context, presumably varies widely across participants, depending on many factors such as age, education, social status and more. Ecological validity as scientific term has been used inconsistently and the TSST has mainly been ascribed ecological validity due to the nature of the stressor used (public speaking, representativeness of real-world experience, Allen et al., 2017), but ecological validity has also been denied in the sense of external validity (Weekes et al., 2006), and studies that set out to further explore "ecological validity" rather provide evidence for external validity in the sense of generalisability to associated functioning in real life (Henze et al., 2017). Such improper application of the term ecological validity seems arbitrary and depending on the definition, ecological validity is either attributed or not to a laboratory paradigm like the TSST. This can lead to confusion for novices and experts alike.

In regard to the NPU-threat test, a first step is to outline what can already be assumed about ecological validity or external validity. A most general view on psychometric criteria reveals that the NPU-threat test has been acclaimed to reliably modulate fear- and anxiety-potentiated startle in healthy individuals as well as clinical populations (Davis et al., 2010; Schmitz & Grillon, 2012) with a good internal validity (Gorka et al., 2017; Lieberman et al., 2017). Face validity has been proclaimed for the use of predictable and unpredictable shock as it evokes anxiety-like symptoms such as subjective anxiety, physiological arousal

and hypervigilance (Grillon & Ernst, 2020). Construct validity in the sense of etiological mechanisms has been indicated from studies on underlying neurocircuits of anxiety and fear in animals as well as humans with and without clinically relevant anxiety (Grillon et al., 2019; Grillon & Ernst, 2020). Furthermore, results from pharmacological studies suggests a good predictive validity as the NPU-threat test has shown to detect anxiolytic, anxiogenic as well as sedative effects of respective drugs (Grillon & Ernst, 2020).

In regard to the aforementioned dimensions of ecological validity, the nature of the stimulus "threat of a potential harm" could be considered natural whereas the geometric shapes, as well as the research context are more artificial. Studies applying context-dependent predictability of threat of shock in virtual reality paradigms as a modification to the NPU-threat test, increase the ecological validity or representativeness of fear- and anxiety-potentiation (Kastner-Dorn et al., 2018). However, the research context itself remains artificial. Especially for anxiety-potentiation, clinical validity in the sense of the degree to which patients can be discriminated from non-patients has been attested (Grillon et al., 2017; Grillon & Ernst, 2020). It is tempting to assume clinical validity as an implicit measure of functioning and behaviour in real-life and therefor a positive indicator for external validity and generalisability (Stevens et al., 2019).

Taken together, there exists some evidence for assuming ecological validity of different dimensions of the NPU-threat test. However, to the best of our knowledge, there exist no studies on ecological validity with direct measures of functioning, behaviour or emotion indicating external validity and generalisability of the NPU-threat test in either healthy nor clinical samples.

2.6 Ambulatory Assessment and Anxiety

Research practices for studying people in their natural environment are encompassed with the term Ambulatory Assessment (AA). Its toolset comprises self-report, observational and biological, physiological as well as behavioural methods, which are also relevant to design studies on the evaluation of ecological validity of laboratory experiments (Trull & Ebner-Priemer, 2013). AA-inspired study designs, also known as daily-diary studies, experience sampling method or ecological momentary assessment, have been used for decades. Yet, the increasing digitisation has led to a rediscovery and a rise of popularity

of AA, in clinical and non-clinical contexts (Trull & Ebner-Priemer, 2020).

The application of AA study designs, including multimodal approaches, has become more affordable and necessary advanced statistical analysis are becoming more and more accessible. Taken together, AA provides the necessary tools for designing lab-field studies to investigate associations between biomarkers derived from the laboratory and real-world experiences (Trull & Ebner-Priemer, 2013).

There already exists a wide variety of studies, using AA in anxiety research, where healthy participants are studied as control group to clinical populations. This, however, implies a dichotomous approach to anxious pathology. In these designs, AA items are often selected to yield differences between health and disease or to measure variance within clinical populations, for example in response to treatment (Hall et al., 2021; Trull & Ebner-Priemer, 2020; Walz et al., 2014). Most studies and AA scales are therefore not designed to measure variability within and between healthy participants.

A recent meta-analysis on AA for mood and anxiety symptoms found considerable heterogeneity in reporting and describing items, even between studies measuring the same constructs (Hall et al., 2021). Although the authors expected to see this heterogeneity, as studies investigated these constructs from different angles and with different research questions, they conclude that the lack of transparency and clarity about items and designs represent a large gap in the field. The analysis further yielded that response scales vary drastically and it is criticised that psychometric properties are rarely reported. A thoughtful selection and wording of AA items has been recommended and open-source data bases might help to make important design decisions based on previous research (Hall et al., 2021; Trull & Ebner-Priemer, 2020).

It is interesting to note, that established and validated psychometric scales seem to be rarely applied in AA studies. Often, only a selection of items is used as researchers try to keep assessments as short as possible to lower burden of the participants and prevent low compliance (Hall et al., 2021; Palmier-Claus et al., 2011). Addressing this shortcoming seems essential for a better understanding of a dimensional approach to anxiety in health and disease. Therefore, applying psychometric mood or anxiety measures proven to detect changes across contexts seems most feasible for use in healthy individuals.

Taken together, investigating the association between NPU-threat test measures and

everyday anxious experiencing of non-clinical individuals can yield valuable information on the generalisability of laboratory biomarkers and real-life functioning, behaviour and emotion.

Part II

Contributions

3 HPA Axis Responses to Psychological Challenge Linking Stress and Disease: What do We Know on Sources of Intra- and Interindividual Variability?

Zänkert, S., Bellingrath, S., Wüst, S. & Kudielka, B. M. (2019). HPA axis responses to psychological challenge linking stress and disease: What do we know on sources of intra- and interindividual variability? *Psychoneurendocrinology, 105*, 86-97. https://doi.org/10.1016/j.psyneuen.2018.10.027

Sandra Zänkert, Brigitte Kudielka and Stefan Wüst conducted the literature search. Sandra Zänkert drafted the manuscript and Brigitte Kudielka, Stefan Wüst and Silja Bellingrath provided critical revisions.

3.1 Abstract

Stress is an ubiquitous phenomenon with significant impact on human physiology when it lasts too long, when it is too intense, or when it hits vulnerable individuals. Examining the mechanisms linking stress exposure with health and disease is an important endeavor in psychoneuroendocrine research. Empirical evidence so far revealed large intra- as well as inter-individual variability in hypothalamic-pituitary-adrenal (HPA) axis responses to acute psychosocial stress, showing that the HPA axis is a highly adaptive system. Thus, the characterization of intra- und inter-individual patterns of HPA axis reactivity is of high scientific interest and forms the basis on which mechanistic links between stress response (dys)regulation and health impairments can be examined. To date, basic knowledge has been, and still is, accumulated on demographic, biological (including genetic and epigenetic) factors, lifestyle behavioral variables, consumption of substances and medication, psychological and personality factors, as well as on methodological aspects. Besides this, there is also very recent progress in respect to the development of laboratory stress paradigms that can be applied in virtual reality or inside an MRI-scanner. In sum, the present review updates our current knowledge on moderating and intervening factors as sources of intra- und inter-individual variability in human cortisol stress responses and offers recommendations for future research designs.

3.2 Introduction

Chronic stress is a major risk factor for several disorders, including highly prevalent diseases such as depression, anxiety disorders, cardiovascular diseases, and the metabolic syndrome (see 3.4.8 and 3.4.9). Since the finding of a disinhibited hypothalamic-pituitary-adrenal (HPA) axis in depressed patients (Carroll et al., 1980), it was assumed that alterations in the activity of this system may be a close correlate of stress-related pathology.

The HPA axis is a core component of the neuroendocrine stress response. When encountering a challenge, neural stimulation of the paraventricular nucleus of the hypothalamus (PVN) leads to the release of the peptide corticotropin-releasing hormone (CRH). CRH initiates the cleavage of pro-opiomelanocortin (POMC) into adrenocorticotropin (ACTH), beta-endorphin, and other peptides and their subsequent release from the anterior pituitary gland into the blood stream. The primary target of ACTH is the adrenal cortex, where it triggers the secretion of glucocorticoids and adrenal androgens. The main glucocorticoid in humans is the steroid hormone cortisol, exerting its various metabolic, immunological, cardiovascular, affective, cognitive and behavioral effects as well as its effects on the HPA axis itself via the ubiquitous low affinity glucocorticoid receptor (GR) and the high affinity mineralocorticoid receptor (MR) (de Kloet et al., 2005).

In the past decades, major efforts have been made in psychoneuroendocrinology to develop a variety of methods allowing a detailed phenotyping of an individual's HPA axis regulation. One important line of progress was based on the assumption that, in addition to the assessment of basal HPA axis activity under chronic stress, the measurement of HPA axis responses to acute psychological stress would be of particular predictive value. It was assumed that a neuroendocrine system may appear fully functional under rest but it may show significant dysregulation under challenge. Basically, this is the main reason why investigating HPA axis responses to acute psychological challenge generated substantial interest over the last decades. Reviewing the literature as present today, it can be concluded that this approach was remarkably fruitful.

A striking and consistent feature of HPA axis responses to acute psychosocial stress is their distinct intra- as well as inter-individual variability. In order to understand the

mechanisms linking HPA axis regulation and disease risk, it is of vital importance to identify the factors contributing to this variation. Which of the revealed variables are considered a confounder that should be controlled for and which factors are conceptualized as variables of interest completely depends on the given research question.

The aim of the present paper is to provide a brief description of current standardized psychological stress paradigms (3.3) and to give an updated overview on important determinants of cortisol responses to psychological stress in humans (3.4). The current knowledge on the role of demographic and biological (including genetic as well as epigenetic) factors, lifestyle behavioral variables, consumption of substances and medication, psychological and personality factors, chronic stress and psychopathology as well as relevant methodological aspects will be summarized. While this review covers a relatively broad spectrum of factors, it is nevertheless not comprehensive. For example, the important influences of prenatal and early life experience on later HPA axis stress responses are not discussed as this topic is presented in the paper by Christine Heim, Claudia Buss and Sonja Entringer (2019).

3.3 Standardized Psychological Stress Paradigms

To further unveil psychoneuroendocrine pathways connecting altered stress regulation with ill health, HPA axis response variability constitutes a certain challenge that can best be controlled for in standardized experimental settings. Naturally occurring stress responses can certainly also be studied outside the laboratory with the potential advantage of an increased ecological validity. However, to allow for highly controlled stress exposures across subjects and to facilitate experimental study designs, laboratory stress paradigms have been established (for more details see Zänkert & Kudielka, 2018). Actually, most findings on intra- und inter-individual differences in human cortisol stress responses so far stem from laboratory studies and in this review, we mainly focus on these empirical studies. In respect to ecological settings, a recent review summarizes empirical examples for ambulatory psychobiological stress research (Rodrigues et al., 2015).

To date, several standardized psychological stress paradigms are available aiming at eliciting an emotional, cardiovascular, or endocrine stress response. These protocols can be differentiated regarding the psychological domain they are - at least predominantly –

addressing, for example, cognitive stressors (e.g., mental arithmetic tasks), social stressors (e.g., social evaluation, peer rejection) and emotional stressors (e.g., presentation of emotional pictures or videos). In terms of this classification, the Trier Social Stress Test (TSST) is an example for a hybrid paradigm as cognitive load in combination with social evaluation are used as stress-eliciting components. The original TSST is composed of a free speech and a mental arithmetic task in front of an audience (Kirschbaum et al., 1993; for a detailed description see Kudielka et al., 2007).

Meanwhile, a variety of modified versions of the TSST for different populations has been developed, like the TSST for children or elderly subjects (see Allen et al., 2017; Zänkert & Kudielka, 2018). Further, to overcome the resource-intensive protocol of the standard TSST and to be able to stress several subjects simultaneously, von Dawans et al. (2011) developed the TSST for groups for up to 6 participants (TSST-G). Moreover, a placebo version, a parallelized non-stress control condition that lacks the main stress-inducing components of the TSST (i.e., no committee, no video camera) was established (Het et al., 2009). Also, a 'friendly' version, the so-called f-TSST that can be used as control condition, was introduced. Subjects interact with a friendly committee as opposed to the neutral and reserved behavior shown in the original TSST (Wiemers et al., 2013).

While for many psychobiological research questions a psychological stress protocol is preferable, HPA axis responses can certainly also be triggered by other means. For example, cortisol responses can be provoked in a highly standardized manner by physical stressors (intense physical exercise, physical pain, e.g. the Cold Pressor Test, CPT). Researchers should also be aware of the fact that meal intake can potentially elicit cortisol increases. Finally, pharmacological provocation tests systematically act at different levels of the HPA system and operate in a dose-dependent manner (see Kudielka et al., 2009; Seeman & Robbins, 1994). In this report, we will primarily focus on cortisol responses to standardized psychological stress paradigms.

Laboratory psychological stress tasks have different potencies in their ability to reliably evoke salivary cortisol responses (Biondi & Picardi, 1999). In a meta-analysis covering 208 laboratory stress studies, Dickerson and Kemeny (2004) investigated conditions capable of eliciting HPA axis stress responses. They concluded that motivated performance tasks

reliably elicit ACTH and cortisol responses if they were uncontrollable or characterized by social-evaluative threat. Tasks containing both elements, like the TSST, were associated with the largest hormonal changes and the longest recovery times.

Recently, Skoluda et al. (2015) investigated whether there exists a 'stimulus-response specificity' comparing the TSST with other commonly used laboratory stressors (namely the Stroop task, CPT), bicycle ergometry) and a resting control condition. All paradigms provoked increases in self-reported stress, reaching the highest scores in the TSST, followed by ergometry, Stroop, and CPT. The highest HPA axis response was found in the TSST, followed by ergometry, CPT, and Stroop.

Finally, there are other established psychosocial stress paradigms such as public speech tasks like the Leiden Public Speaking Task (Westenberg et al., 2009), which demands a speech prepared at home and given in front of a pre-recorded audience, or combined tasks like the Maastricht Acute Stress Test (MAST) (Smeets et al., 2012), which recombines the mental arithmetic elements of the TSST with the physical aspects of the CPT under a condition of social evaluation. The Yale Interpersonal Stressor (YIPS) represents an interpersonal social rejection paradigm that targets the stimulation of negative mood and the activation of the HPA axis (Stroud et al., 2002). A less resource-intensive alternative might be the socially-evaluated cold pressor test (SECPT), combining the thermal pain component of the classical CPT with a social-evaluative component. Evidence shows that the SECPT, indeed, has the potential to provoke significant subjective as well as HPA axis stress responses (Schwabe et al., 2008).

In respect to responder rates, the original TSST robustly induces a two-to-three fold increase in cortisol levels in approximately 70 – 80% of the participants with a reported average effect size of d' = .93 (Dickerson & Kemeny, 2004; Goodman et al., 2017; Kirschbaum et al., 1993; Skoluda et al., 2015). While comparably high responder rates are reported for the MAST (Smeets et al., 2012), medium responder rates were observed for the Leiden Public Speaking Task and the YIPS (Stroud et al., 2017; Westenberg et al., 2009). While relatively low responder rates were reported for the classical CPT (Skoluda et al., 2015), medium responder rates are reachable using the socially-evaluated cold pressor test (SECPT) (Schwabe & Schächinger, 2018).

In psychoneuroendocrine stress research, two recent methodological developments

deserve special attention in respect to psychological stress paradigms. These concern stress exposures in virtual reality (VR) and adaptations of stress paradigms for the application inside an MRI-scanner.

Firstly, during the last decade there have been several attempts to transfer the TSST into virtual reality environments (VR-TSST). This methodological development promises a reduction of required manpower while increasing experimental control for stress-evoking elements of the task like evaluator characteristics (e.g., by standardization/adjustment of sex, race, age, and physical attractiveness of the avatar), control of between-participant session replicability, and location of the task. For example, Fich et al. (2014) reported a difference of cortisol responses depending on the design of the virtual environment. Still, the type of employed VR-technology seems to be a critical issue in the capability to induce a robust stress response (Allen et al., 2017). For instance, the level of immersion did not elicit differences in HPA axis activation (Montero-López et al., 2015) whereas adding a virtual competitor more likely leads to a robust cortisol response (Shiban et al., 2016).

So far, VR-TSSTs have been conducted via monitor or projection (Fallon et al., 2016), head mounted displays (Kelly et al., 2007; Shiban et al., 2016), or inside a VR-CAVE system, an immersive virtual reality environment with projectors being directed to the walls of a room-sized cube (Jönsson et al., 2015; Wallergård et al., 2011). So far, paradigms in a VR-environment seem to be less capable than classical laboratory stress paradigms to elicit cortisol increases. Thus, important issues for future research on VR paradigms are the testing of their respective stress-eliciting potency and ecological validity (e.g. Shiban et al., 2016).

Secondly, progress in neuroimaging techniques now offers insight into regulatory networks and processing of the central nervous system during acute stress. Several methods have been applied to elicit stress inside an MRI-scanner. Yet, first approaches hardly induced robust cortisol responses (see Dedovic et al., 2009). In order to enable an investigation of central nervous HPA axis regulation, a prerequisite however is, that an applied neuroimaging stress paradigm reliably elicits HPA axis responses to acute stress. Likewise, significant cortisol responses are essential as they validate that the observed brain activation changes are a response to psychological stress and do not merely reflect

the processing of the task itself, for example cognitive load.

Thus, Pruessner and colleagues established the Montreal Imaging Stress Task (MIST) which contains a sequence of computerized challenging mental arithmetic tasks under an experimental condition of induced failure (Dedovic et al., 2005). By default, subjects receive negative feedback regarding their performance by the test jury after each run. Meanwhile, the MIST has been employed in several fMRI as well as PET (positron emission tomography) studies, and has also been adapted successfully by other laboratories (Kogler et al., 2015; Lederbogen et al., 2011). This paradigm appears to be less capable than classical laboratory stress paradigms to elicit cortisol increases, with about 50% to 100% elevations relative to baseline in about 50% of subjects. The MIST uses a typical fMRI block design and it appears plausible that the frequent interruption of the stress inducing task by control blocks without stress contributes to this difference in mean cortisol responses.

A more recently developed paradigm for the induction of acute stress inside the scanner is the ScanSTRESS paradigm which is conceptually closely linked to the original TSST. ScanSTRESS particularly translates the 'Mason factors' uncontrollability and ego-involvement as well as a component of social-evaluative threat. These factors are operationalized by pressure to perform a given forced-failure task (with adaptive difficulty) communicated by an observer panel. This jury is presented to the participant via a live video stream during the scanning procedure. Like the TSST, ScanSTRESS combines two different tasks, starting with a serial subtraction task followed by a mental rotation task (Streit et al., 2014). Meanwhile, it could be empirically confirmed that the ScanSTRESS protocol has the potential to induce robust neuronal, heart rate, and cortisol stress responses (Dahm et al., 2017; Streit et al., 2014, 2017). Although ScanSTRESS uses a block design like the MIST, first studies suggest that cortisol responder may be somewhat higher, with up to two-third of subjects showing increases > 1.5 nmol/l (Dahm et al., 2017; Streit et al., 2017).

3.4 Sources of Intra- and Interindividual Variability in HPA Axis Responses to Acute Psychological Stress

3.4.1 Sex and Sex Steroid-Related Factors

A very recent meta-analysis based on 34 studies encompassing 1350 participants (710 men and 640 women) corroborates the view that men show higher salivary cortisol responses to the TSST than women at peak times and during recovery (Liu et al., 2017). This effect could be observed in younger as well as older adults (see Kudielka et al., 2009; Lopez-Duran et al., 2009). Already earlier reviews and meta-analytic reports concluded that sex is a prominent source of variability for HPA axis stress responses (Kajantie & Phillips, 2006; Kudielka et al., 2009; Kudielka et al., 2004a; Kudielka & Kirschbaum, 2005; Otte et al., 2005).

Regarding underlying mechanisms, there has been a discussion whether, in particular, the psychological nature of the TSST promotes such sex effects since the cortisol response to the YIPS (see above) on the contrary has been reported to be greater in females than males. This raised the assumption that women might be more biologically reactive to interpersonal stress such as social rejection challenges while men might be more responsive to achievement stressors such as the TSST (Stroud et al., 2017, 2002). However, this finding was not consistently confirmed in other studies.

Another line of evidence indicates that intake of oral contraceptives in females and the menstrual cycle might be crucial. It appears to be a relatively consistent finding that women medicated with oral contraceptives (OC) show reduced free salivary cortisol responses to acute stress (see Kudielka et al., 2009). This effect might, at least in part, be explained by a moderating role of corticosteroid-binding-globulin (CBG) since oral contraceptives containing an ethinyl-estradiol component can alter endogenous steroid-binding-globulins in the blood, including CBG concentrations (Wiegratz et al., 2003). This view is supported by own data from Kumsta et al. (2007), reporting a significant negative correlation between CBG and salivary cortisol levels after TSST exposure in 115 women taking OC while no such correlation was found in 93 men.

To further elucidate the role of the female menstrual cycle phase, we applied the TSST in total study sample of 81 composed of men, women in the follicular phase, women

in the luteal phase, and women using oral contraceptives in equal parts (Kirschbaum et al., 1999). While no sex differences emerged for total plasma cortisol, salivary cortisol responses differed significantly between groups. Women in the luteal phase had saliva cortisol stress responses comparable to those of men whereas women in the follicular phase or women taking oral contraceptives showed significantly lower salivary cortisol responses. Other studies replicated the finding of comparably high salivary cortisol stress responses in men and women during the luteal phase (see Kudielka et al., 2009) and recent data by Stephens et al. (2016) corroborated a more robust activation of the HPA axis in men compared to women tested during the follicular phase. However, there also exist other recent studies that failed to detect HPA axis response differences across the follicular, ovulatory, or luteal phase (Duchesne & Pruessner, 2013; Herbison et al., 2016).

In this context, it should also be of note that sex-steroid supplementation may alter cortisol reactivity to acute stress and should be considered as potential source of HPA axis response variability (see Kudielka et al., 2009). However, available evidence on the impact of hormonal replacement therapy in humans is still fragmentary and heterogeneous.

In sum, we strongly suggest to control for the use of oral contraceptives and menstrual cycle phases in female participants when investigating HPA axis reactivity. Also, any sex steroid treatment in men and women should be excluded or at least reported. Finally, as a note of caution, it also cannot be ruled out that the premenstrual syndrome in cycling women as well as postmenopausal status in elderly women may impact on HPA axis responses to acute stress. Therefore, these issues should guide a substantiated composition of study samples.

3.4.2 Age

It was proposed that aging might come along with changes in HPA axis resiliency (see Seeman & Robbins, 1994). However, studies applying psychosocial stress paradigms showed no or only somewhat higher responses in older adults, primarily in men (Almela et al., 2011; Kudielka et al., 2009; Otte et al., 2005). For example, in a reanalysis of five independent studies with a total of 102 children, younger as well as older adults who were exposed to the TSST (Kudielka et al., 2004a), we found elevated salivary cortisol

responses in the group of elderly men (for sex difference see also above).

From a mechanistic point of view, two opposing pathways have been proposed to explain age-related alterations in HPA axis regulation. First, the so-called 'glucocorticoid cascade hypothesis', that mainly stems from animal research, attributes age-related changes in HPA axis functioning to a decrease in the ability of hippocampal neurons to maintain sufficient negative feedback, leading to a vicious cycle of continuously increasing HPA axis responses (Sapolsky et al., 1986).

Acknowledging contradicting evidence, the 'corticosteroid receptor balance theory' proposes a similar endocrine response to stress in younger and older adults. Within this theory, it is argued that even with older age homeostatic control could be maintained by a new balance between GR and MR, leading to a propensity for unchanged HPA axis responses (de Kloet et al., 1998). Empirical results are rather heterogeneous and it remains still unclear if old age is characterized by a 'cascade of events' or a new 'compensatory receptor balance'. With this, we recommend to either assess the participants age and to control for it when it is statistically associated with the outcome, or to use predefined age restrictions for participant recruitment.

3.4.3 Factors Related to Pregnancy

It is well-known that pregnancy is accompanied by increases in CRH, ACTH, cortisol, and CBG levels. While basal cortisol levels appear to be increased, salivary cortisol responses to the TSST are dampened (Entringer et al., 2010). There is also some indication for a progressive attenuation of psychobiological stress responses with advancing gestation (see La Marca-Ghaemmaghami & Ehlert, 2015). From a mechanistic point of view, it is assumed that heightened circulating CRH or glucocorticoid levels during pregnancy act by negative feedback to blunt HPA axis responses to challenge, corticotrophic cells in the pituitary might be desensitized or, at least in part, the presence of CRH-binding proteins in maternal plasma reduces the concentration of circulating potentially bioactive CRH. After giving birth, lactation has been associated with dampened hormonal responses to different stressors in rodents (Carter & Altemus, 1997).

On the contrary, in humans there does not seem to be a difference in cortisol responses to psychosocial stress in lactating versus non-lactating mothers (Altemus et al., 2001).

However breast-feeding directly before confrontation with the TSST reduces the salivary cortisol stress response (Heinrichs et al., 2001). As underlying mechanisms, a potential inhibitory impact of the lactogenic peptides oxytocin and prolactin on different levels of HPA axis regulation are discussed (see Heinrichs et al., 2002). In sum, lactation in women (in contrast to rodents) does not result in generally suppressed HPA axis responsivity to acute psychosocial stress (see Kudielka et al., 2009). Rather, breast feeding seems to exert a short-term suppression of the cortisol response to psychosocial stress in women.

Long-lasting changes in HPA axis functioning, including cortisol responses to acute stress in adulthood, can also be ascribed to factors that date back to childbearing. Indicators of prenatal development (like birth weight and length of gestation), but also pre- and early postnatal environmental adversity (like prenatal substance exposure or psychosocial adversity during early childhood), have been shown to be related to potentially lifelong alterations of HPA axis responses to stress (Bunea et al., 2017; A. L. Hunter et al., 2011; Kajantie & Räikkönen, 2010).

As indicated earlier, the impact of prenatal and early life experiences on later HPA axis stress responses are discussed elsewhere in this issue (see Heim et al., 2019).

From a methodological point of view, we advise not to admit pregnant and lactating/breast feeding women as study participants unless it is the central topic of the study. In addition, it might be informative to inquire if study volunteers had been exposed to severe pre- or postnatal childhood adversity.

3.4.4 Genetic and Epigenetic Factors

A significant influence of genetic factors on different markers of basal HPA axis activity was repeatedly shown in twin studies as well as in genome-wide association studies (Bartels et al., 2003; Bolton et al., 2014; Rietschel et al., 2017; Velders et al., 2011; Wüst et al., 2000). A few twin studies do also exist on the heritability of HPA axis responses to acute psychological stress, suggesting a significant, though moderate, heritability of cortisol stress responses (Federenko et al., 2004; Ouellet-Morin et al., 2008; Steptoe et al., 2009).

Another approach to identify potential sources of interindividual variability in HPA axis stress responses are candidate gene studies. Significant associations between cortisol

regulation and sequence variation in candidate genes belonging to the HPA axis pathway in a narrow sense as well as with variation in more 'distant' genes have repeatedly been reported.

For example, we found GR and MR variants to be related to ACTH and cortisol responses to the TSST, partly in a sex-specific manner (Kudielka et al., 2009; Kumsta et al., 2013). Variation in the gene coding for FK506 binding protein (FKBP5), an important GR regulator, was repeatedly shown to be associated with altered cortisol stress reactivity (e.g. Ising et al., 2008; Luijk et al., 2010) and cortisol responses to the TSST were also found to be associated with variation in the CRH receptor gene (CRHR1) (Mahon et al., 2013).

Genetic variation in other neurotransmitter systems, like the serotonergic system (e.g., 5HT transporter-linked polymorphic region, 5-HTTLPR) and the dopaminergic system (e.g., catechol-O-methyltransferase gene, dopamine D4 receptor gene) was also shown to be associated with psychosocial stress responses (Allen et al., 2017; Foley & Kirschbaum, 2010; R. Miller et al., 2013b). Moreover, associations with variation in genes coding for neuropeptides or their receptors like brain-derived neurotrophic factor, alpha-2B adrenergic receptor and monoamine oxidase A were found (Allen et al., 2017). For example, own studies point to an association between sequence variants in the neuropeptide S receptor gene, a novel candidate gene for anxiety disorders, and the cortisol stress response to psychosocial challenge in the laboratory and MRI-scanner environment (Kumsta et al., 2013; Streit et al., 2017).

Overall, it is well known that effects of single gene variants are inherently small, a phenomenon that, for example, has been demonstrated recently for the association between 5-HTTLPR and cortisol stress reactivity (R. Miller et al., 2013b). Nevertheless, there is no doubt that studying variability within a single gene or a circumscribed gene system can be of substantial relevance for psychological stress research. However, genetic effects of interest in our field cannot be adequately explained by a single gene variant in a single gene. Therefore, a sufficient number of sequence variants across the gene (or the gene system) has to be genotyped in order to achieve an adequate coverage and to capture the genetic variability that can be ascribed to the gene (system) of interest.

Meanwhile, several biostatistical tools for the processing and integration of information

from larger numbers of genetic variants are available. A promising example for a strategy that already has been successfully applied in stress research are multi-locus approaches like biologically informed multi-locus scores or polygenic scores derived from genome-wide association studies (Di Iorio et al., 2017; Utge et al., 2018).

Of particular interest for psychobiological stress research is the joint analysis of genetic and environmental factors. Gene-environment (GxE) interactions are presumed to be highly relevant for the understanding of mechanisms linking stress and disease as they are proposed to contribute significantly to the 'missing heritability' (Manolio et al., 2009; Uher, 2014). Furthermore, they can guide the search for epigenetic modifications.

For instance, in subjects with a significant history of stressful life events who were homozygous for the s-allele of the 5-HTTLPR, an elevated cortisol secretion in response to the TSST was observed (Alexander et al., 2009). Further, research on FKBP5 also points to an interaction between childhood trauma and sequence variants in this gene on cortisol reactivity in different age groups using different stress paradigms (Buchmann et al., 2014; Luijk et al., 2010; Zannas & Binder, 2014).

Altogether, an individual's HPA axis regulation is a highly complex phenotype that is only in part directly accessible and measurable. In order to adequately describe relevant domains of this complex phenotype, several methods have been developed in psychoneuroendocrine research, including paradigms to assess HPA axis responses to acute psychosocial stress. The findings from candidate gene and candidate GxE studies presented in this chapter suggest that the assessment of HPA axis stress responses indeed can serve as a valuable intermediate phenotype for HPA axis regulation in toto, which, in turn, is a close correlate of stress-related pathology. However, to tap the full potential of this approach, future candidate studies may be advised to focus not only on a thorough assessment of the target phenotype but also of relevant environmental variables.

An even more sophisticated option would be to plan study designs allowing a systematic variation of environmental variables in GxE experiments (van Ijzendoorn et al., 2011) and to use longitudinal designs. Overall, modern candidate (GxE) studies are a fruitful approach complementing genome-wide strategies and they offer great potential to reveal stress-related disease mechanisms in humans.

Initial findings suggest that also epigenetic mechanisms might be important modulators

of the neurobiological stress response. Epigenetic processes modify gene activity and expression by influencing the accessibility of the DNA without changing its sequence. To date, mechanisms that have mainly been studied in behavioral science are DNA methylation and histone modification. These epigenetic modifications can be altered by environmental influences including psychological factors such as prenatal maternal stress and early life adversity (Allen et al., 2017; Isles, 2015; Serpeloni et al., 2016; Turecki, 2016).

For example, findings on 5-HTTLPR by environment interactions have been extended through epigenetic research, investigating methylation and gene expression profiles (Alexander et al., 2014; Duman & Canli, 2015). GR methylation was shown to be associated with the cortisol recovery slope after stress exposure (van der Knaap et al., 2015) and a moderating role of GR methylation on the association between childhood trauma and cortisol stress reactivity was reported (Alexander et al., 2018).

Consistent with the development in genetics, it also became quickly evident in epigenetic research that studying single genes can be informative but that research on a genome-wide level is additionally required. For example, a recent study on the association between genome-wide DNA methylation profiles and HPA axis regulation identified a novel and presumably relevant pathway. Methylation at the locus of the Kit ligand gene significantly mediated the relationship between childhood trauma and cortisol stress reactivity later in life (Houtepen et al., 2016). While it is known from animal research that stress can induce epigenetic marks in a variety of brain regions, including hippocampus, amygdala, and prefrontal cortex, it is not yet well understood whether these alterations are maladaptive or whether they rather contribute to a proper dynamic regulation (R. G. Hunter et al., 2015).

3.4.5 Lifestyle and Behavioral Variables

Meanwhile, (quasi-)experimental studies have been accumulated, elucidating the potential influence of some lifestyle and behavioral variables on HPA axis responses to stress, ranging from the consumption of alcohol, nicotine, coffee or dietary energy supplies, and intake of medication to physical exercise, body composition and sleep habits (Herbison et al., 2016). In the following, existing evidence will be briefly discussed.

Acute as well as chronic alcohol consumption, alcohol dependency and even a positive family history of alcohol dependency is potentially related to altered HPA axis responses to psychosocial stress (Foley & Kirschbaum, 2010; Kudielka et al., 2009; Van Hedger et al., 2017). However, existing evidence is not unanimous with reports on dampened as well as unaltered HPA axis stress responses (Bibbey et al., 2015). Heterogeneous findings might, at least in part, be attributed to different ethanol dosages in some studies or insufficient statistical power in others. Based on this evidence, we recommend to exclude heavy alcohol users from basic research. Also, subjects should be instructed to refrain from acute alcohol intake on study days and the day before their laboratory appointment. It might also be advisable to assess regular and recent alcohol consumption in study participants.

Smoking, either acute or habitual, can significantly modulate HPA axis responses to acute stress. Smoking itself acutely activates free cortisol increases since nicotine acts as a potent stimulator of the HPA axis through induction of CRH release after binding to cholinergic receptors. After the consumption of only two cigarettes, significant salivary cortisol increases were observed (see Kudielka et al., 2009). Thus, we would advise to strictly prevent acute smoking before and during stress testing. Importantly, habitual nicotine consumption could lead to chronically elevated ACTH and cortisol levels and, in consequence, dampened HPA axis responsiveness to acute psychosocial stress (Herbison et al., 2016; see Kudielka et al., 2009; Rohleder & Kirschbaum, 2006; Van Hedger et al., 2017). As a further note of caution, although some empirical findings support the view that nicotine abstinence does not alter salivary cortisol responses to psychosocial stress, it is still possible that acute nicotine craving affects cortisol stress responses in smokers. Thus, we recommend to exclude habitual smokers or, at least, to control (statistically) for smoking status.

There exist some empirical evidence that caffeine consumption potentially stimulates basal cortisol levels. However, its pure stimulatory potency for HPA axis activation is not unequivocal because several other studies did not report such enhancing effects. Experimental evidence raised the idea that there might at least be a combined stimulatory effect of acute coffee consumption and psychosocial stress exposure (see Kudielka et al., 2009; Van Hedger et al., 2017). Recently, habitual caffeine consumption was reported

to be associated with a greater cortisol stress reactivity to the TSST (Vargas & Lopez-Duran, 2017). With this, we advise to instruct study participants to refrain from coffee consumption before and during a laboratory testing and to potentially exclude volunteers with heavy habitual coffee consumption.

In respect to nutritional state, cortisol does not only affect energy metabolism but is itself influenced by energy intake. For example, low endogenous glucose levels have been associated with blunted free cortisol stress responses whereas in glucose-treated subjects stress exposure triggered larger salivary cortisol responses (with unchanged basal cortisol levels) (see Kudielka et al., 2009). At first glance, this empirical evidence seems to speak against the classical view that glucocorticoids function to provide the individual with energy in stress situations. Presently, it is assumed that a central mechanism may be responsible for regulation of energy balance and HPA axis activation rather than peripheral mechanisms (see Rohleder & Kirschbaum, 2006). Based on these empirical findings, it appears reasonable to standardize blood glucose levels when studying salivary cortisol in response to stress, for example by providing a standardized meal or administration of a glucose-containing standard beverage about 45 min before stress exposition (Kudielka et al., 2009).

Further, it is important to acknowledge that chronic as well as short-term medication, vaccines, or intake of dietary supplements (irrespective of route of administration) potentially impact on salivary cortisol responses to psychosocial stress. This applies to patient groups as well as healthy controls. Highly relevant substances are, for example, synthetic glucocorticoids and psychotropic drugs (Houtepen et al., 2015; see Strahler et al., 2017; Zorn et al., 2017). Very recently, Van Hedger and colleagues (2017) summarized evidence on the effects of single doses of typical pharmacological agents on subjective as well as HPA axis stress responses including anxiolytics, antidepressants and sedatives, analgesics, and beta blockers. Obviously, the spectrum of relevant pharmaceutical ingredients is widespread, and thus, underlying chemical pathways are manifold (see Granger et al., 2009; Holsboer & Barden, 1996; Pariante & Miller, 2001).

Considering the high number of available active substances, typically prescribed dosages as well as interactions with other drugs, it cannot come as a surprise that our knowledge on their respective effects on HPA axis regulation is fragmentary and

selective. Since various pharmaceutical agents interact with HPA axis regulation, it is advisable to exclude subjects with long-term as well as acute medication intake and recent inoculation/vaccination. If medication cannot be precluded, we recommend to inquire on subjects' medication intake. However, it might be impossible to statistically control for such effects if the study population is heterogeneous. At least, researchers should be aware of the half-life of the substance as indicated in the package insert.

Body composition and physical fitness have repeatedly been discussed as potential sources of HPA axis alterations. While several empirical studies points to an increased cortisol stress responsivity to psychosocial stress in obesity, at least in abdominal obesity (Rodriguez et al., 2015), there are also reports on normal salivary cortisol responses to stress in samples composed of only slightly overweight but otherwise healthy participants (Herbison et al., 2016; Jayasinghe et al., 2014). As shown in between-subjects designs (group comparisons) as well as in within-subject designs (interventional studies), physical fitness and intense physical exercise potentially comes along with a blunted cortisol response to acute psychosocial stress (Klaperski et al., 2013; Rimmele et al., 2009; Strahler et al., 2016). Such evidence points to the necessity to make a reasonable decision about the targeted study population, e.g., researchers should decide if obesity qualifies as exclusion criteria. Further, study participants should be instructed to refrain from heavy physical exercise before study participation (even the day before testing) and researchers might want to assess and (statistically) control for physical fitness.

Sleep variables have been repeatedly proposed as influential factors for HPA axis functioning. Although this reasoning appears intuitively plausible, empirical evidence remains surprisingly contradictory. For example, studies investigating the effects of sleep length the night before testing on cortisol stress responses do not support strong effects (for review see van Dalfsen & Markus, 2018). At least, for low sleep quality and excessive daytime sleepiness (van Dalfsen & Markus, 2018) as well as sleep deprivation (Vargas & Lopez-Duran, 2017; but see also Schwarz et al., 2018) there is some indication for potentially altered acute cortisol responsivity the day after. Thus, the assessment of sleep habits could be reasonable. Related to this topic and as a final note of caution, researchers should be reminded to ensure that laboratory appointments do not interfere with the cortisol awakening response (CAR), day-time napping, shift work, or jet-lag

(Stalder et al., 2016).

3.4.6 Psychological Factors and Interventions

It is plausible to assume that the social environment exerts modulating effects on endocrine stress responses. Indeed, psychosocial stress paradigms like the TSST heavily rely on contextual factors to trigger HPA axis activation. Empirical evidence supports this view. For example, social support, at least in men, dampened the HPA axis response to the TSST (Ditzen et al., 2008; Kirschbaum et al., 1995; Kudielka et al., 2009). In order to unveil underlying mechanisms, the neuropeptide oxytocin (OT) has been scrutinized as one biological causal link (see Hostinar et al., 2014). Indeed, OT administration enhanced the buffering effect of social support on salivary cortisol stress responsiveness in young men (Heinrichs et al., 2003). In accordance, young women who obtained a massage before they performed the TSST (presumably increasing endogenous OT levels) showed significantly reduced cortisol responses compared to women who received social support without positive physical partner contact or had no social interaction (Ditzen et al., 2007). Also, other social factors such as the position in the social hierarchy appears to be relevant as shown in an earlier study in army recruits by Hellhammer and coworkers (1997).

Beside this, psychological interventions like group-based cognitive-behavioral stress management, mind-body exercises including progressive muscle relaxation, Taiji practice and some forms of meditation, or even relaxing music have been shown to have the potency to reduce endocrine stress responses to subsequent acute psychological stress exposure (see Kudielka et al., 2009). Thus, in order to avoid any unintended social interactional effects between subjects and investigators or environmental influences that might (un)systematically alter subjects' stress responses, we recommend to adhere to given standardized instructions as strictly as possible.

3.4.7 Personality

The psychoendocrine response to psychological stress can be viewed as a close interaction between person and situation variables within a given context. Thus, it was repeatedly assumed that stable personality traits are closely related to salivary cortisol stress

responses (see Kudielka et al., 2009). However, a meta-analysis by Chida & Hamer (2008) could not find any evidence for an association between negative psychological states or traits (e.g., negative affect, neuroticism, hostility, anxiety, aggression, etc.) and acute HPA axis stress responses whereas some evidence for associations between decreased HPA axis reactivity and positive psychological states or traits (e.g., happiness, positive mood, internal locus of control, self-esteem, empathy, spirituality, active coping, etc.) was reported.

Only personality traits that have traditionally been associated with greater psychopathology (like high neuroticism or lower extraversion) do occasionally show an effect on HPA axis regulation after onetime stress exposure (Oswald et al., 2006). Also, in respect to trait rumination empirical evidence is inconsistent. While some studies have shown that rumination can prolong the salivary cortisol response to stress (Shull et al., 2016; Stewart et al., 2013; Zoccola et al., 2010), other studies do not support this view (Young & Nolen-Hoeksema, 2001; Zoccola & Dickerson, 2015).

How can such results be explained? The novelty of a stress situation appears to cover the impact of personality on HPA axis regulation on first time exposure. This idea is supported by studies showing that the relationship between salivary cortisol responses and personality factors became apparent after repeated stress exposures. A given cortisol response to acute stress is certainly determined by both trait and state factors, with the latter changing over repeated exposures (e.g., novelty, predictability, uncontrollability, learning, memory, etc.).

Data aggregation over repeated stress sessions appears to enhance the chance to uncover otherwise or initially masked relationships between personality traits as assessed by self-report and salivary cortisol stress responses. For example, Pruessner et al. (1997) reported a considerable increase in the correlation between psychological trait measures and mean cortisol stress responses when aggregating over several test days. Consistently, the heritability of HPA axis responses to the TSST increased over repeated exposures, probably due to a relative decrease of state effects (Federenko et al., 2004). Therefore, the pattern of habituation in itself may be an important marker worth investigating (see also Rohleder, 2019).

Thus, we advise to apply multiple stress exposure sessions if researchers are inter-

ested in studying associations between stable personality traits and acute cortisol stress responses. As a further note, there might meanwhile be other promising approaches, like the use of implicit measures instead of self-reports (e.g. Schultheiss et al., 2014), to gain valuable insight into associations between personality factors and endocrine stress responses.

3.4.8 Chronic Stress and Burnout

As stated above, the aim of the present paper is to provide an updated overview on moderating and intervening factors as sources of intra- und inter-individual variability in human cortisol responses to psychological stress. From this perspective, chronic stress and stress-related psychopathology (4.4.9) can, of course, be listed as significant modulators. However, it should be pointed out that basically, the interest of psychoendocrinological research in HPA axis responses to acute stress is founded on the assumption that studying these responses is a fruitful approach contributing to our understanding of the development of chronic stress and stress-related pathology. From that angle, it would in fact be more appropriate to conceptualize stress and stress-related pathology as major outcome variables and not as modulators. However, it is self-evident that it depends on the specific research question which of the two perspectives appears more suitable.

Either way, it is reasonable to assume that continuing stress leads, in the long run, to enduring changes in HPA axis regulation (for meta-analysis see G. E. Miller et al., 2007). There is also empirical evidence for altered HPA axis stress reactivity in individuals suffering from chronic stress, states of exhaustion, and burnout (for overviews and meta-analysis see Chida & Hamer, 2008; Eddy et al., 2018; Heim et al., 2000; Kudielka et al., 2006b). In this field of research, one major challenge is that conceptualizations of chronic stress (and related assessment tools) differ substantially, ranging for example from family caregiving to effort-reward imbalance, job strain, unemployment (including financial strain), etc. This might, at least in part, explain the great variability in reported results ranging from HPA axis hyper- to hyporesponsivity in chronically stressed individuals.

In their seminal review, Heim and colleagues (2000) reported evidence for hypocortisolism in individuals living under conditions of chronic stress. As potential mechanistic

pathways, they discuss a reduced biosynthesis or depletion of CRH, ACTH, and cortisol, CRH hypersecretion and an adaptive down-regulation of pituitary CRH receptors or changes in receptor sensitivity, increased feedback sensitivity of the HPA axis, or morphological changes.

Studies on chronic work stress and burnout are somewhat mixed, reporting either hyper- or hyporesponsivity (for review see Kudielka et al., 2006a). This might not come as a surprise, considering that firstly, there still exists no consistent definition of burnout, and secondly, the burnout syndrome shows a large symptom overlap with different forms of depression. At least, the few studies that assessed chronic work stress according to the effort-reward-imbalance model appear to be more consistent, merely pointing to HPA axis hyporeactivity to acute stress (for review see Bellingrath & Kudielka, 2016; but see also Eddy et al., 2018). In accordance, more recent studies on severe burnout, clinical cases of burnout or insufficient long-term rescovery after exhaustion disorder also merely point to blunted salivary cortisol responses to psychosocial stress (Bellingrath & Kudielka, 2016; de Vente et al., 2015; Eddy et al., 2018; Jönsson et al., 2015; Lennartsson et al., 2015).

Taken together, the results picture would be in accordance with a time-course or two-stage model as proposed earlier (Hellhammer & Wade, 1993; see also Bellingrath & Kudielka, 2016): An early state of chronic stress (characterized by hyperactivity of the HPA axis) could, in the long run, lead to a hyporeactive state as result of a functional adaptation to excessive exposure to stress hormones. In consequence, such changes over time could then blur results pattern. Thus, in group analysis hyper- and hypocortisolemic effects in different individuals could cancel each other out. In sum, long-term dysregulations in HPA axis functioning due to chronic stress and states of exhaustion or burnout, in turn, affect acute psychosocial stress responses. Thus, researchers might want to inquire into the subjects' experience of not only acute but also chronic stress.

3.4.9 Psychopathology

Stress plays a crucial role in the pathogenesis, onset, and progression of various illnesses. In turn, numerous somatic as well as psychiatric diseases come along with altered HPA

axis responses to acute stress (Chrousos, 2009). Detailed reviews and meta-analysis can be found elsewhere, focusing for example in particular on somatic illnesses (Strahler et al., 2017), autoimmune disorders (Buske-Kirschbaum et al., 2002; Tsigos & Chrousos, 1994), psychiatric diseases (Bradley & Dinan, 2010; Ciufolini et al., 2014; Knorr et al., 2010; Zorn et al., 2017), or pathophysiological conditions in children (Jessop & Turner-Cobb, 2008). Empirical findings on particular diseases are usually not unambiguous regarding the direction of HPA axis dysregulation. For example, depression was reported to be related to either hyperresponsiveness as well as hyporesponsiveness, depending on depression subtype (e.g., major depression or melancholic depression versus atypical or seasonal depression), comorbidity with other diseases like anxiety, or depending on sex.

However, so far, available evidence predominantly suggests HPA axis hyporesponsiveness in, for example, schizophrenia, adult PTSD, chronic fatigue syndrome, fibromyalgia and atopic dermatitis and, possibly, in anxiety disorders. Hyperresponsiveness was, for example, observed in anorexia nervosa, panic disorder and PTSD in children. Mechanistically, it is not always clear, whether a disease leads to altered HPA axis alterations, or if HPA axis alterations contribute to health impairments, or both. In general, we advise to screen subjects carefully for disease states and to define eligibility clearly by health-related exclusion criteria. Irrespective of the heterogeneity of findings and evident methodological differences across studies, results support the view that differences between patients and healthy controls are more likely to be observed when the system is challenged.

3.4.10 Methodological Aspects

In the following, we summarize evidence regarding some methodological aspects like time of testing, the (complementary) collection of blood samples, habituation to repeated testing, anticipation effects, and the assessment of stress appraisal. Finally, we will briefly discuss the issue of inter-laboratory variations. It is well-known that the secretion of cortisol follows a typical circadian rhythm. To account for regular diurnal changes, and at the same time, to avoid any interference with the CAR (Stalder et al., 2016), we advise to schedule acute stress sessions in the afternoon. In their recent comprehensive meta-analysis, Goodman et al. (2017) did not observe pronounced differences in effects sizes regarding cortisol responses at different times of day, but cortisol responses were

slightly lower and more variable during morning sessions.

In a reanalysis of own data based on five independent studies, we analysed cortisol stress responses to the TSST in the morning versus afternoon (Kudielka et al., 2004b). Data showed that net salivary cortisol stress responses could be assessed with comparable reliability in morning and afternoon sessions, taking into account that pre-stress cortisol levels are systematically higher during the morning. However, we found that higher basal cortisol levels were slightly (but significantly) associated with lower acute stress responses pointing to the presumption that higher baseline levels might, to some degree, reduce a superimposed net stress response.

Thus, to increase the likelihood of stronger cortisol responses, stress sessions should ideally be scheduled during the afternoon. However, other time windows may still present feasible alternatives, at least if avoiding meal times and interference with the CAR. Of course, experimenters should ensure that all test sessions of a study are performed during the same time window to avoid any bias caused by circadian rhythm effects.

If a researcher intends to take saliva and blood samples concomitantly, it should be acknowledged that a venepuncture elicits a cortisol response in more than one-third of subjects. Thus, cannula insertion should be followed by an extended relaxation period in order to allow cortisol levels to return back to baseline levels before experimental blood samples are drawn (Weckesser et al., 2014).

In case of repeated acute stress exposures, mean HPA axis stress responses typically show a rapid habituation across sessions. With this, the HPA axis is different from the sympathetic nervous, immune and blood coagulation system as well as indices of hemoconcentration which all show rather uniform activation patterns after repeated acute stress exposures (see Kudielka et al., 2009).

In an own study, we set out to scrutinize this phenomenon in more detail (Wüst et al., 2005). Data showed that there is substantial variability of salivary cortisol response habituation patterns in healthy young individuals. The majority of our participants (52%) exhibited the well-known response habituation across three TSST test sessions, about a third (30%) did not show obvious response alterations and almost 16% of participants even showed a response sensitization. It can be speculated that habituation of the HPA axis to psychosocial stress by many subjects is due to decreasing experience of task

novelty, unpredictability and uncontrollability, leading to a reduction in context variables across sessions.

The phenomenon of habituation should influence the researchers' choice for an adequate study design. For example, a simple within-subjects design with repeated pre- and post-interventional TSST exposures would not qualify as valid proof of stress-reducing effects of a given treatment (e.g., psychotherapy, stress management training, etc.) on acute HPA axis stress regulation. In such a design, it would remain unclear whether the treatment or the familiarization with the TSST exposure had caused a potential reduction of the stress response.

It was argued, however, that pure habituation effects in studies with repeated stress exposition might potentially be prevented, or at least reduced to a considerable extent, by large enough between trial intervals and/or changing test settings (see Foley & Kirschbaum, 2010). As already discussed above, habituation to a repeated stressor can, on the other hand, also help to unveil associations with factors that might otherwise be masked at first stress exposure, such as personality traits, genetic factors or work-related exhaustion (see Kudielka et al., 2009). In this case, a within-subjects design might be the study design of choice.

As already known, cortisol stress responses can be evoked, at least in men, by the sole annunciation of an upcoming psychosocial stress task, pointing to a pure anticipation effect (Kirschbaum et al., 1992). Thus, researchers should have in mind that anticipation might impact on reactive cortisol stress responses (Engert et al., 2013). This underlines the importance of standardized study instructions as well as adequate relaxation periods in which subjects should not ruminate about the upcoming stress task (see also Goodman et al., 2017).

Related to this, Gaab and colleagues (2005) reported that anticipatory stress appraisals, but not retrospective appraisals, of a psychosocial challenge explain up to 35% of the variance of the salivary cortisol stress response. With this, we recommend to psychometrically assess not only the participants' subjective stress experience after stress exposition but also directly before stress exposure. In line with this, a close correspondence between subjective emotional and biological stress responses has been rarely reported (see Campbell & Ehlert, 2012).

This seems to speak for a lack of covariance between subjective and physiological stress response markers. At first glance, this appears to be surprising. Acute stress elicits multiple psychological as well as physiological responses in humans. Theoretically, such different responses to a given stressor represent indicators of the same construct. Therefore, clear associations between acute psychological and physiological responses, and thus a high psychoendocrine covariance, should be expected. However, empirically this does not seem to be the case. Obviously, reduced correspondence could be explained by the effects of multiple confounding variables (as reviewed in this chapter) and, at least in part, by measurement error but it also reflects imperfect coupling of the different stress response systems.

Schlotz and coworkers (2008) indeed showed that lowered interrelations can be ascribed to the different dynamics of these systems. Acute subjective psychological stress responses occur within seconds and may change dynamically during a prolonged stress situation whereas cortisol responses reach their peak approximately 15 to 20 minutes after the onset of stress exposure and change less dynamically. According to the hypothesis that associations between an acute psychological and endocrine stress response should be higher when response correlations are computed at similar system-specific stages relative to the onset of the stressor, it was observed that subjective psychological responses precede HPA axis responses and that high levels of cortisol are associated with lower later levels of anxiety and activation using a cross-correlational analytic approach (see Kudielka et al., 2009).

In sum, it could be shown that psychoendocrine responses are indeed coupled with cortisol levels if time-lagged correlations are applied. Results indicate that the so-far described lack of covariance might be, at least in part, explained by the different time courses of psychological and endocrine responses to stress, with subjective psychological responses preceding HPA axis responses. Therefore, if we want to draw valid conclusions about psychoendocrine covariance in response to acute stress, the different time courses of psychological and biological responses need to be accounted for.

Although stress paradigms like the TSST are principally highly standardized protocols, there is variation between laboratories. Some researchers decide to apply modified versions of the TSST, other deviations are caused by practical considerations. Goodman

et al. (2017) recently analysed various protocol modifications of the original TSST and provided a list of recommendations that ensure a robust activation of the HPA axis. Fortunately, the effectiveness of the TSST appears to be relatively robust to (some) methodological variability. Nevertheless, we recommend adhering to the original protocol specifications to ensure maximized cortisol responses and to enable better comparability across studies.

3.5 Conclusions

Identifying determinants of inter- and intra-individual variability in cortisol regulation as well as understanding the mechanisms underlying pathologically relevant dysregulation of cortisol activity are key topics in psychobiological stress research. However, the phenotyping of markers of HPA axis reactivity in humans is a challenging, laborious, and time-consuming task.

To date, we know that numerous moderating and intervening factors, carefully described in different laboratories and summarized in the present paper, can have an impact on cortisol responses. Knowing relevant modulators increases the chance to detect true effects and to improve, technically spoken, the signal to noise ratio. This is crucial as the effects that can be expected in psychobiological stress research are usually of modest size (although they can well be of psychobiological or clinical relevance). This is certainly a somewhat challenging situation as it is not possible to control for countless potential confounders in each study and this holds particularly true when sample sizes are relatively modest. Therefore, in this review we aim at giving specific recommendations on how researchers might handle the respective variables. Of course, researchers should be aware of the fact that this list is still selective and new insight is accumulating continuously.

The presented knowledge might be helpful at different stages of a research project (see Kudielka et al., 2009). First, when planning an experiment, it might guide the researchers' decision on exclusion criteria, eligibility and selection of subjects (depending on the study question), further information that should preferably be provided by participants (e.g., in accompanying demographic or psychometric assessments), factors that could be held constant across subjects and issues that are relevant for the instruction

of subjects before and during the assessment period. For example, smoking or the intake of oral contraceptives can be defined as exclusion criteria, can be held constant across different study groups, can afterwards be used as covariate, or defined as the experimental manipulation in a (quasi-) experimental study design. Such decisions consequently influence the theoretically optimal sample size of a study, apart from considerations of feasibility.

Second, when it comes to data analysis, knowledge about moderating and intervening factors can help to select potentially relevant control variables to be used, for example as covariates in statistical models. However, researchers should be aware of the fact that the appropriate number of covariates depends on the sample size since model overfitting might lead to spurious results (Babyak, 2004). Furthermore, Miller and Plessow (2013a) offer a discussion on transformation techniques of cortisol data to meet distributional criteria for use in general linear model-based analysis.

Third, the acknowledgement of potential sources of variance is finally essential when it comes to data interpretation. This might be important, for example, for studies based on small sample sizes, quasi-experimental designs, studies with limitations in randomization, or studies conducted under ambulatory settings and field conditions, etc. A discussion of potential sources of variance might contribute to the explanation of contradictory or conflicting results across different studies and might trigger the exploration of further yet unknown sources of variance. Another important aspect is the issue of generalizability of given results.

Finally, in case of secondary analyses and reanalyses taking advantage of preexisting samples or data sets, researchers should be particularly aware of potential differences between samples, for example due to the specific study aim, design characteristics or due to possible variance in cortisol concentrations obtained with different biochemical assays. However, we should also bear in mind that even in highly controlled studies results might be sample-specific for unknown reasons. Therefore, replications in diverse study samples are always a necessary requirement.

To conclude, with the present review we aim to provide an updated overview on moderating and intervening factors as sources of intra- und inter-individual variability in human cortisol responses to psychological stress. We hope that this knowledge will be

helpful for further research investigating pathways leading from individual psychobiological stress regulation to health and disease.

So far, laboratory stress paradigms have proven useful tools in the field of experimental basic, applied, and clinical stress research. However, there is still a pressing need of studies investigating the ecological validity of psychological stress protocols by comparing real-life stress responses to those observed in the laboratory (see Henze et al., 2017). Similarly, the predictive validity of laboratory stress paradigms should be further evaluated by using them in prospective longitudinal studies on chronic stress or stress-related clinical outcomes. A major future task will be to translate more and more our basic knowledge to clinical application, for example, in order to predict disease susceptibility, symptom severity and/or to develop therapeutic approaches and monitor the efficacy of practical interventions.

4 Effect of Sugar Administration on Cortisol Responses to Acute Psychosocial Stress

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Sandra Zänkert and Stefan Wüst developed the study concept and study design. Sandra Zänkert supervised the data collection. Sandra Zänkert performed data analysis and drafted the manuscript. Stefan Wüst and Brigitte Kudielka provided critical revisions.

4.1 Abstract

Sugar administration prior acute psychosocial stress exposure was shown to enhance subsequent salivary cortisol responses. However, this finding is based on studies that have administered high doses of glucose to male subjects after long fasting periods. Therefore, in the present study, we investigated the effect of different sugar-containing drinks on acute cortisol stress responses under experimental conditions that are commonplace in stress research and our sample included females and males. Our primary aim was to derive feasible recommendations for a standardized sugar administration in future studies. Of the 103 healthy young participants (49 females, 54 males), 72 were confronted with the Trier Social Stress Test after being randomly assigned to one of three sugar conditions (200 ml of grape juice, a 75 g glucose or a 75 g maltodextrin drink); 31 subjects served as control sample and were exposed to the TSST without sugar administration. Cortisol stress responses were significantly enhanced in the grape juice as well as the glucose group as compared to the control group. Post hoc analysis revealed that this effect seemed to be more pronounced in males than in females. We did not find a significant effect of maltodextrin. Cortisol responder rates in all three experimental groups were higher than in the control group. Our results suggest that, at least in males, the administration of 200 ml of grape juice is sufficient to facilitate HPA axis reactivity and to minimize confounding effects due to interindividual differences in energy availability while being exposed to a laboratory stress paradigm. The unexpected gender-specific effect is of

potential relevance and should be scrutinized in future studies.

4.2 Introduction

Cortisol responses to psychosocial stress are an important biomarker of hypothalamus-pituitary-adrenal (HPA) axis reactivity. A consistent feature of HPA axis stress responses is their distinct intra- as well as inter-individual variability. To understand the mechanisms linking HPA axis regulation and disease risk, it is of vital importance to identify the factors contributing to this variation. A well-established paradigm to induce moderate psychosocial stress in standardized experimental settings is the Trier Social Stress Test (TSST, Kirschbaum et al., 1993). Psychobiological research revealed several factors influencing cortisol responses to the TSST, including genetic factors, gender, age, psychopathology, medication, and the consumption of several substances (Zänkert et al., 2019). A further relevant factor is the nutritional state prior stress exposure (Strahler et al., 2017).

The influence of glucose on cortisol stress responses was first studied in males who fasted for 8 – 11 h and ingested either 100 g glucose or a placebo before being either confronted with the TSST or a control setting (Kirschbaum et al., 1997). Results showed that glucose administration per se did not affect cortisol levels but that stress induced a larger cortisol response in glucose-treated subjects compared to controls. Additionally, energy administration through protein or fat consumption was not found to amplify cortisol responses (Gonzalez-Bono et al., 2002). The underlying mechanisms are poorly understood, but a role of hunger and saturation regulating neuropeptides has been discussed (Rohleder & Kirschbaum, 2007).

Based on these findings, it has been recommended to avoid major differences in blood sugar levels between participants (Gonzalez-Bono et al., 2002) to minimize confounding effects of the variability in energy availability on cortisol stress responses. However, published studies that applied the TSST or similar stress paradigms since then show a remarkable diversity regarding the implementation of these recommendations. Implementations range from no (reported) consideration to strict fasting periods prior testing (e.g., 3 h fasting in Duncko et al., 2006) and from administration of grape juice (e.g., 250 ml grape juice in Steudte-Schmiedgen et al., 2017) or other sugary drinks (e.g.,

300 ml regular sugared cranberry juice in Rohleder et al., 2003) to the administration of standardized meals (e.g., 125 g tomato risotto in Boyle et al., 2016).

Moreover, as they did not aim at investigating the influence of energy availability on cortisol responses per se, these studies did not include control conditions without any nutrient administration. To date, this lack of standardization between studies significantly impairs the comparability of reported findings. Therefore, we conducted a study with three sugary drink groups and a control group to investigate the effect of sugar administration on cortisol responses to the TSST.

Our primary aim was to derive feasible recommendations for a standardized sugar administration in future studies. This aim guided the present sample composition, the study protocol and the selection of sugary drinks. First, and in contrast to the early studies, we included both females and males. We had no specific hypothesis on gender-specific effects. However, in principle, females should be excluded from study samples in stress research only in well-founded exceptional cases. Moreover, (unexpected) differences in cortisol responses between males and females are a well-known phenomenon (Kumsta et al., 2007; Liu et al., 2017).

Secondly, instead of a long fasting period, our protocol included the widely-used instruction to refrain from eating major meals 3 h before testing. Thirdly, we selected grape juice for one of the experimental conditions as it has been used in several previous studies and because it is easy to obtain and relatively palatable. Grape juice has the highest sugar content among natural fruit juices with a sugar mixture of about 49 percent glucose, 50 percent fructose and 1 percent sucrose (Souci et al., 2016)(Souci et al., 2015). However, as it is a natural juice, sugar content differs between products. We inspected 13 different off-the-shelf juices containing 14.6 to 19 g sugar/100 ml (mean = 16.23, SD = .94, median = 16). Exact data on glucose/fructose ratios were often not specified. A higher degree of standardization can be reached with a self-mixed drink with defined glucose content. As mentioned earlier, an effect of glucose administration on TSST cortisol responses has been shown before (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997) but only after longer fasting periods and only in males.

Another carbohydrate with promising characteristics regarding improved feasibility is the oligosaccharide maltodextrin. Maltodextrin with a dextrose equivalent (DE) of 19,

the form that has been used in the present study, shows a high glycaemic index (GI $\sim$ 85) and is being digested, absorbed and used as a fuel source as rapidly as glucose (GI = 100) (Gonzalez et al., 2017; Hofman et al., 2016). The specific advantage of maltodextrin is the moderately sweet taste that is usually perceived as being more palatable than glucose. For comparison, grape juice has a GI of 52, indicating that over time total blood glucose levels show a lower increase than after glucose or maltodextrin consumption.

4.3 Materials and Methods

A total of 106 healthy students were recruited at the University of Regensburg. Seventy-five participants were randomly assigned to one out of three sugar conditions and a sample of 31 participants, who did not receive any drink, served as control group (CTRL). As three participants were excluded because of extremely high baseline cortisol levels ($>$ 2 SD), the final sample consisted of 103 participants (49 women, 54 men; age: M = 23.9 years, SD = 3.8 years). In women, a self-reported stable menstrual cycle was an inclusion criterion and tests were performed during the luteal phase, defined as $\leq$ 10 days to expected onset of next menstruation. All participants had a BMI $<$ 30, were non-smokers, did not take any medication and reported to be in good health.

The TSST protocol was approved by the ethics committee of the University of Regensburg and participants gave their written informed consent at the beginning of the experiment. They received either 10 Euro or course credit as reimbursement.

Participants were asked to abstain from eating major meals or drinking caffeinated beverages 3 h before the laboratory procedure. Sessions were scheduled between 1:00 and 5:00 pm (TSST onset not before 1:50 pm) to control for diurnal effects. Sugar drinks were administered prior a 50 minutes resting period, followed by the TSST exposure.

In brief, the TSST consists of a preparation phase (3 min), followed by a mock job interview and a mental arithmetic task (5 min each) in front of a panel consisting of two observers and a camera (for a detailed description see Kudielka et al., 2007). Saliva samples were collected repeatedly at -50, -1, +1, +10, +20 and +30 min relative to the TSST using Cortisol Salivettes (Sarstedt, Nümbrecht, Germany). For the CTRL group we used non-published data from a study that was recently conducted by the same research team in the same rooms and with the same TSST protocol including identical

inclusion criteria for participants. The only differences were that no -50 min saliva sample was collected and that no drink was administered. Hence, the -50 min saliva sample was excluded from all data analysis. The CTRL group was not exposed to any additional tasks.

Subjects in the GRAPE group drank 200 ml off-the-shelf grape juice containing 32 g sugar (8 g glucose and 8 g fructose per 100 ml). The dosage of 75 g customary glucose in group GLUCO was adopted from the oral glucose tolerance test protocol. Consistently, 75 g maltodextrin (DE 19) was used in group MALTO. Glucose and maltodextrin were mixed with 200 ml of chamomile tea and served cold for a tolerable taste.

Saliva samples were stored at -20 °C until analysis. They were assayed in duplicate using a time-resolved immunoassay with fluorometric detection (DELFIA) by the biochemical laboratory at the University of Trier. Inter- and intra-assay coefficients of variation were below 10 %.

4.4 Results

To investigate our main question, we conducted a series of repeated measures ANOVAs to test the effect of each sugar condition individually (GRAPE, GLUCO, MALTO) compared to the CTRL group with salivary cortisol as dependent measure. A first analysis without between subject factor revealed a significant effect of time on salivary cortisol ($F(1.65, 168.33) = 60.5$, $p < .001$, $\eta^2 = .37$). The analysis of GRAPE vs. CTRL ($F(1,51) = 4.55$, $p = .038$, $\eta^2 = .082$) as well as GLUCO vs. CTRL ($F(1, 55) = .4.57$, $p = .037$, $\eta^2 = .077$) revealed significant main effects of group. On a descriptive level, lowest mean cortisol responses to the TSST were observed in the CTRL group whereas groups GRAPE and GLUCO showed the highest mean responses (see Figure 1, A). For the analysis MALTO vs. CTRL, no significant main effect of group emerged ($F(1, 53) = 1.54$, $p = .22$, $\eta^2 = .028$). A series of three single ANOVAs results in a cumulated alpha error probability. To control for multiple comparisons, we thus conducted an additional repeated measures ANOVA including all four conditions with subsequent post hoc analysis using Dunnett's test for comparisons against the CTRL group (one-sided). At the first level, this analysis showed no significant group effect ($F(3, 99) = 2.22$, $p = .091$, $\eta^2 = .063$) or group x time effect ($F(4.93, 162.7) = .846$, $p = .517$, $\eta^2 = .025$). However, the post hoc analysis

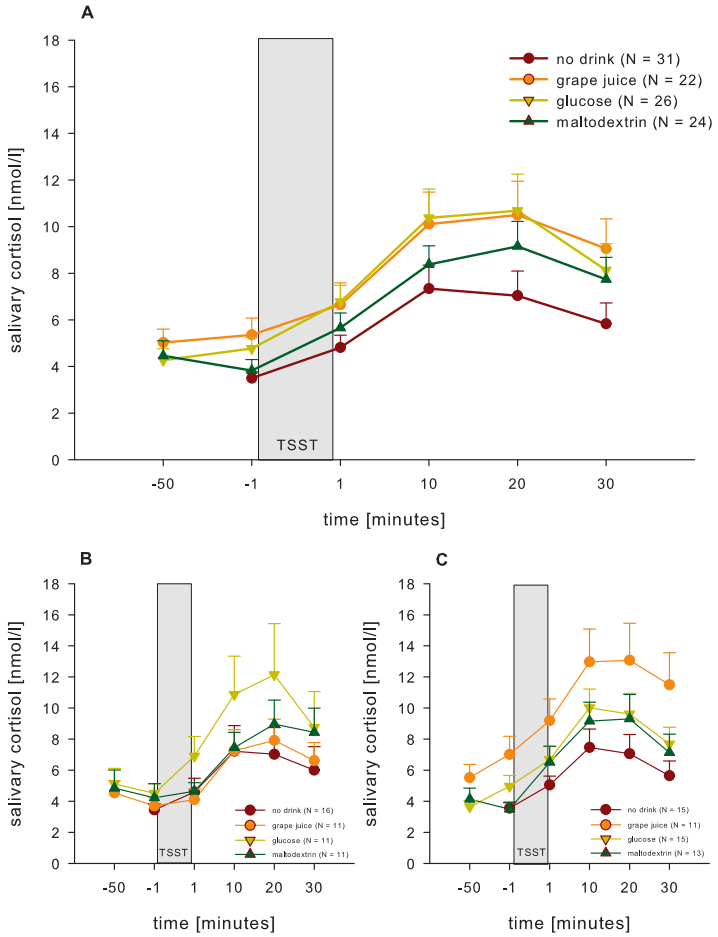

Figure 1: Mean salivary cortisol responses (+/- SEM) to the Trier Social Stress Test in groups in (A) total sample, (B) women and (C) men

revealed significant differences for CTRL vs. GRAPE (p = .04) and CTRL vs. GLUCO (p = .046) but not for CTRL vs. MALTO (p = .308), thus confirming the single ANOVA results.

Next, we entered gender as between subject factor in our models. The analysis of GRAPE vs. CTRL revealed a main effect of gender ($F(1, 49) = 4.4$, $p = .04$, $\eta^2 = .08$) and a significant gender x group effect ($F(1, 49) = 4.06$, $p = .049$, $\eta^2 = .077$) with males showing higher cortisol levels in group GRAPE (see Figure 1, B and C). In the other analyses, we did neither detect a main effect nor a significant interaction (gender x time, gender x group, gender x time x group; all $F < 1.5$, $p > .2$); the number of males and females in our experimental conditions are shown in Figure 1 B and C. As supplementary illustrative analysis, we categorized 72 (69.9%) out of the 103 participants as responders

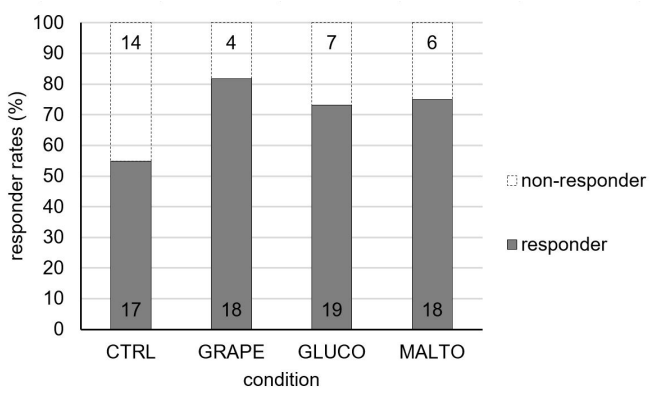

Figure 2: Responder rates (1.5 nmol/l criterium) for each condition in % (bars containing absolute numbers)

according to the cortisol response criterium of 1.5 nmol/l increase proposed by Miller et al. (2013a). Responder rates were lowest in the CTRL group (54.8%) and ranged between 73.1% in group GLUCO, 75% in group MALTO and 81% in group GRAPE (see Figure 2). Statistical comparisons using Chi-squared tests revealed significant distribution differences between responders vs. non-responders in CTRL vs. GRAPE ($X^2(1) = 4.18$, $p = .041$) but not CTRL vs. GLUCO ($X^2(1) = 2.38$, $p > .1$) and CTRL vs. MALTO ($X^2(1) = 2.02$, $p > .1$).

4.5 Discussion

There is convincing evidence that the administration of large doses of glucose after long fasting periods amplifies cortisol responses to TSST exposure. Moreover, the administration of grape juice has been occasionally reported (e.g. Steudte-Schmiedgen et al., 2017) but the effects of these pre-treatments remained unclear, since these studies did not include a control group (as they did not focus on this comparison). Therefore, we studied the effect of sugar administration on cortisol stress responses under more typical conditions (no major meals within 3 h prior testing).

When compared to controls, we found significantly higher cortisol levels after administration of grape juice (with 16 g glucose and 16 g fructose) or a 75 g glucose drink, but not after a 75 g maltodextrin drink. Overall, we thus successfully replicated the augmenting effect of sugar (GRAPE and GLUCO) on cortisol responses to the TSST. It is noteworthy that group differences in cortisol levels already began to emerge prior

TSST onset at time point -1 min (see Figure 1).

On a descriptive level, a similar difference was found in the early study by Kirschbaum et al. (1997). It could be speculated that this effect is mediated by an influence of sugar administration on adrenocortical activity due to anticipation of the upcoming challenge.

Moreover, to the best of our knowledge, this is the first study that included women. We detected a significant group x gender interaction for GRAPE vs. CTRL. Men who drank grape juice showed the highest cortisol responses while in women no difference between GRAPE and CTRL was found (Figure 1B and C). On a descriptive level, cortisol responses in women were higher in group GLUCO than in CTRL subjects. However, the relatively high mean peak at +20 minutes was driven by only two women with peak cortisol > 25 nmol/l. As this is a post hoc observation, this gender-specific effect remains highly speculative and needs to be replicated. Moreover, future studies might consider estradiol mediated differences in storage, breakdown and utilization of carbohydrates between men and women (Wismann & Willoughby, 2006).

Our findings support the view that sugar administration does amplify salivary cortisol stress responses not only when blood sugar levels are markedly low (i.e., after a long fasting period) but also under experimental conditions that are commonplace in stress research. This result extends the methodological relevance of the effect of sugar on cortisol responses originally described by Kirschbaum and colleagues (1997).

Remarkably, cortisol responses after administration of 75 g glucose were not larger than after administration of grape juice containing only 32 g of sugar. Consistently, responder rates were similar in all sugar groups with, at least on a descriptive level, lower responder rates in the CTRL group. A significant difference was found for the comparison GRAPE vs. CTRL group (see Figure 2). This illustrates that not only mean cortisol responses were more pronounced in groups GRAPE and GLUCO than in CTRL but that also the actual number of subjects who showed a distinct psychoendocrine response was higher.

The rationale for testing the effect of maltodextrin was that it a) has a high glycaemic index and it can used by the organism as a fuel source as rapidly as glucose (Hofman et al., 2016) and b) that it usually is perceived as being more palatable. However, we

failed to find support for the assumption that the augmenting effect of 75 g maltodextrin (DE 19) on cortisol responses is similar to that of 75 g glucose. As, in contrast, grape juice with 32 g of sugar and a lower GI did have a significant effect on cortisol responses, our data do not support the assumption of a simple linear relationship between the effect of a sugar form on cortisol stress responses and its influence on blood glucose and insulin. A speculation offering a partial explanation is that blood sugar levels above a certain threshold might have no additional augmenting effect on cortisol responses.

Moreover, as discussed by Rohleder and Kirschbaum (2007), the effect of blood sugar levels on HPA axis activity might be mediated by regulation of orexigenic and anorexigenic neuropeptides. They conclude that although neuropeptides involved in energy homeostasis have been shown to impact on HPA axis activity, observed effects are inconclusive when it comes to suppression in states of low energy availability (Rohleder & Kirschbaum, 2007). It could also be speculated that differences in the feeling of satiety after maltodextrin and glucose consumption might play a role (Hofman et al., 2016; Rohleder & Kirschbaum, 2007).

Our study design showed at least two limitations. First, we did not measure blood sugar and insulin levels. One could argue that this measurement is dispensable as blood sugar and insulin levels after sugar administration have been studied extensively and it is not necessary to answer the major question of our study. However, we admit that blood sugar and insulin levels over the course of the test session would have been an interesting additional predictor. Second, data for our control condition were derived from an independent study conducted in the same laboratory and did not include a placebo drink. However, the study protocol and research team were identical except for the initial -50 min prestress cortisol sample and it thus appears relatively implausible that unknown factors acted as significant confounders. Furthermore, as protein or fat consumption was not found to increase cortisol responses to TSST exposure (Gonzalez-Bono et al., 2002), it is unlikely that a placebo drink administration would have had a major effect.

4.6 Conclusion

In summary, our findings support the view that in studies including laboratory stress paradigms, sugar administration does amplify mean salivary cortisol responses and leads

to higher responder rates in men. Administration of 200 ml of commercially available grape juice (with 8 g glucose and 8 g fructose per 100 ml) seems to be sufficient to facilitate HPA axis reactivity. However, in a way, this study failed to derive general and easy recommendations as cortisol responses after sugar administration in women seem to differ from those in men. This effect might be relevant for psychoendocrine stress research as gender-specific effects of the nutritional state could contribute to the well-known but still only partly understood difference in cortisol responses to acute psychosocial stress in females and males. Therefore, this unexpected and interesting preliminary observation should be further investigated in future studies.

5 Sustained threat and phasic fear in the laboratory and cognitive-emotional processes of anxiety in everyday life - an ambulatory assessment study

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Sandra Zänkert, Stefan Wüst and Anja Schmitz developed the study concept and study design. Sandra Zänkert supervised the data collection. Sandra Zänkert performed the data analysis and drafted the manuscript. Stefan Wüst, Brigitte Kudielka, Alfred Lindl and Andreas Mühlberger provided critical revision.

5.1 Abstract

Fear is a phasic state of apprehension to an imminent threat, whereas anxiety is a more sustained state of expecting a potential threat leading to tension and worry. The NPU-threat test is a laboratory startle paradigm allowing a reliable and valid assessment of both, fear- and anxiety-potentiated reactions. It is suggested to differentiate between anxiety disorders, but little is known on associations with everyday life experiences of cognitive-emotional processes regarding anxiety in non-clinical samples. In the present project, the NPU-threat test was applied in three studies with (1) unselected healthy individuals, (2) subjects with extreme manifestations of trait anxiety (low vs. high) and (3) individuals preparing for a high-stakes exam. Self-reported states of emotionality and worry were assessed during a four-day ambulatory assessment (AA). Overall, NPU-threat test measures did not significantly differ between studies, while the AA dependent measures were sufficiently sensitive to capture differences between groups. However, there was no significant association between psychophysiological measures of the NPU-threat test and AA state measures across participants. In participants recruited for low vs. high trait anxiety we found an association with AA worry and emotionality, but no interaction with potentiated startle. The present findings do not support the idea of a link between our laboratory biomarker and adaptive regulation of cognitive-emotional

states in everyday life in healthy individuals with average till high manifestations of trait anxiety. We speculate that an association between laboratory physiological measures and everyday experience of anxious states may be detectable in clinical samples.

5.2 Introduction

Anxiety disorders are a major public health burden not only in western societies but worldwide (Stein & Craske, 2017). Yet the understanding of mechanisms of anxiety remains limited and treatment remains a significant challenge (Grillon et al., 2019). While there has been progress in our understanding of the neurobiology and neurochemistry of threat processing in animal models, there was only limited advancement regarding the translation to new treatments and improved clinical outcomes in humans over the last decade. A more elaborate understanding of fear and anxiety as distinct features of anxiety disorders is key to elucidate the nature of these disorders and to adapt optimized treatment strategies for better effectiveness (Grillon et al., 2019; Wilkinson et al., 2016). This also includes a better understanding whether and how possible biomarkers for clinical outcomes derived from laboratory paradigms show associations to everyday life in non-clinical individuals.

Although the distinction and definition of fear and anxiety has been controversial, accumulating evidence support the notion that they differ in a number of key dimensions (Schmitz & Grillon, 2012). Psychometric analysis of symptomatology distinguishes characteristic elements of anxiety like anxious apprehension, enhanced vigilance and general distress from features of fear like fight/flight, focused attention and enhanced arousal (Brown et al., 1998; Davis et al., 2010; Grillon et al., 2019; Schmitz & Grillon, 2012; Zinbarg & Barlow, 1996). Studies suggest that especially internalizing disorders presumably sharing a negative affectivity factor can be cut across two core psychopathological processes or sub-dimensions, one representing fear disorders (e.g., specific phobia, social phobia, agoraphobia, panic disorder (PD) and other 'anxiety-misery' disorders (e.g., depression, dysthymia, generalized anxiety disorder (GAD), posttraumatic stress disorder (PTSD) (Cox et al., 2002; Davis et al., 2010; Krueger, 1999; Vollebergh et al., 2001). Further evidence derives from experimental studies that used context conditioning paradigms to elicit either fear or anxiety and, for example, found differences in

psychophysiological markers like differences in startle responses between PD and GAD (Davis et al., 2010; Grillon et al., 2008, 2009b).

Recent reviews on translational and human research conclude that the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BST) as major subdivisions of the extended amygdala both covary with signs and symptoms of fear and anxiety. Both areas show phasic responses to short-lived threat as well as heightened activity during sustained exposure to diffusely threatening contexts (Robinson et al., 2019; Shackman et al., 2016).

A study on neuronal connectivity of the extended amygdala using a threat of shock paradigm reports less strong coupling of the CeA and BST with the ventromedial prefrontal cortex, the cingulate cortex, and nucleus accumbens during threat. Additionally, the CeA became more strongly coupled with the thalamus (Torrisi et al., 2018). However, there remains a gap between knowledge especially derived from animal models (Siminski et al., 2021) and translation from basic science to clinical research (Grillon et al., 2019; LeDoux & Pine, 2016).

Acknowledging the importance of human experimental models on fear and anxiety seems crucial to understand the complex cognitive and emotional processes, which eventually result in affective disorders. Moreover, it might help to adapt psychological and pharmacological treatment strategies (Gorka et al., 2017; Grillon et al., 2019; Grillon & Ernst, 2020). From an evolutionary perspective, fear and anxiety represent basic defensive responses that motivate an organism to detect, react and cope with threat and danger (Schmitz & Grillon, 2012). These responses vary with the nature of the threat, depending on whether it is proximal and requires immediate action or if it is temporally uncertain or distal, calling for sustained vigilance. The duration (phasic vs. sustained) and predictability of the threat (predictable vs. unpredictable) are seen as two distinct dimensions and are reflected by the operational terms sustained anxiety and phasic fear (Davis et al., 2010; Schmitz & Grillon, 2012).

In laboratory settings, a fear can be induced with discrete cues that are predictably paired with an aversive event whereas anxiety can be induced using more diffuse cues or cues less predictably associated with aversive events (Davis et al., 2010). The threat of the predictable and unpredictable aversive events test (NPU-threat test) (Schmitz &

Grillon, 2012) is a startle paradigm that has been widely used to differentiate startle potentiation to predictable (fear-potentiation) and unpredictable (anxiety-potentiation) threat. In brief, the NPU-threat test consists of three conditions: no aversive events (N condition), predictable aversive events (P condition) and unpredictable aversive events (U condition). Aversive events can be unpleasant shocks (Brown et al., 1998) or blasts of air to the neck (Schmitz et al., 2011) and the participants' reaction is measured using the startle reflex by an electromyogram (EMG) of the orbicularis oculi to capture the blink component of the reflex (Schmitz & Grillon, 2012).

The potentiation of the startle reflex in general is a cross-species measure of aversive states (Davis et al., 2010). The NPU-threat test is designed to evoke a fear-potentiated startle response in the P condition and an anxiety-potentiated startle response in the U condition. Several studies suggest good reliability of NPU-threat test measures (Grillon & Ernst, 2020; Kaye et al., 2016; Lieberman et al., 2017; Nelson, Hajcak, et al., 2015; Shankman et al., 2013). Validity of the NPU-threat test can be derived from studies in clinical and non-clinical samples underpinning the usefulness of the physiological measures of the NPU-threat test as possible biomarkers for anxiety pathology (Davis et al., 2010; Grillon & Ernst, 2020).

For example, elevated anxiety-potentiated startle is reported in patients with PD and PTSD compared to healthy controls while there were no differences in fear-potentiated startle (Davis et al., 2010; Grillon et al., 2008, 2009b). Patients with GAD, on the other hand, did not show an alteration of either anxiety- or fear-potentiated startle compared to healthy controls (Grillon et al., 2009b). It is suggested that differences between PD and GAD derive from different cognitive processes regarding worry and rumination in GAD and anticipatory fear and anxiety in PD (Davis et al., 2010).

Other fear-based anxiety disorders like social anxiety and specific phobias have also been reported to elicit a greater startle potentiation to unpredictable threat, but not predictable threat, compared to individuals with GAD, major depressive disorder and controls, suggesting that these anxiety disorders are related to a hypersensitivity to uncertain aversive events (Gorka et al., 2017). Additionally, reactivity to unpredictable threat has been shown as a possible treatment target in fear-based anxiety disorders (PD, SAD, PTSD) with decreased startle magnitudes during unpredictable threat post

cognitive-behavioural therapy (Gorka et al., 2017).

Furthermore, results on medication sensitivity of the NPU-threat test components in humans support results from translational animal models on the neurological basis of fear and anxiety. The CRH1 antagonist GSK561679 has been shown to increase fear but not anxiety in a non-clinical female-only sample (Grillon et al., 2015), whereas the benzodiazepine alprazolam was shown to preferably reduce anxiety in a non-clinical mixed-sex sample (Grillon et al., 2006). Increasing levels of serotonin, via chronic administration of the selective serotonin reuptake inhibitor citalopram for two weeks, was found to reduce contextual anxiety but not cued fear (Davis et al., 2010; Grillon et al., 2009a). Consistently, acute tryptophan depletion was associated with a reduction of serotonin increased anxiety- but not fear-potentiated startle (Grillon et al., 2009a; Robinson et al., 2012; see also Davis et al., 2010).

Finally, the NPU-threat test has also been acknowledged in the Research Domain Criteria (RDoC, Insel et al., 2010), a research framework for investigating and capturing the interface between neurobiology and psychopathology. However, knowledge on the ecological validity of the NPU-threat test seems limited. Particularly, there are no studies yet relating intra- and interindividual differences in NPU-threat responses to differences in everyday experience of fear and anxiety.

To address this question a combination of a laboratory and an ambulatory assessment (AA) study is a promising approach as it allows investigating ecological validity in regard to every day inner experience and human behaviour (Trull & Ebner-Priemer, 2013; Wilhelm & Grossman, 2010; Wrzus & Mehl, 2015). Especially in the field of anxiety pathology, research on mood, behaviour and symptomatology in everyday life is well established (Trull & Ebner-Priemer, 2020; for review on anxiety pathology see Walz et al., 2014; also Chun, 2016). Moreover, ambulatory monitoring of patients becomes more and more important in clinical treatment research (Alpers, 2009; Trull & Ebner-Priemer, 2009; Walz et al., 2014).

However, this research is almost exclusively focused on pathology, with a few exceptions (see e.g. Wegerer et al., 2013) and assessment scales usually selected for daily measurement of anxiety or worry have been tailored to differentiate between healthy participants and patients. Often only single items have been used, for example “How worried do you feel?”

to assess worry or "How anxious do you feel?" to assess anxiety (e.g. Helbig-Lang et al., 2012), apparently resulting in a floor effect for healthy control groups displaying only little variance (e.g. Kirchner et al., 2017). To keep assessments short and to minimize the participants' burden, some AA studies used a selection of items from full scales to assess momentary affect and symptoms (Naragon-Gainey, 2019; Starr & Davila, 2012) or short self-developed scores lacking any psychometric properties (Hall et al., 2021; Pfaltz et al., 2010).

Considering the hypothesis of a dimensional conceptualization of psychopathology assuming a continuum from normal to pathological anxiety (Grillon et al., 2019), a more precise assessment of everyday inner experience of anxiety and fear appears appropriate for a better understanding of interindividual differences in non-clinical samples. Research approaches combining laboratory measures and ambulatory assessment designs, like a study on the relationship between fear conditioning and aversive memories in the following 2 days (Wegerer et al., 2013), are still sparse. Recently, Grillon and colleagues (2019) stressed the importance to understand anxiety as a spectrum and they concluded that especially a better understanding of intraindividual differences in non-clinical samples and the association with trait measures should be more taken into account. Therefore, combining AA to assess mood and behaviour in everyday life with laboratory measures seem crucial for a better understanding of fear and anxiety as well as affective disorders.

To the best of our knowledge, only few studies from the field of education research on test anxiety investigated sustained anxiety and phasic components of fear in non-clinical samples in longitudinal study designs. Here, situation-specific worry (cognitive) and emotionality (affective) were assessed, for example, four times over the course of one semester (Lotz & Sparfeldt, 2017; Roos et al., 2020). Laux and colleagues (2013) adapted measures for situation-unspecific worry and emotionality for the assessment of trait and state anxiety. The main focus of the present analyses was to investigate the ecological validity of the NPU-threat test using an AA setting.

Therefore, we assessed cognitive-emotional processes regarding anxiety in everyday live in three independent non-clinical samples. For everyday measures of anxiety, we utilized a two-component approach with emotionality as a short-lived perception of heightened bodily arousal and worry as long-lasting cognitive concerns about negative expectations

regarding the future. A first study was conducted to investigate the association between every day worry and emotionality with physiological measures of the NPU-threat test from the laboratory in unselected healthy volunteers. In a second and third study, we extended our design to allow for more variability in our dependent measures within and between participants. Therefore, in study 2 we conducted an online screening and subsequently included only participants with high vs. low trait anxiety. Additionally, as a natural alteration in the variability of everyday anxiety and fear and therefore responses to worry and emotionality, we recruited participants preparing for a high-stakes academic exam in study 3 (Hodapp et al., 2011; Laux et al., 2013).

5.3 Materials and Methods

5.3.1 Participants

Participants (aged 18 – 46 years, M = 21 years) for all three studies (N = 76) were recruited at the University of Regensburg via flyer and social media. Exclusion criteria were ascertained via self-report and included disorders of the central or peripheral nervous system or acute affective disorders, psychopharmacological medication, wearing a heart pacemaker and pregnancy in women. All participants provided informed consent and the NPU-threat test was approved by the ethics committee of the University of Regensburg. All participants received a monetary compensation of 25 Euro or research course credit. For the first study, we recruited N = 23 psychology students. We had to exclude one participant who withdrew from the laboratory session and one with bad startle data quality leaving N = 21 for analysis.

For the second study, a total of N = 563 participants took part in an online screening to identify high and low anxious participants according to the State-Trait Anxiety-Depression Inventory (STADI, T-value less than or equal to 40 for low and greater or equal to 60 for high anxiety, according to Laux et al., 2013). Of the 183 high and 39 low anxious participants, we invited N = 15 of each group to take part in our study. Participants of the online questionnaire who were not selected for the laboratory procedure could enrol for 20 lottery drawings of 25 Euro vouchers as compensation.

For the third study we recruited an initial sample of 24 participants, who were tested during a period while preparing for a high-stakes exam of subjective personal

importance. Twelve participants were preparing for state examinations, five participants had to take re-examination that had to be passed to prevent de-registration from university, one participant prepared to take the Graecum, two participants prepared for the preliminary medical examination, two participants prepared for oral examination in clinical psychology, one participant prepared for his PhD defence and one participant for his quarterly examination in music. We had to exclude five participants due to low compliance with the AA protocol ($> 50\%$ missing values) and three participants due to bad startle quality.

Therefore, our final sample for study 3 comprised only N = 16 participants. Hence, the final analyses were based on N = 67 participants (47 female).

5.3.2 General Procedure

The design of laboratory and daily assessment was the same in all three studies. All eligible participants were invited for a laboratory session and took part in a four-day ambulatory assessment which was scheduled either a few days before or after the NPU-threat test. On their first visit, participants received detailed study information on both parts of the study and written informed consent was obtained.

During the laboratory session participants had to complete a set of questionnaires on trait and state affect and anxiety. Once finished, they received a detailed description of the NPU-threat test and electrodes were placed to measure the eye-blink startle via electromyogram along with additional electrodes for measurement of the electrocardiogram and skin-conductance level (data of the latter not reported here). The NPU-threat test was presented in two blocks of 14 minutes each and a short break of 5 – 10 minutes in between. State anxiety was assessed before and after the task. Participants were asked to rate threat value and shock expectancy of each NPU condition after the first and second block. The entire session took about 80 minutes. For a detailed description see Schmitz & Grillon (2012).

For the ambulatory assessment, participants were equipped with a study smartphone preinstalled with the app movisens XS, Version 0.6.3658 (movisens GmbH, Karlsruhe, Germany). They received verbal and written detailed information on the assessment procedure, how to use the app and how to contact the researchers if there were any

problems. Additionally, all items of the assessment were explained shortly to ensure a common understanding between participants. The exact date of the assessment was scheduled to start either on a Monday or Tuesday till Thursday or Friday, respectively. Participants were asked to keep the smartphones on vibration and at least minimum volume at all times, to ensure appropriate attention to the alarms.

5.3.3 Psychometric Measures

To assess measures that reflect phasic fear and sustained anxiety, we chose the subscales emotionality and worry of the anxiety scale of the State-Trait Anxiety-Depression Inventory (STADI, Laux et al., 2013; Renner et al., 2018). The STADI consists of a state and trait version with 20 items each, measuring depression and anxiety. Studies suggest substantial construct validity as well as discriminant and convergent validity (Laux et al., 2013; Renner et al., 2018). The subscales of the state version have shown to be sensitive to change. For the trait version, the authors report a long-term stability (14 months) of r = .85 for the global score and r between .50 and.60 for anxiety and depression (Laux et al., 2013). The state scales seemed suitable for application in an AA design (see below). The construct emotionality is defined as emotional component of anxiety and asks for physiological apprehension and level of arousal whereas worry is seen as cognitive component directed towards negative expectations or concerns in the future.

It has been shown that especially patients with GAD score higher on worry scales (trait and state) whereas emotionality is correlated with symptoms associated with a physiological state of apprehension as it can be found for example in PD (Laux et al., 2013). Studies on the validity of the STADI state scales investigated sensitivity regarding imagined and real-life challenging examination scenarios, leading to the conclusion that the worry scale represents negative long-term expectations while emotionality measures capture immediate responses to a potential threatening challenge.

We further assessed the anxiety-related traits anxiety sensitivity (AS) using the 18-item revised version of the Anxiety Sensitivity Index-3 (ASI-3, S. Taylor et al., 2007; German version: Kemper et al., 2009), intolerance of uncertainty (IU) using the 18-item German Intolerance of Uncertainty Scale (IU-18, Gerlach et al., 2008) and test anxiety using the German version of the Test Anxiety Questionnaire (Prüfungsangstfragebogen,

PAF, Hodapp et al., 2011) in study 2 and study 3.

5.3.4 Laboratory Threat Task

For the implementation of the NPU-threat task we used the protocol published by Schmitz and Grillon (2012). In short, the task included three within-subject conditions (including respective visual cues): No shock (N condition, cued by green circle), predictable shock (P condition, red square, shock possible during cue presentation) and unpredictable shock (U condition, blue triangle, shock possible at any time). In each condition acoustic startle probes were administered during cue presentation (CUE) and during inter-trial intervals (ITI). Electric shocks (1 – 5 mA) were used as aversive stimuli and a shock work-up procedure was completed as described by Schmitz & Grillon (2012) to find an individual shock level that was rated uncomfortable but not painful.

Participants were given detailed instructions on the experimental conditions and were informed via a note on top of the screen about the contingency of aversive shock events during each condition. Conditions were presented in two blocks with a 5 – 10 minute break in between as reported by Schmitz and Grillon (2012). After each block, participants were asked to rate threat value and shock expectancy for each condition (CUE and ITI). State anxiety after each block was measured using the State-Trait Anxiety Inventory (STAI, German version: Laux et al., 1981). Ratings on treat value, shock expectancy and state anxiety for both blocks were averaged.

5.3.5 Psychophysiological Recording and Processing

The acoustic startle stimulus consisted of a 40 ms duration 102-dB(A) burst of white noise presented through headphones. The electromyography (EMG) signal was amplified, filtered (28 – 499 Hz), rectified and integrated (100 ms time constant) using the BrainVision Analyzer 2.1 (Brain Products GmbH, Gilching, Germany). Peak amplitudes of the blink reflex were determined in the 20 – 120 ms time frame following stimulus onset relative to the average EMG level for the 50 ms immediately preceding stimulus onset. Peak magnitudes were averaged separately for the aforementioned conditions over blocks. Baseline startle was defined as the mean startle magnitude during ITI. To assess fear- and anxiety-potentiated startle (Schmitz & Grillon, 2012), t-scores were calculated in

order to control for baseline startle differences. Fear- and anxiety-potentiated startle were defined as difference scores between mean startle magnitudes (t-scores) during P_{CUE} and P_{ITI} for fear and during U_{ITI} and N_{ITI}. for anxiety.

5.3.6 Ambulatory Assessment

During AA, assessments were triggered via the app movisens XS from 9 am till 21 pm. Each participant received eight alarms per day on four consecutive work days that were triggered pseudo-randomly with at least 30 minutes between each assessment resulting in 32 assessments per subject. Participants had the option to delay an alarm for 5 times but no longer than 15 minutes. Altogether, the assessment was comprised of 21 items. Fear and anxiety were assessed with 5 items each reflecting the state anxiety subscales of the STADI emotionality and worry. The STADI state version has shown to be a sensitive measure of change (Laux et al., 2013).

Context information was assessed with 6 items asking about the current location, other present people and if there were any special events since the last assessment. Up to 5 follow-up questions were presented when appropriate. A full list of items can be found in Appendix A. Data sets with more than 50% missing assessments were excluded. With a Level 2 sample size of N = 67 and a Level 1 sample size of theoretically N = 32 observations per subject (with $N_{80\%} = 25.6$ assuming average compliance of 80%) and a total of N = 2144 observations ($N_{80\%} = 1715.2$), our sample size seemed appropriate to detect hypothesized effects using multilevel analysis (Scherbaum & Ferreter, 2009).

5.3.7 Statistical Analysis

Statistical software SPSS (IBM SPSS Statistics 26) and R (version 3.6.3; R Core Team, 2020) were used for data analyses. To test for group differences in demographics and trait measures, we conducted a series of chi-square tests, t-tests or one-way analysis of variance (ANOVA) in accordance with data level. To confirm that the NPU-threat task elicited startle potentiation as reported elsewhere (Schmitz & Grillon, 2012), we conducted a 3 x 2 repeated measures (RM) ANOVA (condition: N, P, U x cue: cue, no cue) across all participants and with study as a between-subject factor as well as with $\text{anxiety}_{[\text{low vs. high}]}$ (low = 0; high = 1) in the subsample of study 2. RM ANOVAs were

also applied to test for differences in reported threat values and shock expectancies (Table C.1). Here, values for both blocks were averaged. Anxiety-potentiated (U_{ITI} – N_{ITI}) and fear-potentiated startle (P_{CUE} – P_{ITI}) were calculated accordingly (Schmitz & Grillon, 2012),. Greenhouse-Geisser correction was applied when necessary. Pearson correlations were calculated to test for associations between trait measures (Appendix C).

To evaluate the association between repeated measures of emotionality or worry from the AA with laboratory and trait measures we applied intercept-only Linear and Generalized-Linear Mixed Models (LMM and GLMM) using the R packages lme4 (v1.1-26, Bates et al., 2020) and glmmTMB (v1.0.2.1, Magnusson et al., 2020). Intraclass correlations (ICCs) were calculated for emotionality and worry for each study separately, for the low and high anxious groups of study 2 and the pooled sample, using the R package ICC (v2.3.0, Wolak, 2015). Differences in between- (varb) and within-person variances (varw) will be reported on a descriptive level. The ICC package calculates the ICC based on a simple analysis of variances and therefor varb and varw represent mean square residual variances. Density plots of emotionality and worry suggested a non-normal, right-skewed distribution. We applied empty GLMMs, which indicated best fit for a negative binomial distribution with linearly increasing variances with the mean (Brooks et al., 2017) for both emotionality and worry. To avoid non-convergence of GLMMs, we adjusted the scale ranges of state emotionality and worry to 0 – 20.

For the final analysis we first applied the less complex LMMs. In a second step, we reanalysed models indicating significant fixed effects using GLMMs assuming a negative binomial distribution to test the robustness of these effects. The physiological measures (EMG startle amplitudes) of the NPU-threat test were evaluated in four different models, entering (1) only the startle amplitudes (2) the interaction of startle:$\text{condition}_{[N,\ P,\ U]}$ (3) the interaction of startle:$\text{condition}_{[N,\ P,\ U]}$:$\text{cue}_{[cue,\ no\ cue]}$ and (4) fear- and anxiety-potentiated startle in comparison to an empty model. For study 2, analyses were conducted in the corresponding subsample with addition of the fixed factor $\text{anxiety}_{[low\ vs.\ high]}$, including main and interaction effects. Models on exam preparation were conducted including the factor $\text{exam}_{[yes]}$ for participants of study 3 and $\text{exam}_{[no]}$ for studies 1 and 2. All continuous predictors were grand-mean centred. Finally, we conducted a sensitivity analysis for our overall model using the simr package (v1.0.5, Green & MacLeod,

2019). A complete list of R packages used can be found in the external file, linked in Appendix C.

5.4 Results

5.4.1 Sample Characteristics

Sample characteristics are presented in Table 2. Overall, 47 female and 20 male participants were included and the sex distribution did not differ significantly between studies (X^2 (2, N = 67) = 4.7, p = .95). Participants were aged between 18 and 46 years (M = 22.01, SD= 4.35). Oneway ANOVA and posthoc Tukey's HSD test revealed a significant difference regarding age, with study 3 > study 2 (see Table 2). On the total group level, trait measures of anxiety were not significantly different between our three study samples (Table 2). As expected, the $\text{anxiety}_{[high]}$ subgroup in study 2 scored significantly higher in all trait measures compared to subgroup $\text{anxiety}_{[low]}$ (Emotionality (trait): t(27.57) = -4.5, p < .001, d = 1.7; Worry (trait): t(20.04) = -6.55, p < .001, d = 2.5; AS: t(22.17) = -2.66, p = .014, d = 1.1; test anxiety: t(27.05) = -2.49, p = .02, d = .94; IU: t(21.98) = -5.01, p = .005, d = 1.9, Table 2).

Compared to study 1 and 3, we found a significant difference in trait anxiety (F(3, 63) = 12.47, p < .001, η^2 =.37), IU (F(3, 63) = 6.94, p < .001, η^2 = .25) and ASI (F(3, 63) = 3.24, p = .03, η^2 = .13) with $\text{anxiety}_{[low]}$ scoring lowest, $\text{anxiety}_{[high]}$ highest and study samples 2 and 3 scoring in between. Test anxiety (PAF) was only assessed in studies 2 and 3. Mean test anxiety scores in study 2 subgroup $\text{anxiety}_{[low]}$ were significantly lower than in subgroup $\text{anxiety}_{[high]}$ with the latter not differing significantly from mean scores in study 3 (F(2,43) = 4.29, p = .02, η^2 = .17).

5.4.2 NPU-Threat Test

Regarding the NPU-threat test, a significant main effect of condition (F(1.8, 120.17) = 103.4, p < .001, η^2 = .61) (NPU) and cue (F(1, 66) = 153.47, p < .001, η^2 = .7) was detected as well as a condition x cue interaction across all participants in the overall analysis (F(1.77, 16.77) = 27.38, p < .001, η^2 = .29). Posthoc Tukey-Test revealed significant differences (p <.0.01) with $U_{ITI} > N_{ITI}$ (anxiety potentiation) and $P_{CUE} > P_{ITI}$ (fear-potentiation) on a descriptive level (see Table 2). We therefore concluded that the

NPU-threat task had been applied successfully. Next, startle amplitudes have been compared between study samples. Adding study as between-subject factor did not reveal a significant main ($F(2, 64) = 1.93$, $p = .15$, $\eta^2 = .06$) or interaction effect ($p > .1$).

Furthermore, comparison of potentiated startle amplitudes revealed a significant difference between potentiated startles ($F(1,64) = 5.27$, $p = .03$, $\eta^2 = .076$) with anxiety < fear, but no main effect of study as between-subject factor ($F(2, 64) = 1.69$, $p = .19$, $\eta^2 = .05$) nor an interaction ($F(2, 64) = 1.55$, $p = .22$, $\eta^2 = .05$). Regarding study 2, we did not find significant differences between the low and high anxiety subgroups in startle amplitudes ($F(4.04, 113.02) = .54$, $p = .71$, $\eta^2 = .02$). However, there was also a significant difference between potentiated startles ($F(1, 28) = 12.98$, $p = .001$, $\eta^2 = .32$) with anxiety < fear, but no main effect of anxiety group ($F(1, 28) = .08$, $p = .78$, $\eta^2 = .003$) or interaction ($F(1,28) = .18$, $p = .67$, $\eta^2 = .007$). Due to technical difficulties, habituation startle is missing for two participants from study 1, leaving 65 participants for analysis. Information on subjective ratings can be found in Appendix C, Table 9.

5.4.3 Ambulatory Assessment

Altogether, we were able to record 1752 valid everyday ratings from 67 participants across all studies with compliance rates ranging between 53% – 100% (exclusion of participants with < 50% compliance). Average compliance rates varied between 94% in study 1, 88% in study 2 and 82% in study 3. Some assessments were missing in the first and second study due to technical problems causing fewer alarm triggers (28 instead of 32 in total). We therefor achieved a Level 2 sample size of $N = 67$ and a Level 1 sample size of $N = 26$ per subject with a total of $N = 1752$ valid observations for our overall analysis. Post hoc sensitivity analysis showed that we had a power of 80% to detect small till medium effect sizes ($r = .15$ – $r = .24$) in our overall analysis (see supplementary material B).

5.4.4 Multilevel Models

For multilevel analysis, we first inspected ICCs for our state scales for the total sample and each study separately. An ICC expresses the resemblance between micro-units (daily assessments for emotionality and worry) belonging to the same macro-unit (person). The

Table 2: Sample characteristics, startle amplitudes (t-scores), potentiated startles and traits across studies

			Study 2			
	Total	Study 1	Total	$anxiety_{[low]}$	$anxiety_{[high]}$	Study 3
Sample characteristics						
N (female)	67 (47)	21 (11)	30 (23)	15 (12)	15 (11)	16 (13)
Age[1]	22.01 (4.4)	22.2 (6.4)	20.4 (2.3)	20.1 (1.5)	20.6 (2.9)	24.9 (2.2)
EMG startle amplitudes[2]						
N_{CUE}	47.27 (0.27)	46.82 (0.56)	47.63 (0.38)	47.72 (0.59)	47.55 (0.49)	47.19 (0.52)
N_{ITI}	46.27 (0.21)	46.19 (0.40)	46.39 (0.29)	46.10 (0.39)	46.69 (0.43)	46.15 (0.50)
P_{CUE}	54.23 (0.33)	54.64 (0.70)	54.12 (0.45)	53.72 (0.72)	54.53 (0.53)	53.90 (0.61)
P_{ITI}	48.50 (0.29)	48.15 (0.58)	48.4 (0.32)	48.54 (0.43)	48.26 (0.50)	49.14 (0.74)
U_{CUE}	52.81 (0.39)	53.07 (0.84)	52.79 (0.50)	53.27 (0.78)	52.3 (0.64)	52.49 (0.85)
U_{ITI}	50.55 (0.40)	51.87 (0.68)	49.67 (0.47)	49.55 (0.4)	49.78 (0.86)	50.48 (1.03)
Potentiated startle[3]						
fear	5.73 (0.47)	6.49 (0.99)	5.73 (0.55)	5.18 (0.90)	6.27 (0.65)	4.75 (1.07)
anxiety	4.28 (0.49)	5.68 (0.94)	3.27 (0.53)	3.46 (0.48)	3.09 (0.97)	4.33 (1.07)
Trait						
Anxiety[4]	19.46 (0.64)	19.86 (1.1)	19.00 (1.07)	14.33 (0.81)**	23.67 (1.76)**	19.81 (1.03)
Emotion	9.75 (0.33)	9.76 (0.58)	9.50 (0.56)	7.53 (0.58)**	11.47 (1.00)**	10.19 (0.53)
Worry	9.72 (0.38)	10.1 (0.64)	9.50 (0.64)	6.80 (0.35)**	12.20 (0.74)**	9.62 (0.60)
ASI-3	12.07 (1.18)	9.81 (1.63)	13.20 (1.96)	8.47 (1.76)*	17.93 (3.09)*	12.94 (2.59)
PAF	45.39 (0.91)		44.47 (1.16)	41.80 (1.37)*†	47.13 (1.65)*†	47.12 (1.40)†
Emotion	9.52 (0.42)		9.17 (0.51)	7.93 (0.67)*	10.40 (0.63)*	10.19 (0.74)
Worry	13.20 (0.57)		12.70 (0.69)	11.00 (0.93)*	14.40 (0.82)*	14.12 (1.00)
IU-18	42.52 (1.58)	41.05 (2.58)	42.47 (2.52)	33.13 (1.82)**	51.80 (3.25)**	44.56 (3.25)

[1] mean (SD) in years;
[2] mean t-scores (SEM);
[3] fear = P_{CUE} - P_{ITI}, anxiety = U_{ITI} - N_{ITI}
[4] STADI: State-Trait-Anxiety-Depression-Inventory; ASI-3: Anxiety-Sensitivity-Index-3; PAF: Prüfungsangstfragebogen; IU-18: Intolerance-of-Uncertainty-Scale
* p .05, ** p .01 for t-test $anxiety_{[low\ vs.\ high]}$, Mean (SEM);
† p .05 for ANOVA $anxiety_{[low\ vs.\ high]}$ vs. $study_{[3]}$

ICCs with respective 95% CI and estimates for within- (varw) and between-variance (varb) for state emotionality and worry for each individual study are summarized in Table 3. For state emotionality, we identified an overall ICC of .32 (95% CI = .25 – .42), thus 32% of the variance was accounted for by between-person differences and the remaining variance was due to within-person variability. Regarding state worry, we identified an overall ICC of .49 (95% CI = .4 – .58). Thus, between-person variability in state worry was higher compared to state emotionality and almost equal to within-person variability.

On a descriptive level, within- and between-person variances differed across studies. Regarding state emotionality, there were similar estimates for within- and between variance for participants from study 1, whereas for participants in study 2 and study 3, estimates for within-variance were considerably higher than for between-variance. In study 2, the high-anxiety group showed higher within- and between-variance compared

to the low-anxiety group as well as highest within-person variance compared to studies 1 and 3. For state worry, a different pattern emerges. In study 1, we saw higher between variance, in study 2 between and within-variances were almost equal (ICC = .50, CI = .38 – .65) and in study 3 there seemed to be more within-variance. Although ICCs for the low- and high-anxiety groups of study 2 were almost equal, between- and within-variance in the high-anxiety group was overall higher. To sum up, within- and between-variances seemed to differ between study samples and subsamples for both state emotionality and worry. The main objective of our analyses was to investigate

Table 3: Intra-class correlations and variances for state emotionality and worry (AA)

	ICC (95% CI)	k	varw	varb
Emotionality				
Total	.32 (.25 - .42)	26.14	4.66	2.23
Study 1	.47 (.33 - .66)	26.13	4.10	3.65
Study 2	.26 (.18 - .41)	26.15	4.62	1.68
$\text{anxiety}_{[\text{low}]}$	.24 (.13 - .46)	26.30	3.22	1.01
$\text{anxiety}_{[\text{high}]}$	.24 (.13 - .47)	25.95	6.03	1.95
Study 3	.24 (.13 - .45)	26.09	5.46	1.69
Worry				
Total	.49 (.40 - .58)	26.14	5.67	5.39
Study 1	.56 (.42 - .73)	26.13	5.72	7.38
Study 2	.50 (.38 - .65)	26.15	4.58	4.66
$\text{anxiety}_{[\text{low}]}$	.50 (.33 - .72)	26.30	3.31	3.28
$\text{anxiety}_{[\text{high}]}$	.46 (.30 - .69)	25.95	5.88	5.00
Study 3	.35 (.21 - .58)	26.09	7.64	4.14

Note:
ICC (intra-class correlation) from variance components (mean squared errors) of a one-way analysis of variance as specified in the R package ICC [Wolak, 2015]; CI = 95% confidence interval, varw = within-person variance, varb = between-person variance, k = number of measurements per individual per group.

the association between the NPU-threat test as a standard laboratory measure with worry and emotionality in everyday life across three different samples. Therefore, we applied random-intercept fixed-slope LMMs and GLMMs with startle amplitudes or fear- and anxiety-potentiated startle as predictors. A complete overview of the models is given in the supplemental material B. LMMs were built as described in the method section, separately for emotionality and worry as dependent variable. None of the analyses, investigating fixed factors of the NPU-threat test (consecutively adding startle, $\text{startle:condition}_{[\text{N, P, U}]}$ and $\text{startle:condition}_{[\text{N, P, U}]}\text{:cue}_{[\text{cue, no cue}]}$) or models including only startle potentiation for fear and anxiety, revealed significant associations with state worry or emotionality in either the overall sample or in any of the three studies separately.

Only startle potentiation for fear and anxiety as fixed factors slightly increased the amount of total variance explained compared to corresponding empty models. Fixed factors of the NPU-threat test did not contribute to the total variance explained by their respective models (all marginal $R^2 < .001$).

Only anxiety- and fear-potentiated startle modestly improved the explained variance of the respective models across studies and in the overall sample. Last, adding the fixed effect of exam preparation$_{[yes\ vs.\ no]}$ to the overall sample did not reveal additional information. Only in the analysis on study 2, the fixed effect anxiety$_{[low\ vs.\ high]}$ showed a significant association with emotionality and improved explanation of variance in emotionality and worry in models including interaction with fear- and anxiety-potentiated startle (tables 5 – 8, see also Figure 3).

To follow up on this effect, the more complex GLMMs had been applied (external file, see Appendix C). We analysed if the fixed effect anxiety$_{[low\ vs.\ high]}$ itself added information compared to an empty model. For emotionality, the fixed factor anxiety$_{[low\ vs.\ high]}$ failed to reach significance whereas the intercept for worry in high anxious participants was predicted to be significantly higher ($\beta = 2.04$, CI = 1.08 – 3.88, p = .029). However, the effects were only moderate as the fixed effects only added little to the total variance of the respective model (for worry: marginal $R^2 = .08$, conditional $R^2 = .60$). Adding the interaction of anxiety$_{[low\ vs.\ high]}$ with potentiated startle for fear and anxiety as fixed factors did not reveal additional information.

Taken together, none of these models provided evidence for significant associations between physiological measures of the NPU-threat test and the dependent variables emotionality or worry. Only the fixed factor anxiety$_{[low\ vs.\ high]}$ in study 2 showed significant associations with AA emotionality as well as AA worry but this effect should be interpreted with caution due to statistical considerations.

5.5 Discussion

The present analyses aimed to investigate the association between the well-known NPU-threat test, a laboratory paradigm to elicit fear- and anxiety-potentiated startle (Schmitz & Grillon, 2012), with everyday experience of emotionality and worry, assumed to partly reflect fear and anxiety, in healthy individuals. Participants went through a laboratory

protocol and took part in a four-day ambulatory assessment. We conducted three studies with different sample characteristics to facilitate variance in our dependent variables emotionality and worry. A first study included unselected healthy participants (with no further prerequisites), for a second study we recruited two groups with extreme manifestations of trait anxiety (low vs. high) within the normal range and for a third study we recruited students preparing for a high-stakes exam with subjective high relevance.

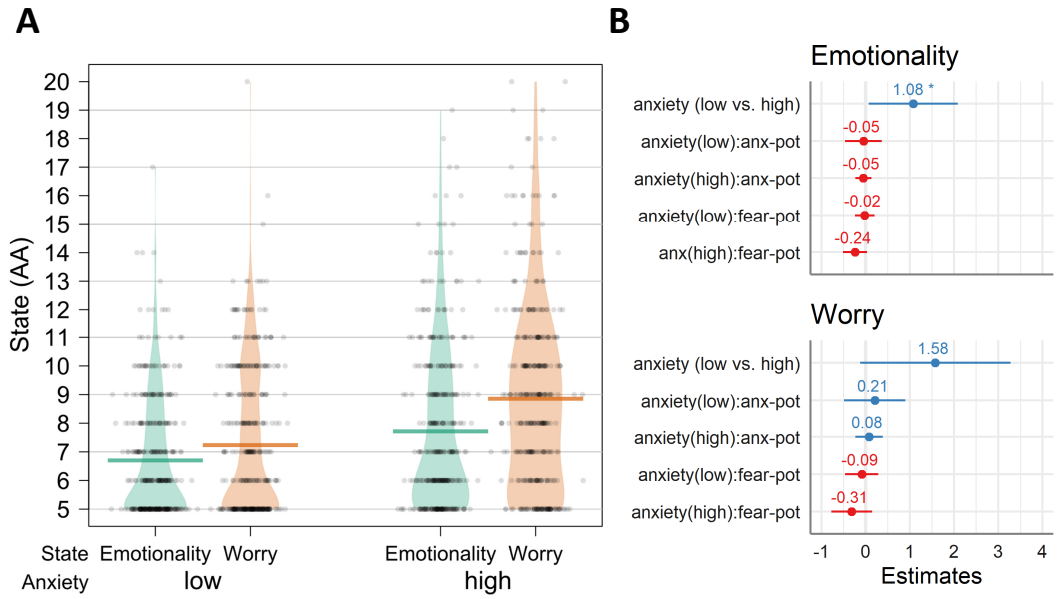

Figure 3: Association of NPU-threat test and AA states with anxiety (low vs. high) A Density of AA state emotionality and worry in study 2 for low and high anxiety including group means (thick line). B Illustration of estimates for association of anxiety[low vs. high] and interaction with anxiety- (anx-pot) and fear-potentiated startle (fear-pot) of the NPU-threat test as fixed effects with emotionality or worry as dependent variable in LMM for study 2 (see Appendix B, tables 5 till 8 for complete model overview).

We conducted multilevel analyses on both dependent variables, worry and emotionality, [1] including NPU-threat test startle amplitudes, their respective interaction with [2] condition$_{[N,\ P,\ U]}$ and [3] condition:cue$_{[cue,\ no\ cue]}$ as well as [4] for corresponding anxiety- and fear-potentiated startles. These analyses did not reveal a significant association between laboratory measures and everyday ratings on emotionality or worry in an overall

analysis. Moreover, none of our predictors showed significant associations with our dependent variables. A subsequent analysis of study 2 data (comparing participants with low vs. high anxiety) suggested an association of trait anxiety and AA dependent variables. Upon closer inspection, the high anxiety group showed higher everyday ratings compared to the low anxiety group but there was no interaction with fear- or anxiety-potentiated startle.

Overall, we did not see meaningful differences in NPU-threat test EMG amplitudes or startle potentiation, but found alterations in everyday ratings on emotionality and worry between samples. We conclude that the present analyses do not provide evidence for an association between psychophysiological measures of the NPU-threat test and everyday experience of worry and emotionality in healthy participants.

The NPU-threat test is undoubtedly a well-established laboratory paradigm. It balances the dimensions duration (phasic vs. sustained) and nature of threat (predictable vs. unpredictable) to measure fear- and anxiety and it has proven its validity, amongst others, by differentiating between anxiety-related disorders (Davis et al., 2010; Gorka et al., 2017). For example, findings have led to the proposal that differences in the association with anxiety-potentiation between PD and GAD could be attributed to differences in cognitive processes with pronounced anticipatory fear (anxiety) in the first and rumination and worry in the latter disorder (Davis et al., 2010). Also, patients with SAD and specific phobias have shown hypersensitivity mainly to unpredictable threat (Gorka et al., 2017). Unpredictable threat but not predictable threat amplitudes decreased after cognitive behavioural therapy in SAD, PD and PTSD patients (Gorka et al., 2017).

Beyond the well-established association between sensitivity to unpredictable threat with symptoms of anxiety, a recent study also found a longitudinal relationship between both, sensitivity to unpredictable and predictable threat as neurobiological vulnerability factors and clinically relevant functional impairment, especially cognition (understanding, communicating) and getting along with others (Stevens et al., 2019). Since an association was primarily expected with sensitivity to unpredictable but not predictable threat, the authors speculate on possible mechanisms on shared neuronal mechanisms that might be more vulnerable to disruption and thus are involved in pathways leading from

neurophysiological vulnerability to functional impairment (Stevens et al., 2019). Together, this might indicate an association between laboratory measures and domains affecting everyday life.

The lack of significant associations in the present study could be explained by assuming that we failed to properly apply the NPU-threat test resulting in a lack of variance in our predictor variables. However, our data do not support this view as we successfully elicited fear- and anxiety-potentiated startle modulation with $P_{CUE} > P_{ITI}$ and $U_{ITI} > N_{ITI}$ (Schmitz & Grillon, 2012), which showed to be normally distributed in all three study samples. Interestingly, we did not detect any significant differences in EMG amplitudes or startle potentiation for fear and anxiety between our three samples or between groups with low vs. high anxiety of study 2 (s. Table 2).

To the best of our knowledge, there are no studies yet investigating the association between NPU-threat test laboratory measures and everyday cognitive-emotional processes of anxiety. On a conceptual level, we chose to assess the components worry and emotionality of the state anxiety scale of the STADI, which show different temporal activation patterns (Laux et al., 2013). Emotionality is reflecting perception of bodily arousal, rising immediately upon confrontation with a threating encounter and might be seen as response to short-lived (phasic) events. Items for worry reflect self-reported beliefs about future events and cover a more long lasting (sustained) cognitive component (Renner et al., 2018).

We applied a short-term yet extensive four-day assessment with eight assessments per day. Although there have been differences in compliance and completeness of the daily assessments between studies (see below), we achieved acceptable average compliance with $> 80\%$ completeness rates in all three studies. We were able to observe variation in ratings on emotionality and worry within and between participants. However, there was a tendency to positive skewed distributions for both scales, i.e., participants often rated their everyday experience of emotionality or worry rather low. We tried to minimize this tendency by giving precise instructions and explanations regarding all items and answer options (see e.g. Roekel et al., 2019) and by a careful study design including different sample characteristics to achieve additional trait (study 2) and context variability (study 3).

The relatively low mean ratings might be of some concern when applying LMM and we tried to account for that by also applying GLMM. A first inspection of the data reassured that there was no general floor effect in our dependent variables. Moreover, we found differences between mean ratings on emotionality and worry across assessments with more variability in worry ratings, which is also reflected in different ICCs for our dependent variables. The ICCs (expressing the relation between within- and between-person variance with ICC $< .50$ indicating more within-person variance and ICC $> .50$ indicating more between-person variance) suggested more within-person variance across assessments in emotionality (ICC = .30) whereas variance in worry ratings seemed to be attributable equally to fluctuations within-person as well as differences between participants (ICC = .49). This finding is in line with the assumption of a different temporal activation pattern of both states. It was suggested that worry occurs relatively early and lasts long due to the anticipation of an event (e.g. an important exam) while emotional arousal is assumed to rise just before the confrontation with a threat and quickly declines thereafter (Renner et al., 2018). Therefore, emotionality might have been more context dependent and differences between participants might have contributed only moderately to the total variance. On the other hand, worry as cognitive component directed towards negative expectations or concerns in the future, seemed to add a more stable source of variance between participants.

To get an impression of how variances in emotionality and worry differed between our three study samples, we compared the variance estimates within and among participants (see Table 3). In study 2, high-anxious participants showed increased within- as well as between-person variance for state worry as well as emotionality compared to low-anxious participants. In study 3, participants preparing for a high-stakes exam, showed by far the highest within-person variance compared to the other samples regarding worry, which can be interpreted as future directed cognitive component of anxiety (Renner et al., 2018). We conclude that our dependent variables were sensitive to capture meaningful differences between our study samples with additional trait (study 2) and context variability (study3). Although there was a tendency to positively skewed distributions in both dependent variables, we did not see a general invariance.

Taken together, we successfully elicited predictable and unpredictable threat and

derived fear- and anxiety potentiated startle EMG amplitudes using the NPU-threat test. Furthermore, we were able to gather meaningful everyday ratings on state worry and emotionality in a four-day AA in three different samples. However, we failed to detect a significant association between EMG startle amplitudes or anxiety- and fear-potentiated startle and our dependent AA variables. The explained variance of our models relied predominantly on differences within participants (random effects) and NPU-threat test measures as fixed factors added only little information.

5.5.1 Other Possible Explanations for Non-Association

Based on the validation concept of the present study we have to acknowledge that our data do not provide much evidence for ecological validity of the NPU-threat test. However, as each study can only probe for a limited number of facets regarding the broad concept of validity, we certainly cannot conclude that the NPU-threat test lacks ecological validity in general. In support of this view it has been shown that healthy controls elicit lower startle magnitudes compared to exaggerated responses in clinical samples suggesting an increased biological vulnerability in the latter (Gorka et al., 2017; Grillon et al., 2017).

Additionally, there are no reports on associations between trait anxiety and physiological measures of the NPU-threat task in non-clinical samples (Grillon et al., 1993; Grillon & Baas, 2003; Schmitz et al., 2011; Schroijen et al., 2016). Associations between average trait anxiety and self-reported states of apprehension, however, can be seen as adaptive immediate reaction to a threat of shock paradigm (Grillon et al., 1993; Haddad et al., 2012; Schroijen et al., 2016; Stegmann et al., 2019). Compared to anxiety disorders that are characterized by high and persistent levels of anxious states in particular contexts, a lower biological vulnerability and a more adaptive regulation of anxious states (lower frequency and level) can be assumed in healthy individuals with average trait anxiety (Raymond et al., 2017). We were able to see differences in AA measures of cognitive-emotional processes regarding anxiety, especially in high trait anxious individuals with increased levels of everyday worry.

The high anxiety group could also be seen as the most vulnerable group, with high trait anxiety and comparatively high values for AS and IU, which are additional potential

risk factors for developing anxiety-related disorders (Carleton et al., 2007). However, fear- and anxiety-potentiated startle did not differ between study samples, nor between low and high anxious participants from study 2. Thus, we assume equally distributed biological vulnerability as measured by startle response to predictable and unpredictable threat across samples which on average can be considered low derived from studies on anxiety disorders (Gorka et al., 2017; Grillon et al., 2017). Everyday anxious emotional processing (Renner et al., 2018) is not only shaped by context and emotion but also by multiple cognitive processes. Although there exists a body of research on the interaction between anxiety and cognition including threat of shock studies, the neural mechanisms behind differences in adaptive and maladaptive processing in the continuum of normal to pathological anxiety are far from clear (Grillon et al., 2019; Robinson et al., 2019). In this project, the lack of association between physiological measures of the NPU-threat test and everyday states of cognitive-emotional processes might be explainable by a low biological vulnerability in healthy individuals with average as well as high manifestations of trait anxiety.

5.5.2 Limitations

For the sake of economy, we have chosen a brief, yet extensive ambulatory assessment. Furthermore, we modulated study sample characteristics to get a first impression on the association between NPU-threat test amplitudes and everyday experiences of anxiety. Although our AA measures have shown to be sensitive to capture fluctuations within and between persons, our AA design resulted in limited variance for worry and emotionality. On a statistical level, LMMs did not always converge and we applied GLMMs when appropriate. This might indicate that our relatively short yet extensive AA design in these specific samples was not optimal for covering a wider range of potentially fearful situations, contexts or cues, facilitating a more complex emotional processing and perception of emotionality and worry. We assume that a mixed time- and event-based design over a longer period of time could be preferable to allow for more situation-specific variation across assessments and days (Trull & Ebner-Priemer, 2020). Moreover, it has been shown that short questionnaires (around 30 items) with high-sampling frequencies are not associated with negative consequences like increased burden or compromised data quantity and quality in healthy participants (Eisele et al., 2020).

We did not assess physiological arousal like heart rate or skin conductance level during AA. A recent review on test anxiety conducted a metanalytical evaluation on the relationship between the components worry and emotionality with physiological arousal. Although the authors mentioned several methodological issues regarding the assessment of physiological arousal across studies, they concluded that physiological measures can provide valuable additional information (Roos et al., 2020). As especially heart rate monitoring in daily life gets more and more convenient due to dissemination of consumer devices, a multimodal ambulatory assessment combining self-report and biological measures would be an interesting extension for future projects.

Last, there have been considerable differences regarding compliance and completeness between study samples. Participants of study 3 experiencing increased burden due to preparing for a high-stakes exam, showed lowest compliance reflected by low data quantity and quality in general. This might have resulted in a bias to a certain extent. Further, we do not know if assessments were missed in general due to high demands or stressful situations or just because participants payed less attention on alarms while studying. Here, additional devices like wristbands could trigger non-audible but tactile alarms. End-of-day assessments could help to collect general information about stress and demands during the day to better control for sources of low compliance.

5.6 Conclusion

In this study we set out to investigate the ecological validity of the NPU-threat test combining laboratory sessions with a four-day ambulatory assessment. However, although we successfully assessed sensible fluctuations in state emotionality and worry in everyday life of differently characterized study samples, we did not detect significant associations with physiological measures from the NPU-threat test in an overall analysis.

Nevertheless, we do not conclude in general that the NPU-threat test is not ecological valid. Our results possibly reflect a weak link between NPU-threat test measures and the experience of adaptive states of worry and emotionality in everyday life in healthy individuals with low biological vulnerability for anxiety disorders. This assumption certainly needs to be tackled in future studies e.g., in settings with increased variance in AA measures via manipulation of exposure to threatening or stressful contexts in healthy

individuals, in ‘high-risk’ individuals (e.g., with pronounced intolerance of uncertainty or anxiety sensitivity) or in clinical populations with exaggerated startle responses.

Part III

Discussion and Conclusions

6 General Discussion

6.1 Stress Research and the TSST

The narrative review presented in chapter 3 aimed to provide an extensive overview on sources of intra- and interindividual variability of the stress response (Zänkert et al., 2019). The manuscript elaborated on factors that alter cortisol responses to standardised psychological stress paradigms, while not only focusing on studies using the TSST. Thus, to introduce the reader to additional standardised protocols that are currently used to study HPA axis response to stress in the laboratory, a short overview on a variety of psychological stress paradigms was provided first. The different protocols were briefly discussed, for example as different types of psychological stressors can elicit either cognitive, social or emotional stress. It became apparent, that each protocol has its own advantages and disadvantages for studying emotional, cardiovascular or endocrine changes in response to stress (Skoluda et al., 2015). However, in this list, the TSST remained the most reliable tool to activate the HPA axis (Dickerson & Kemeny, 2004; Skoluda et al., 2015) and thus took a central role in this review. Additionally, current implementations using virtual reality technology (Fallon et al., 2016; Jönsson et al., 2010; Kelly et al., 2007; Shiban et al., 2016) and adaptations for MRI environments (Dedovic et al., 2005; Lederbogen et al., 2011; Streit et al., 2014) were shortly introduced.

In the second and main part, an updated overview of important sources of variance of the cortisol response to psychosocial stress in humans was provided. This complements previous reviews, that summarised sources of variance of acute stress responses to psychological challenge such as the TSST (Allen et al., 2017; Foley & Kirschbaum, 2010; Kudielka et al., 2007; Kudielka & Wüst, 2010) or in combination with reaction to pharmacological challenge (Kudielka et al., 2009). The current manuscript began with an overview of the role of demographic and lifestyle behavioural factors, including consumption of substances and medication, followed by psychological and personality factors, chronic stress and psychopathology as well as knowledge of biological factors including genetics as well as epigenetic mechanisms involved in HPA axis reactivity. Especially epigenetic studies were still sparse as the necessary methodology had only just been implemented in biopsychological research in humans.

Valuable information also derived from recent meta-analyses and reviews on different factors such as sleep (van Dalfsen & Markus, 2018), early life adversity (Bunea et al., 2017) and sex differences (Liu et al., 2017) but also methodological aspects like the effect of venepuncture (Weckesser et al., 2014) and protocol variation (Goodman et al., 2017). At the end of each section, a summary was provided and when appropriate recommendations were given on how to address the respective factors when conducting a study on acute stress. Taken together, it became apparent that knowledge on the stress system was accumulating fast and continues to hold scientific interest.

It must be noted, apart from affecting the stress response individually, the mentioned modulators of the stress response do also intersect and form individual patterns of factors that might results in an acute or chronic alteration in HPA axis activity. A theoretical framework that describes the cost of chronic exposure to fluctuating or heightened neural and neuroendocrine responses that result from repeated or chronic challenges an individual reacts to for being particularly stressful, is the concept of allostatic load (Guidi et al., 2021; McEwen, 1998, 2003). In short, allostasis is defined as the ability of an organism to achieve stability through change and for healthy functioning, certain biological systems like the HPA axis need to adjust to challenge. Repeated or prolonged stress is believed to exceed an individual's ability to cope and adapt to these, e.g., environmental strains and thus result in allostatic overload and dysregulation of the respective bodily systems (Guidi et al., 2021; McEwen & Stellar, 1993). Besides major stressful events, chronic stress or environmental demand may also be due to experiences in daily life, like ordinary daily stressful events, that are subtle but long-lasting life situations and thus become frequent stressors (Guidi et al., 2021; McEwen, 2003).

Thus, it is not surprising that ethnicity, perceived racial discrimination, social inequalities or acculturation stress have shown to be associated with allostatic load (Guidi et al., 2021). It has also been recognised that cultural factors might contribute substantially to susceptibility and variation in mental illness experience and expression (Shattuck, 2019). Therefore, if research on stress, as global health burden, wants to succeed in concern for global mental health, it needs to pursue expanding investigations beyond mostly western, educated, industrialised, rich and democratic (so-called WEIRD) populations (Ghai, 2021; Kuhlman et al., 2019; Shattuck, 2019). This topic was not covered in the

review, but as it warrants more attention, a brief outline will be provided here.

Cultural factors can be conceptualised on different levels, from a global (e.g., migration patterns, environmental disasters) and political-social level (e.g., immigration policies, attitudes towards immigration) to microsystems (e.g., neighbourhood, school, family cultural values) and individuals (e.g., demographic variables, cultural identity) (Haft et al., 2021). Regarding stress reactivity, studies exploring effects of cultural background also exist. For instance, one study suggested that bicultural identity integration is an important construct to consider in biopsychosocial studies on immigrant stress and health (Yim et al., 2019) and another found different patterns of cultural adaptation in Latino youth to be associated with different patterns in HPA reactivity to acute stress (Gonzales et al., 2018). On an individual level, sensitivity to ethnic microagressions was associated with a blunted cortisol reaction (Majeno et al., 2021) and induction of acute racial and ethnic discrimination has shown to elicit a biological stress response in immigrants (Fischer et al., 2017). Reduced cultural mismatch was associated with a lower cortisol reactivity to the TSST (Sladek et al., 2020). On a more global level, Miller and Kirschbaum (2019) reported between-country differences in cortisol stress response to the TSST and argue that these might be attributable to variability in cultural values. Ethnic differences in reactivity to the TSST have also been reported (e.g. R. Y. Chong et al., 2008; Hostinar et al., 2014).

Laboratory paradigms like the TSST, that are easily adaptable to different research questions, can be used to further investigate common cultural experience and their effect on health disparities in ethnic minority groups. In one study, for instance, Mexican American adolescents were asked to translate a difficult medical document from English to Spanish for their parents as alternative speech task to simulate language brokering (Kim et al., 2018). This modified version of the TSST elicited a stress response that varied according to participants' perceptions of language brokering (parental dependence and efficacy), parental hostility, and discrimination experiences (Kim et al., 2018).

Though growing in interest, research on possible separate and joint effects of ethnicity, culture and nationality on dysregulation in HPA axis functionality and risk to develop stress-related diseases are still sparse (Scholaske et al., 2021; Shattuck, 2019). One reason, that has only been discussed recently, is that psychoneuroendocrine research in diverse

and under-represented populations involves a number of challenges, ranging from the recruitment process, to collecting biological samples as well as data analysis, interpretation and dissemination (Kuhlman et al., 2019). However, especially investigations on the intersection of known mediators and individual characteristics in the context of ethnicity, culture and nationality seem crucial for a comprehensive understandings of inter- and intraindividual sources of variability of the stress response (Busse et al., 2017; Keenan et al., 2021; Kuhlman et al., 2019; Scholaske et al., 2021). Special emphasis must be put on the proper use of constructs and precise differentiation between culture, nationality, intersectionality and/or social context (Scholaske et al., 2021; Shattuck, 2019).

In the last part of the review, methodological aspects like time of day, collection of blood samples, anticipation effects and assessment of stress appraisal, as well as habituation to repeated testing, were summarised. After many years of research with the TSST, there seemed to be a wide variety of protocols that were applied across different laboratories (Goodman et al., 2017). So far, there has not yet been taken any action to establish a more standardised protocol, or more consistent way of reporting from a meta-methodological perspective, like there has been for the cortisol awakening response (Stalder et al., 2016). However, aiming to standardise protocols for data collection, analysis and interpretation could be seen as a foundation of empirical research and a sign of a discipline's maturity (Elson, 2019; Moriarity & Alloy, 2021).

This degree of standardisation has not yet been established in research utilising the TSST and, for example, missing meta information on study protocols still results in limitations for analysis conducted in meta-analyses (Goodman et al., 2017; Liu et al., 2017; Narvaez Linares et al., 2020). A study by Liu and colleagues (2017) mentions "obtaining data from fellow researchers" as "an important methodological challenge". From 66 articles that were identified as relevant for their meta-analysis, only 22 contained data that could be extracted right away. From the remaining 44 articles, that did not provide the necessary information for analysis, authors had to be contacted. This however only resulted in 12 responses providing usable data for inclusion to the analysis. So, of 66 potential articles, necessary data was only available for 36 articles, of which 34 studies with independent samples were included in the final analysis (Liu et al., 2017). It is concluded that, due to missing information, possible analysis on sex differences

and moderating factors, that could otherwise have been provided by this valuable meta-analysis, had to be limited or skipped. The authors thus stressed the importance of making data available in a more open, acessible and useful way (Goodman et al., 2017; Liu et al., 2017; Narvaez Linares et al., 2020).

A final remark refers to the importance to fully grasp the dynamic of the cortisol stress response. During the past years of research, it was often considered sufficient to analyse the course of mean averages between groups with traditional statistical approaches like repeated measures ANOVA (Lopez-Duran et al., 2014). But alternative statistical methods like growth curve models or linear mixed models have advantages in regard to power and sensitivity but also in modeling the (individual) dynamic of the stress response (Lopez-Duran et al., 2014). Among other advantages, growth curve modeling also allow to investigate the rate of change or time-latency of post-stress peak concentration in cortisol on the individual as well as on group level (Felt et al., 2017; Lopez-Duran et al., 2014). The benefit obtained by applying new statistical approaches will, of course, vary depending on the research question. However, they also allow to investigate new research questions, like the evaluation of change over time, which might allow a more detailed inspection of intra- and interindividual differences in the stress response over time (Felt et al., 2017; Segerstrom et al., 2017).

6.2 Glucose and Stress

The second aim of this thesis was to further elucidate the association between glucose availability and HPA axis reactivity to acute challenge. One objective was to derive improved and more valid recommendations on, if and how glucose availability should be standardised with a caloric drink at the beginning of stress experiments. General recommendations on standardising energy availability have been around for some time (Kirschbaum et al., 1997; Kudielka et al., 2007). Application of a glucose drink, however, was only mentioned by one(Labuschagne et al., 2019) but not the other (Birkett, 2011) of two recent pulications providing a more detailed protocol, or step-by-step guide respectively, on how to conduct the TSST. This variation can lead to confusion and inconsistencies in protocol application, as well as reporting, by novices and experts alike. And, as already mentioned in the introduction, taking a look in method sections

across random or preselected samples of publications revealed a wide range of possible implementations on how to control for energy availability with no discernible standard (see 2.3).

Thus, the study presented here compared the effect of a natural dose of glucose (200 ml grape juice), a high dose of glucose (75 g per 200 ml) and a high dose of non-sweet maltodextrin with the same GI as the latter (75 g per 200 ml) on salivary cortisol levels in response to the TSST in a mixed sex sample. In short, results showed elevated cortisol levels after administration of a grape juice (lower dose of sugar) as well as a glucose drink (high dose of sugar) compared to a control group. Cortisol levels after administration of maltodextrin (same GI like glucose drink) were slightly elevated compared to controls, on a descriptive level only (n.s.). Therefore, the expected dose-dependent relation between administration of sugar and the cortisol stress response could not be confirmed. Additionally, explorative post-hoc analysis revealed different associations between sugar drinks and cortisol stress response in women and men. Highest cortisol levels were observed in women in the GRAPE condition whereas in men highest cortisol levels where seen in the GLUCO condition (for general discussion see Zänkert et al., 2020).

The following paragraph extends the discussion by inclusion of more recent publications and additional thoughts to the overall aim of the study. Updated recommendations as well as a discussion on future directions are provided.

6.2.1 Updated Discussion

There has been an update to the literature since publication of the manuscript (chapter 4, Zänkert et al., 2020) with two additional studies that also investigated the association between glucose and HPA axis reactivity in response to the TSST (Bentele et al., 2021; von Dawans et al., 2021). It seems appropriate to take the opportunity to expand the discussion on the present research question and to integrate the more recent findings.

The first study investigated HPA axis reactivity in response to the TSST (psychological stress) or the CPT (physiological stress) after administration of either 75 g glucose, sweetener or water (300 ml each) to explore the association between HPA axis and metabolic regulation in men (von Dawans et al., 2021). Participants were asked to fast

for four hours prior testing and blood glucose levels were measured at four time points across the session. They were randomised to one of six groups ($\text{stress}_{[\text{TSST vs CPT}]}$ by $\text{drink}_{[\text{glucose vs. sweetener vs. water}]}$).

In general, the results support the observation of elevated cortisol in response to stress after glucose intake. The observed effect was more pronounced in participants confronted with the TSST compared to the CPT, which was discussed as an effect of the nature of the stressor (psychosocial vs. physical) (von Dawans et al., 2021). However, the study did not observe a difference in responder rates between conditions, contrary to previous findings (Zänkert et al., 2020). Interestingly, HPA axis reactivity to neither, the TSST or the CPT, was completely blunted as all groups showed an increase in cortisol levels (von Dawans et al., 2021). The authors therefore conclude that glucose substitution after only four hours of fasting might not be a prerequisite for a significant cortisol response (von Dawans et al., 2021).

A second study investigated cortisol responses to a group version of the TSST (TSST-G) after administration of either 400 ml of grape juice (64 g sugar) or the same volume of water, in women. It is important to note that experimental sessions were scheduled between 8 – 10 am which is justified with the idea to facilitate the long fasting time of 8 hours for all participants. But of course, this might have conflicted with the cortisol awakening response (Bentele et al., 2021). However, they found that cortisol levels in response to acute stress were significantly elevated after administration of sugar compared to the control condition. This is in line with previous results in women who consumed a high dose glucose drink, but not women who received grape juice (Zänkert et al., 2020). The high dose glucose condition (75 g sugar) might be more comparable to the condition of Bentele and colleagues (2021) as they used twice the volume of grape juice and therefore double the amount of sugar (64 g sugar). Interestingly, they observed a more blunted cortisol reaction in the water control condition, which stands in contrast to results in the control condition of previous results in women (Zänkert et al., 2020). But it remains up to speculation if this was a pure effect of the longer fasting time or due to early testing hours (Bentele et al., 2021).

For a better overview, a summary of the relevant literature, including information on samples, fasting times, experimental conditions and results, can be found in Table 4.

All studies reported elevated cortisol levels after administration of glucose, compared to respective control conditions, in response to acute stress. This effect was seen in men (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997; von Dawans et al., 2021; Zänkert et al., 2020) as well as women (Bentele et al., 2021; Zänkert et al., 2020) although the effect might have been depending on type of glucose/sugar drink (Zänkert et al., 2020). A boost in cortisol levels was reported independent of preceding fasting time (3 – 4 hours vs. > 8 hours). However, longer fasting hours (> 8 hours) might more likely result in a suppression of HPA axis response to acute psychosocial stress whereas short fasting times might not prevent a significant rise in cortisol (von Dawans et al., 2021).

6.2.2 Possible Mechanisms

Though the precise mechanisms for the association between glucose availability (including fasting and energy restoration) and HPA axis reactivity remain unclear, there has been some speculation on and discussion of possible theories and pathways.

A theory that has been discussed as a potential explanation for a connection between central glucose regulation and the HPA axis is the Selfish Brain (Bentele et al., 2021; Peters & McEwen, 2015). In short, the brain has a high demand for glucose and might therefore be able to control the distribution of glucose to ensure its own energy supply, contrary to a passive supply. In times of acute or chronic stress, additional cerebral energy is needed. Activation of the HPA axis and thus elevated cortisol levels not only stimulates gluconeogenesis but also suppresses insulin secretion. This “cerebral insulin suppression” might help to maintain cerebral energy concentration through preferred transport of glucose across the blood-brain-barrier instead of uptake into muscle or fat cells (Peters & McEwen, 2015). Additionally, on a structural and neuronal level, limbic structures like the hypothalamus or the amygdala have been suggested to interact with glucose-dependent insulin increase, as a possible humoral regulator (Ulrich-Lai & Ryan, 2014). There is evidence that the amygdala might be prone to insulin modulation and that glucose or insulin might activate the PVN via the ventromedial nuclei whereas low glucose might inhibit the PVN directly (Choi et al., 1996; von Dawans et al., 2021).

Table 4: Studies on the association between glucose vs. control and acute HPA axis reactivity in response to the TSST

	Sample		Design			Measures		Results	
Study	N	sex	fasting time	energy manipulation	stressor	blood glucose	cortisol	sugar administration x cortisol	blood glucose x cortisol
Kirschbaum et al., 1997	Study 1: 35 Study 2: 12	m	> 8 hrs	Study 1: ml (100 g glucose) x stress[yes vs. no] vs. 400 ml water x stress[yes] Study 2: Within-subject design (2 days); Glucose load x nicotine x stress vs. water x nicotine x stress	TSST	3 times, via capillary blood (baseline, pre and post stressor)	salivary cortisol, Study 1: 10 samples, Study 2: 12 samples	Study 1: glucose[stress] > water[stress] water[stress] similar to glucose[no stress] Study 2: glucose x nicotine > water x nicotine	Study 1: positive correlation between glucose response (pre TSST measure – baseline) and cortisol AUCi Study 2: no significant correlation between rise in glucose and cortisol response
Gonzales-Bono et al., 2002	37	m	> 8 hrs	200 g avocado (80 g fat) vs. 300 ml water (75 g glucose) vs. 300 ml (83 g protein) vs. 300 ml water	TSST	3 times, via capillary blood (baseline, pre and post stressor)	salivary cortisol, 10 samples	glucose > other conditions, fat, protein and water did not differ, but also showed cortisol increase	positive correlation between glucose increase (baseline vs. pre TSST) and cortisol AUCi
Zänkert et al., 2020	103	m f	> 3 hrs	200 ml grape juice (32 g glucose fructose) vs. 200 ml tea (75 g glucose) vs. 200 ml (75 g maltodextrin, DE19) vs. no drink	TSST	none	salivary cortisol, 6 samples	glucose and grape juice did not differ, both sugar drinks > maltodextrin > no drink; exploratory analysis regarding sex: women: highest cortisol levels in glucose, men: highest cortisol levels in grape juice	-
von Dawans et al., 2021	151	m	> 4 hrs	300 ml drink (75 g glucose + 30 g blackcurrant juice*) vs. 300 ml drink (270 ml water + 3 g sweetener + 30 g blackcurrant juice*) vs. 300 ml water *blackcurrant juice contained 2.16 g fructose	TSST CPT	4 times, via capillary blood (baseline, pre and post stressor, end of experiment)	salivary cortisol, 8 samples	overall glucose > sweetener or water, with stronger association in TSST vs. CPT, sweetener and water did not differ, but also show cortisol response	blood glucose change did not predict salivary cortisol stress response (AUCg, AUCi)
Bentele et al., 2021	100	f	> 8 hrs (over night)	400 ml grape juice (64 g sugar) vs. 400 ml water	TSST-G	3 times, via capillary blood (baseline, pre and post stressor)	salivary cortisol, 8 samples	grape juice > water	significant association of blood glucose concentration with time^2 and drink, no information on association with cortisol levels

Note:
f = female, m = male; DE19 = dextrose equivalent of 19

Also, results from knock-out mice suggest the importance of insulin receptors in hypothalamic neurons for crosstalk between energy status and stress reactivity (A. C. N. Chong et al., 2015). However, studies investigating the role of brain insulin on stress reactivity in humans are inconclusive (von Dawans et al., 2021), with reports on suppression (Bohringer et al., 2008) as well as stimulation of cortisol levels (Fruehwald-Schultes et al., 2001).

Lastly, in contrast to other studies (see Table 4), the study presented here included the non-sweet carbohydrate maltodextrin, which has a similar glycaemic index compared to glucose (Zänkert et al., 2020). Consumption of maltodextrin did, however, not result in a similar increase of cortisol levels after acute stress. Unfortunately, assessment of blood glucose levels for a better control of possible differences between digestibility and blood glucose dynamics was not possible. Discussion of possible mechanisms remains thus purely speculative. One possible mechanism might be found in a different orchestration of peptides such as ghrelin (hunger) and leptin (satiation), which have both been associated with HPA axis activity (Allen et al., 2014; Dammann et al., 2013). Further studies using maltodextrin should therefore consider to assess feelings of satiety as the different texture may feel more filling.

6.2.3 Updated Recommendations

Taken together, it can be concluded that sugar administration at the beginning of stress experiments amplifies mean salivary cortisol responses. This effect seems relatively independent of preceding fasting time (3 – 4 hours vs. > 8 hours), when at least euglycemic glucose levels can be assumed at baseline. After long fasting periods (> 8 hours) suppression of HPA axis reactivity might be more likely whereas short fasting periods (3 - 4 hours) on the other hand might not prevent a significant rise in cortisol levels. Substitution of glucose after short fasting might therefore not be a prerequisite for a significant HPA axis response to stress (von Dawans et al., 2021). However, as blood glucose levels have shown positive correlations with cortisol stress response in most studies (see Table 4), short fasting periods should be implemented to at least ensure euglycemic levels across participants. After long fasting periods (> 8 hours), glucose substitution can prevent blunted cortisol responses to stress. In regard to sex, a general

boosting effect of sugar intake, or elevated blood glucose levels respectively, can be seen in men as well as women (see Table 4). However, a possible sex-specific effect of different nutritional states and blood glucose levels should be further investigated (Zänkert et al., 2020). This might contribute to unravel differences in HPA axis responses to acute psychosocial stress seen between females and males.

As already pointed out elsewhere, control of dietary behaviour and energy consumption before stress induction experiments should be obligatory (Kudielka et al., 2007; von Dawans et al., 2021). Additionally, reporting whether or not fasting periods where implemented and whether or not glucose, sugar or other caloric supplements were administered at the beginning of a stress experiment should become the norm.

6.3 Summary and Future Perspectives in Research With and On the TSST

Stress research has come a long way during the last decades and the biopsychological perspective has had a relevant share in this process. The development and the critical review of reliable tools for laboratory research has been an important contribution. Here, especially the TSST can be considered a facilitator not only for research within the community, but also as a common research tool that can easily be applied by novices in other fields of research and can be easily be adapted if necessary. The present work provided a general overview on factors of intra- and interindividual variability in response to psychological challenge and an empirical contribution to a better understanding on the effect of energy availability on the cortisol stress response. In regard to both parts, recommendations for implementation in future research projects on acute stress responses have been derived.

However, there are some remaining challenges that need to be addressed more thoroughly to make sure, that the acclaimed high standardisation of the paradigm will be maintained from a modern research perspective (Narvaez Linares et al., 2020). In the early years of stress research, one main objective was to generate a robust activation of the HPA axis in the laboratory due to psychological stress. Thus, seemingly small changes of the protocol, that still resulted in acceptable cortisol responses and responder rates, were probably not considered a significant issue. And for some research questions this, of course, remains valid until today. However, specific research objectives and

limitations of resources required adaptations from the original protocol (Narvaez Linares et al., 2020). This variation of TSST protocol implementations has been the focus of recent meta-methodological analyses (Goodman et al., 2017; Labuschagne et al., 2019; Narvaez Linares et al., 2020). Among others, they found that some variations from the original protocol, such as shortening the speech preparation time or questionnaires during that time, did not seem to impact the strength of stress response, whereas others, such as the composition of the panel, have affected outcome measures like salivary cortisol (Goodman et al., 2017; Narvaez Linares et al., 2020).

Importantly, these meta-analyses identified a lack of systematic recommendations on detailed reporting of stress protocol implementation. This resulted in missing information, which affected the overall informative value of meta-methodological analyses on one hand but also the replicability of results in general (Goodman et al., 2017; Narvaez Linares et al., 2020). Comparing studies was also difficult due to considerable variability in exclusion criteria (Narvaez Linares et al., 2020). This raised the concern, that protocol variation, as a source of variance, makes it difficult to distinguish if cortisol stress response patterns reflect individual differences in stress experience and consequences (health risks) or lab dependent differences in general (Narvaez Linares et al., 2020). This issue could be tackled with more rigourous recommendations or guidelines for planning and reporting of TSST and similar psychological stress protocols (Goodman et al., 2017; Labuschagne et al., 2019; Narvaez Linares et al., 2020). Although the TSST procedure has been described in great detail (Kirschbaum et al., 1993; Kudielka et al., 2007), the original research material was never published.

To improve administration and rigour of TSST research, but also the scientific standard and reliability of reported stress effects, an even more comprehensive introductory guide has been published recently, with the aim to provide those unfamiliar with the TSST protocol with significant details not included in the original publications (Labuschagne et al., 2019). But if and how these recommendations will be adopted, by novices and experts alike, remains to be seen. An important first step should be a more standardised way of reporting methodological details, as well as providing guidelines for both, authors and reviewers (Labuschagne et al., 2019; Narvaez Linares et al., 2020). Common guidelines should be discussed in the scientific stress community and updated regularly. Standards

in reporting on required methodological details could be set and enforced by journals and expert reviewers. Novice reviewers and authors should have open and easy access to this information. In any case, it seems indisputable that research standards need to be improved in order to further advance understanding of the stress system.

Furthermore, increasing digitisation will have an impact on stress research in the future. A recent meta-analysis attests that virtual versions of the TSST are equivalently robust in activating the HPA axis (Helminen et al., 2021). These approaches might become low-cost alternatives which could be applied even easier by novices, increasing interdisciplinarity. Also, due to the corona virus pandemic, the development and use of a remote version of the TSST has gained momentum (Gunnar et al., 2021; Kirschbaum, 2021). Even in post-pandemic times, this approach has a potential to allow for different study designs and access to populations that would otherwise be hard to test in the laboratory (Kirschbaum, 2021). A modified remote mobile version of the TSST has been used in an ambulatory experimental study design to test usefulness of consumer wearable tracker in naturalistic environments (Pakhomov et al., 2020). But here again, for all that is known about possible sources of variance, special care must be taken for precise documentation of circumstances such as the test situation.

And on a last note, as stress is perceived as a global health burden, it also needs a global approach to investigate effects of stress. This includes an ongoing diversification in regard to non-WEIRD sample populations and researcher perspectives as well as consideration of possible (intersectional) moderators of stress like ethnicity, culture, nationality and migration (Kuhlman et al., 2019).

6.4 The NPU-Threat Test and Ambulatory Assessment

The present study was conducted to investigate the ecological validity of the NPU-threat test. The aim was to extend general knowledge on the generalisability of the acclaimed biomarkers fear- and anxiety-potentiated startle measured via the NPU-threat test to real-life anxious experiencing. However, no significant association between psychophysiological measures from the laboratory and the state scales worry and emotionality in everyday life could be detected. A comprehensive discussion of the present results can be found in in chapter 5. The following paragraphs extend this discussion and provide a

summary on possible mechanisms and possible implications for future investigations on the generalisability and external validity of this laboratory paradigm. In general, it is not concluded that the NPU-threat test lacks ecological or external validity. This would be a false conclusion as it was already established in chapter 2 that the NPU-threat test scores well on different dimensions of validity close to the concept of external ecological validity (Grillon & Ernst, 2020). The present results, however, oblige to take a critical look at the study design and reflect on what was and was not investigated in the sense of external validity and generalisability.

In a first step, the laboratory paradigm and measures that were used as predictors in multilevel models, will be discussed. The NPU-threat test induces fear- and anxiety-potentiation which can be measured via modulation of the startle response as proposed in the original protocol (Schmitz & Grillon, 2012). As the startle responses during the conditions of NPU-threat test show acceptable reliability in healthy controls and clinical samples (Lieberman et al., 2017), it seemed reasonable to adhere to these metrics. Here, mean magnitudes for each trial type were calculated and either raw or t-scores are subtracted per condition accordingly to yield anxiety- and fear-potentiated startle as difference scores. Results reflect a successful replication of the anticipated results for mean startle magnitudes as well as anxiety- and fear-potentiation (Schmitz & Grillon, 2012) in each study. There were no differences in startle magnitudes nor potentiation between study samples. Startle magnitudes for each condition, as well as difference scores for anxiety- and fear-potentiation, were used as level-2 predictors in multilevel models. However, no other NPU-threat test metrics were investigated. Compared to anxiety- or fear-potentiation, for example more general markers for sensitivity to unpredictable (SUT) or predictable threat (SPT) can be computed for average startle magnitudes during the P-condition relative to the N-condition (SPT) and the U-condition relative to the N-condition (SUT), independent of presence of the cue. Regarding these markers, for example, sensitivity to either predictable or unpredictable threat have shown to differ between men and women (Burani & Nelson, 2020) and a positive association with dispositional anxiety sensitivity is reported (Nelson, Hajcak, et al., 2015).

Also, both markers elicit different physiological patterns between anxiety pathologies. For example, a hypersensitivity to unpredictable threat was found in panic disorder,

hypersensitivity to predictable threat in social anxiety disorder and an enhanced baseline startle in generalised anxiety disorder, which might reflect self-generated anxious thoughts in absence of imminent danger (Grillon et al., 2017). Thus, SPT and SUT can be considered useful markers in addition to anxiety- and fear-potentiation. Besides these difference metrics, it has been suggested just recently to calculate residualised change scores instead of difference scores for potentiated startle due to better psychometric properties (Meyer et al., 2017; Stevens et al., 2019).

Furthermore, the startle response was assessed, a well-preserved cross-species reflex with special value for translational research. Changes in the startle magnitude serve as physiological readout of sustained anxiety and phasic fear (Grillon et al., 2019). However, it is also possible to measure higher cognitive processing in response to predictable and unpredictable threat of shock via fMRI and EEG which might help to detect associations with everyday experiencing of anxious emotions. In regard to the latter, e.g. enhancement of the probe-elicited event related potentials (ERP) N100 and P300, that index attentional processes, demonstrated acceptable reliability and it is suggested that both might serve as potentially useful markers of fear and anxiety in addition to the startle reflex (Nelson, Hajcak, et al., 2015). Here, the probe P300 was attenuated in anticipation of predictable and unpredictable threat relative to the N-condition, while the probe N100 was only enhanced in the U-condition (Nelson, Hajcak, et al., 2015). The authors therefore speculate that anticipatory threat cues activate "motivated attention" and result in an aversive system activation (Nelson, Hodges, et al., 2015; Nelson, Hajcak, et al., 2015). But also cue-elicited ERPs might provide useful measures of the temporally distinct stages of unpredictable and predictable threat on stimulus processing and attention (MacNamara & Barley, 2018; Nelson, Hodges, et al., 2015).

Other markers that have been studied in relation to the NPU-threat test are the so called steady-state visual evoked potentials (ssVEP, see Stegmann et al., 2019; Wieser et al., 2016). SsVEPs are oscillatory, electrocortical responses to flickering visual stimuli which can serve as a marker of early stages of visual processing and attention allocation. It has been shown that onset of the unpredictable context (U condition) results in increased ssVEPs compared to the predictable context (U condition), and that predictable cues elicit greater activity than no-threat cues (Wieser et al., 2016). Also, low anxious individuals

exhibited larger ssVEP amplitudes to contextual threat (P_{CUE} and U) compared to safety cues (N and P_{ITI}) while high anxious individuals did not differentiate between contexts (Stegmann et al., 2019). This might indicate that high anxious individuals are less able to differentiate between contextual cues and therefore overestimate the probability and aversiveness of unpredictable threat (Stegmann et al., 2019). In regard to fMRI BOLD, predictable as well as unpredictable threat have shown to increase activation in the amygdala whereas only unpredictable threat has been associated with increased activity in the BNST, which is involved in anxious anticipation (Alvarez et al., 2011). However, the presented modalities require special equipment and know-how of implementation and conduction of studies as well as analysis of results.

Taken together, there exist additional valuable markers and modalities apart from the startle response and potentiation focused on in the present study and analysis. Especially markers derived from EEG and activation of fMRI BOLD could be used to study higher cognitive processing like attentional and neural processes which might be better predictors for anxious experiencing in everyday life.

In regard to AA measures of the present study, sensible differences in within- and between-variances of worry and emotionality between samples could be detected. In short, ratings on emotionality as experience of arousal generated more within-variance suggesting a higher context dependency whereas ratings on worry as cognitive concerns about aversive consequences of failure showed higher between-variance suggesting a more stable component. High compared to low anxious participants showed elevated within- and between variances. The exam preparation sample (study 3) generated by far the highest within-person variance suggesting situation-specific fluctuation of emotionality but especially worry. However, there was a tendency to positive skewed distributions for both scales across samples. This resulted in a limitation of statistical models, which had to be kept simple to avoid non-convergence. In regard to the AA measures, more complex interactions in modelling everyday dynamics of emotional processing between emotionality and worry could have been applied (Naragon-Gainey, 2019). But AA measures of emotionality and worry were already right skewed and therefore the focus was put on intercept variation. To avoid additional non-convergence, more complex modelling was not applied. However, in order to increase variability, several strategies

could be implemented.

A first step towards increased within-variances could be a longer assessment duration with a mixed time- and event-based design to capture a bigger variety of contexts and situations (Trull & Ebner-Priemer, 2020). Additionally, though the AA scales seemed sensitive to illustrate differences between samples, alternative AA measures or constructs could be used. For example, an association between naturalistic worry and increased and sustained anxiety was found in GAD patients with a set of items for worry, anxious arousal, thought valence and worry duration. The items were selected after an experimental validation in an initial pilot study using worry, stress, happy, sad and relaxation induction in a within-design. Selection was based on item effect sizes within a domain and effect sizes suggesting best discrimination from baseline and between domains (Newman et al., 2019). Further, a recent meta-analysis on AA for mood and anxiety symptoms provides an item-specific database of past research as overview of constructs that have been examined so far (Hall et al., 2021). Scales that are reported here might serve as possible resource for item selection and comprise measures for positive and negative affect, mood or symptoms of depression. However, measures which ask for anxious or affective symptomatology seem less suitable as discussed earlier (chapter 2).

Only recently, a study reported an association between startle during threat and functional impairment assessed with the World Health Organization Disability Assessment Schedule 2.0 (WHODAS, Üstün et al., 2010) in participants recruited irrespective of current or life time diagnosis of psychopathology. In more detail, sensitivity to both predictable and unpredictable threat were associated with impairment in cognition (understanding, communicating) and getting along with others after 1 year. Higher threat responding to predictable threat was additionally associated with decreased mobility (Stevens et al., 2019). Even though the WHODAS is considered a tool to asses health and disability, the reported results from its subdomains on cognition and mobility might indicate an association between the NPU-threat test to behaviour in everyday life. Further, cognitive and behavioural avoidance, measured via the Cognitive-Behavioural Avoidance Scale (Ottenbreit & Dobson, 2004), might be additional useful constructs that have, for example, shown divergent associations with rumination, worry as well as sad and anxious affect over the course of a 7-day assessment in adolescents (Dickson et al., 2012). Thus,

assessing behaviour or behavioural patterns in addition to contextual variables seems to be a viable study extension. Especially, mobility or general activity could be measured objectively via actigraphy (Wilhelm & Grossman, 2010).

In general, applying multimodal assessments, integrating information on actual arousal via physiological measures like heart rate or skin conductance levels, could yield additional information to self-report on experiencing arousal (Wilhelm & Grossman, 2010). In regard to worry and emotionality in the context of test anxiety, a recent meta-analysis reports evidence on positive correlations between self-report and physiological arousal in a majority of the reviewed studies. However, they conclude that both modalities do not completely overlap and might therefor explain different variance (Roos et al., 2020). Also, physiological objective modalities could be used to trigger subjective assessments which would provide a valuable extension to self-triggered event-based designs (Trull & Ebner-Priemer, 2020; van Halem et al., 2020).

And on a final note, of course between-person characteristics apart from dispositional anxiety should be put into focus. Traits like for instance, anxiety sensitivity or intolerance of uncertainty have been associated with an increased risk to develop anxious pathology (Carleton et al., 2007). Further, trait worry has been associated with blunted startle response to threat while an association to unpredictable threat was only evident in participants with a history of anxiety disorders (Rutherford et al., 2020). Moreover, different experiences in daily life have shown associations with higher risk to develop affective disorders. For example, being bullied can predispose for depression and anxiety disorders and having experienced bullying in the past year has even shown associations with sensitivity to unpredictable threat in adolescents (Radoman et al., 2019). Addressing these personal high-risk dispositional but also contextual variations might provide strategies in developing AA designs to further investigate external validity and generalisability of a laboratory paradigm like the NPU-threat test in the future.

6.4.1 Conclusion

Taken together, there are several indications that laboratory markers for anxiety show associations to everyday life, not only in clinical but also non-clinical populations. However, the present study design did not provide evidence for an association between physiological

measures of the NPU-threat test and everyday anxious experiencing in healthy individuals. The preceding discussion provided an overview of possible methodological adaptations to address this research question from different angles. A better understanding of how known (risk) factors, or circumstances, mediate the translational value of laboratory markers to alterations in everyday cognitive, emotional and behavioural processing on the dimension from health to disease, will help to close the gap to scientific groundwork on the aetiology of fear and anxiety as well as for the development of novel treatment strategies. The gold standard to address these questions are long-term studies which are, however, challenging and cost-intensive.

7 Final Conclusion

The overall aim of this thesis was to contribute to the methodological development of two laboratory paradigms that are commonly used in biopsychological research. This included a comprehensive general narrative overview as well as empirical work addressing moderators of the stress response and the question of generalisability of biomarkers for fear and anxiety.

In general, even 30 years after the introduction of the TSST, it can still be considered a thorough success for research on stress. The protocol is considered the gold standard for induction of acute psychosocial stress in laboratory settings and has been applied across laboratories in different fields of research. Numerous original articles, as well as a growing number of reviews and meta-analysis, illustrates the accumulation of knowledge on moderating factors of the intra- and interindividual stress response but also on the effect of an activated HPA axis due to psychological stress on emotion, cognition and behaviour. Also, knowledge on the complexity of the biological stress response itself, with alterations in hormones and peptides other than cortisol and how they interact with body and mind, has been advanced over the years. However, there also is a variety of heterogenous results to some research questions in the context of acute stress and the extent to which methodological heterogeneity in the application of the TSST protocol contributes to this divergence, warrants more attention.

But taken together, the biological response to acute psychosocial stress is considered

an important biomarker for HPA axis functioning (Nater et al., 2013). Biomarkers and their corresponding laboratory protocols, like the TSST and the NPU-threat test, are playing an increasingly important role in classification, diagnosis and treatment of psychopathology and are considered possible instruments for early intervention to prevent the development of stress- and anxiety-related diseases in the first place (Hariri & Holmes, 2015; Moriarity & Alloy, 2021). However, biological psychiatry has been accused of falling short of the discovery of novel anxiolytic compounds and treatment strategies that successfully translated from laboratory groundwork to the clinic (Grillon et al., 2019; Hariri & Holmes, 2015; Moriarity & Alloy, 2021). Interdisciplinary and intermediate research, combining basic neuroscience tools, neuroimaging and detailed clinical profiling, has been proposed to move the field forward (Grillon et al., 2019; Hariri & Holmes, 2015). Also, partly due to the replication crisis, more scientific effort should be put on meta-methodological research (Elson, 2019). For example, a better understanding and elaborated research on the basics of measurement properties of biological variables could help to avoid poor measurement and thus misestimation of effects (Elson, 2019; Moriarity & Alloy, 2021). At the same time, more personalised approaches to psychopathology are discussed, where mental disorder is defined as a complex system of contextualised dynamic processes that is non-trivially specific to each individual (Wright & Woods, 2020). Intensive longitudinal data capture and statistical modeling of dynamic processes of individual pathology are more accessible today and psychology is starting to recognise the value of big data (e.g., Renner et al., 2020).

In regard to stress, methodology is currently advancing fast and will have its impact on what questions researchers will be able to investigate in the future. The application of more complex statistical methods like linear mixed models can be observed (Lopez-Duran et al., 2014; Segerstrom et al., 2017) and even machine learning algorithms have been applied to TSST outcome measures (Baird et al., 2019). With a progression in biotechnology, continuous assessment of cortisol and other biomarkers might soon become available and affordable for research purposes (Samson & Koh, 2020; Steckl & Ray, 2018). In combination with additional physiological and other sensors, this opens up possibilities for more advanced research questions and more precise assessment of the individual cortisol reaction. However, in order to benefit from the potential these new developments promise, more rigour must be put in a better standardisation of laboratory protocols

like the TSST, to better account for possible unwanted confounding factors within and between studies. This rigour in standardisation spans from study planning and empirical work, up to reporting and open documentation of methodology and resulting data.

Both, the TSST and the NPU-threat test, are well-known standardised protocols that are applied in basic as well as clinical research. Both paradigms can be easily adapted for different research purposes and contexts and there are promising applications for future research. However, in order to maximize the potential of these laboratory protocols and their applications, an ongoing documentation and better understanding of inter- and intraindividual sources of variance, as well as comprehensive and up-to-date recommendations and guidelines, seem indispensable.

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Appendix

A AA Questionnaires

German version (original)

Bitte beantworten Sie die nachfolgenden Fragen, um die Situation zu beschreiben, in der Sie sich augenblicklich befinden.

Wo befinden Sie sich gerade?
- ☐ Zuhause
- ☐ Arbeit/Universität
- ☐ Freizeit
- ☐ Unterwegs
- ☐ Sonstiges

Bitte geben Sie Sonstiges genauer an:

Wie entspannt fühlen Sie sich gerade?
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Wie angenehm ist Ihnen die Situation gerade?
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Sind Sie gerade allein?
- ☐ ja
- ☐ nein: [FILTER]

Wer ist gerade bei Ihnen?
- ☐ Partner
- ☐ Familie
- ☐ Freunde
- ☐ Arbeitskollege/Kommilitonen
- ☐ Fremde

Ist seit der letzten Abfrage ein für Sie subjektiv belastendes Ereignis aufgetreten?
- ☐ ja
- ☐ nein: [FILTER]

Was für ein belastendes Ereignis ist aufgetreten?

Bitte beantworten Sie die nachfolgenden Fragen, um ihr augenblickliches Befinden zu beschreiben.

Mein Herz schlägt schnell.
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Ich grüble über meine Situation nach.
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Ich bin kribbelig.
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Ich mache mir Gedanken über meine Situation.
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Ich bin unruhig.
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Ich mache mir Sorgen über das, was auf mich zukommt.
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Ich bin nervös.
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Ich bin unsicher, ob alles gut gehen wird.
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Ich bin aufgeregt.
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

Ich mache mir viele Gedanken.
- ☐ überhaupt nicht
- ☐ ein wenig
- ☐ ziemlich
- ☐ sehr

English version (translated)

Please answer the questions below to describe the situation you are currently in.

Where are you right now?
- ☐ at home
- ☐ work/university
- ☐ leisure
- ☐ on the way
- ☐ other

Please specify Other in more detail:

How relaxed are you feeling right now?
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

How comfortable are you with the situation right now?
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

Are you on your own right now?
- ☐ yes
- ☐ no: [FILTER]

Who is with you right now?
- ☐ partner
- ☐ family
- ☐ friends
- ☐ work colleague/fellow student
- ☐ strangers

Has an event occurred that was subjectively stressful for you since the last query?
- ☐ yes
- ☐ no [FILTER]

What stressful event occured?

Please answer the following questions to describe how you are feeling at the moment.

My heart beats fast.
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

I ruminate about my situation.
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

I am jittery.
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

I am concerned about my situation.
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

I am restless.
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

I am worried about what is coming up.
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

I am nervous.
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

I am unsure if everything will be okay.
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

I am agitated.
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

I am doing a lot of thinking.
- ☐ not at all
- ☐ a little
- ☐ quiet
- ☐ very

B Supplementary LMM and GLMM Tables

Table 5: Linear-mixed models (study 2) with emotionality as outcome

	Model 1			Model 2			Model 3			Model 4			Model 5		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
Intercept	6.74	6.09 – 7.39	**<0.001**	6.69	5.97 – 7.40	**<0.001**	6.74	6.09 – 7.39	**<0.001**	6.74	6.09 – 7.39	**<0.001**	6.74	6.08 – 7.39	**<0.001**
$anxiety_{high}$	0.96	0.05 – 1.88	**0.039**	1.08	0.07 – 2.09	**0.036**	0.96	0.05 – 1.88	**0.039**	0.96	0.04 – 1.88	**0.040**	0.97	0.04 – 1.89	**0.041**
$anxiety_{low}$:fear-potentiated startle				-0.02	-0.24 – 0.20	0.868									
$anxiety_{high}$:fear-potentiated startle				-0.24	-0.51 – 0.03	0.087									
$anxiety_{low}$:anxiety-potentiated startle				-0.05	-0.46 – 0.36	0.817									
$anxiety_{high}$:anxiety-potentiated startle				-0.05	-0.23 – 0.13	0.584									
$startle_{tscore.zen}$							-0.00	-0.02 – 0.02	0.975	0.00	-0.05 – 0.05	0.993	-0.00	-0.08 – 0.08	0.999
$anxiety_{high}$:$startle_{tscore.zen}$							0.00	-0.03 – 0.03	0.973	0.00	-0.07 – 0.07	0.987	0.00	-0.11 – 0.11	0.971
$anxiety_{low}$:$startle_{tscore.zen}$:$condition_{P}$										-0.00	-0.07 – 0.07	0.975	-0.00	-0.10 – 0.10	0.991
$anxiety_{high}$:$startle_{tscore.zen}$:$condition_{P}$										-0.00	-0.07 – 0.07	0.981	-0.00	-0.11 – 0.10	0.956
$anxiety_{low}$:$startle_{tscore.zen}$:$condition_{U}$										-0.00	-0.08 – 0.07	0.985	-0.00	-0.10 – 0.10	0.996
$anxiety_{high}$:$startle_{tscore.zen}$:$condition_{U}$										-0.00	-0.08 – 0.08	0.980	-0.00	-0.12 – 0.12	0.980
$anxiety_{low}$:$startle_{tscore.zen}$:$condition_{N}$:Cue_{IT}													0.00	-0.09 – 0.09	0.997
$anxiety_{high}$:$startle_{tscore.zen}$:$condition_{N}$:Cue_{IT}													-0.00	-0.09 – 0.09	0.980
$anxiety_{low}$:$startle_{tscore.zen}$:$condition_{P}$:Cue_{IT}													-0.00	-0.13 – 0.13	0.988
$anxiety_{high}$:$startle_{tscore.zen}$:$condition_{P}$:Cue_{IT}													0.00	-0.11 – 0.12	0.945
$anxiety_{low}$:$startle_{tscore.zen}$:$condition_{U}$:Cue_{IT}													-0.00	-0.17 – 0.17	0.990
$anxiety_{high}$:$startle_{tscore.zen}$:$condition_{U}$:Cue_{IT}													-0.00	-0.11 – 0.10	0.965
Random Effects															
σ^2	4.47			4.47			4.47			4.47			4.48		
τ_{00}	1.61 $_{Person}$			1.63 $_{Person}$			1.61 $_{Person}$			1.61 $_{Person}$			1.61 $_{Person}$		
ICC	0.26			0.27			0.26			0.26			0.26		
N	30 $_{Person}$			30 $_{Person}$			30 $_{Person}$			30 $_{Person}$			30 $_{Person}$		
Observations	4710			4710			4710			4710			4710		
Marginal R^2 / Conditional R^2	0.037 / 0.292			0.067 / 0.316			0.037 / 0.292			0.037 / 0.292			0.037 / 0.291		

Table 6: General-linear-mixed models (study 2) with emotionality as outcome

			Model 1			**Model 2**			**Model 3**	
Predictors	*Incidence Rate Ratios*	*CI*	*p*	*Incidence Rate Ratios*	*CI*	*p*	*Incidence Rate Ratios*	*CI*	*p*
Intercept	1.74	1.31 – 2.30	**<0.001**	1.36	0.93 – 1.98	0.108	1.33	0.89 – 2.00	0.161
$anxiety_{high}$				1.62	0.95 – 2.76	0.074	1.72	0.98 – 3.03	0.060
$anxiety_{low}$:fear-potentiated startle							1.00	0.88 – 1.13	0.999
$anxiety_{high}$:fear-potentiated startle							0.92	0.79 – 1.07	0.255
$anxiety_{low}$:anxiety-potentiated startle							0.97	0.77 – 1.23	0.830
$anxiety_{high}$:anxiety-potentiated startle							0.99	0.90 – 1.10	0.885
Random Effects									
σ^2		0.84		0.84			0.84		
τ_{00}		0.60_{Person}		0.54_{Person}			0.51_{Person}		
ICC		0.42		0.39			0.38		
N		30_{Person}		30_{Person}			30_{Person}		
Observations		4710		4710			4710		
Marginal R^2 / Conditional R^2		0.000 / 0.417		0.041 / 0.416			0.057 / 0.415		

Table 7: Linear-mixed models (study 2) with worry as outcome

	Model 6			Model 7			Model 8			Model 9			Model 10		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
Intercept	7.39	6.32 – 8.45	**<0.001**	7.51	6.30 – 8.72	**<0.001**	7.39	6.32 – 8.45	**<0.001**	7.39	6.32 – 8.45	**<0.001**	7.39	6.32 – 8.46	**<0.001**
$anxiety_{high}$	1.44	-0.07 – 2.94	0.062	1.58	-0.13 – 3.28	0.070	1.44	-0.07 – 2.94	0.062	1.43	-0.07 – 2.94	0.062	1.43	-0.08 – 2.95	0.063
$anxiety_{low}$:fear-potentiated startle				-0.09	-0.46 – 0.29	0.649									
$anxiety_{high}$:fear-potentiated startle				-0.31	-0.77 – 0.14	0.180									
$anxiety_{low}$:anxiety-potentiated startle				0.21	-0.49 – 0.90	0.562									
$anxiety_{high}$:anxiety-potentiated startle				0.08	-0.23 – 0.39	0.616									
$startle_{tscore.zen}$							-0.00	-0.02 – 0.02	0.989	0.00	-0.05 – 0.05	0.999	0.00	-0.08 – 0.08	0.999
$anxiety_{high}$:$startle_{tscore.zen}$							0.00	-0.03 – 0.03	0.984	-0.00	-0.07 – 0.07	0.986	-0.00	-0.11 – 0.11	0.990
$anxiety_{low}$:$startle_{tscore.zen}$:$condition_{P}$										-0.00	-0.07 – 0.07	0.995	-0.00	-0.10 – 0.10	0.994
$anxiety_{high}$:$startle_{tscore.zen}$:$condition_{P}$										0.00	-0.07 – 0.07	0.982	0.00	-0.10 – 0.10	0.988
$anxiety_{low}$:$startle_{tscore.zen}$:$condition_{U}$										-0.00	-0.07 – 0.07	0.992	-0.00	-0.10 – 0.10	0.988
$anxiety_{high}$:$startle_{tscore.zen}$:$condition_{U}$										0.00	-0.08 – 0.08	0.968	0.00	-0.12 – 0.12	0.967
$anxiety_{low}$:$startle_{tscore.zen}$:$condition_{N}$:Cue_{IT}													0.00	-0.08 – 0.09	0.994
$anxiety_{high}$:$startle_{tscore.zen}$:$condition_{N}$:Cue_{IT}													0.00	-0.09 – 0.09	0.999
$anxiety_{low}$:$startle_{tscore.zen}$:$condition_{P}$:Cue_{IT}													0.00	-0.13 – 0.13	0.994
$anxiety_{high}$:$startle_{tscore.zen}$:$condition_{P}$:Cue_{IT}													0.00	-0.12 – 0.12	0.991
$anxiety_{low}$:$startle_{tscore.zen}$:$condition_{U}$:Cue_{IT}													0.00	-0.17 – 0.17	0.972
$anxiety_{high}$:$startle_{tscore.zen}$:$condition_{U}$:Cue_{IT}													-0.00	-0.11 – 0.10	0.974
Random Effects															
σ^2	4.44			4.44			4.44			4.44			4.45		
τ_{00}	4.40 $_{Person}$			4.69 $_{Person}$			4.40 $_{Person}$			4.40 $_{Person}$			4.40 $_{Person}$		
ICC	0.50			0.51			0.50			0.50			0.50		
N	30 $_{Person}$			30 $_{Person}$			30 $_{Person}$			30 $_{Person}$			30 $_{Person}$		
Observations	4710			4710			4710			4710			4710		
Marginal R^2 / Conditional R^2	0.055 / 0.525			0.084 / 0.554			0.055 / 0.525			0.055 / 0.525			0.055 / 0.525		

Table 8: General-linear-mixed models (study 2) with worry as outcome

		Model 1			Model 2			Model 3	
Predictors	*Incidence Rate Ratios*	*CI*	*p*	*Incidence Rate Ratios*	*CI*	*p*	*Incidence Rate Ratios*	*CI*	*p*
Intercept	2.17	1.54 – 3.06	**<0.001**	1.52	0.96 – 2.39	0.072	1.70	1.05 – 2.75	**0.031**
$anxiety_{high}$				2.04	1.08 – 3.88	**0.029**	1.94	0.98 – 3.81	0.056
$anxiety_{low}$:fear-potentiated startle							0.97	0.84 – 1.13	0.685
$anxiety_{high}$:fear-potentiated startle							0.91	0.76 – 1.10	0.339
$anxiety_{low}$:anxiety-potentiated startle							1.17	0.89 – 1.54	0.267
$anxiety_{high}$:anxiety-potentiated startle							1.01	0.89 – 1.14	0.873
Random Effects									
σ^2		0.61			0.61			0.61	
τ_{00}		0.92_{Person}			0.79_{Person}			0.74_{Person}	
ICC		0.60			0.56			0.55	
N		30_{Person}			30_{Person}			30_{Person}	
Observations		4710			4710			4710	
Marginal R^2 / Conditional R^2		0.000 / 0.601			0.083 / 0.601			0.117 / 0.600	

C Supplementary Analysis NPU-Threat Test

Supplementary file [external]: C_results_NPU_AA.html via `https://osf.io/zyqtm/`

Results NPU-threat test: subjective measures

As for ratings of state anxiety, there was a main effect of time (pre vs. post NPU-threat task) (F (1, 5.3) = 6.1, p = .016, η^2 = .02) with higher ratings of state anxiety post NPU-threat task, a main effect of study (F(2, 15.56) = 5.83, p = .004, η^2 = .12) as well as a significant interaction time x study (F(2, 5.34) = 6.33, p = .003, η^2 = .05). Posthoc analysis revealed that participants in study 1 rated their current anxiety significantly lower pre and post NPU-threat task compared to participants of studies 2 and 3 (see Table 9). Additionally, participants in study 3 rated their state anxiety pre vs. post NPU-threat task significantly lower compared to participants in studies 1 and 2. Ratings of threat value and shock expectancy for the conditions of the NPU-threat task are summarized in Table 9 and show an expected pattern with lowest ratings for the N condition (cue and no cue), slightly higher ratings for P_{ITI} and highest ratings for threat value and shock expectancy in Pcue and both U conditions. Fear and anxiety potentiation are also found in subjective ratings. There are no differences between studies (p > .3). Regarding study 2, there was no difference between the low and high anxiety group in state anxiety pre and post NPU, ratings of threat value and shock expectancy of the NPU conditions or fear and anxiety potentiation (p > .05).

Table 9: Ratings on anxiety, threat value and shock expectancy on NPU-threat test across studies

			Study 2			
	Total	Study 1	Total	$\text{anxiety}_{[\text{low}]}$	$\text{anxiety}_{[\text{high}]}$	Study 3
State anxiety[1]						
Prae NPU-threat	1.95 (0.41)	1.76 (0.36)	2.06 (0.31)	2.17 (0.30)	1.96 (0.30)	1.98 (0.55)
Post NPU-threat	2.03 (0.45)	1.82 (0.49)	1.99 (0.28)	2.03 (0.21)	1.96 (0.34)	2.36 (0.49)
Threat value[2]						
N_{CUE}	2.36 (1.42)	2.17 (1.20)	47.63 (0.38)	2.17 (1.05)	2.17 (1.34)	2.59 (1.66)
N_{ITI}	2.16 (1.33)	1.93 (1.13)	46.39 (0.29)	1.93 (1.20)	1.93 (1.08)	2.28 (1.35)
P_{CUE}	5.35 (2.20)	5.81 (2.00)	4.98 (2.20)	4.80 (1.85)	5.17 (2.54)	5.44 (2.40)
P_{ITI}	3.20 (1.76)	3.43 (1.99)	2.72 (1.39)	2.67 (1.16)	2.77 (1.61)	3.81 (1.86)
U_{CUE}	5.70 (2.23)	5.88 (2.02)	5.51 (2.20)	5.30 (1.66)	5.72 (2.66)	5.81 (2.58)
U_{ITI}	5.43 (2.24)	5.86 (2.03)	4.85 (2.25)	4.50 (1.66)	5.21 (2.72)	5.94 (2.30)
Potentiated threat[3]						
fear	2.15 (0.21)	2.38 (0.34)	2.27 (0.33)	2.13 (0.42)	2.40 (0.45)	1.63 (0.43)
anxiety	3.27 (0.26)	3.48 (0.45)	2.29 (0.39)	2.4 (0.53)	3.27 (0.64)	3.66 (0.56)
Shock expectancy[4]						
N_{CUE}	2.86 (9.60)	1.07 (3.71)	1.88 (4.94)	2.43 (5.50)	1.33 (4.34)	7.03 (17.50)
N_{ITI}	1.57 (6.20)	0.31 (1.57)	1.70 (5.87)	2.73 (7.83)	0.67 (2.54)	2.97 (9.58)
P_{CUE}	60.0 (22.9)	53.2 (20.4)	59.3 (23.20)	56.60 (23.70)	62.00 (22.90)	70.20 (22.40)
P_{ITI}	5.66 (13.9)	3.69 (13.0)	5.07 (11.00)	3.80 (7.71)	6.33 (13.50)	9.38 (18.80)
U_{CUE}	52.6 (26.0)	42.9 (22.7)	49.6 (25.30)	48.10 (28.20)	51.20 (22.30)	70.90 (22.70)
U_{ITI}	50.9 (26.6)	43.3 (24.0)	45.5 (25.40)	46.0 (28.30)	45.00 (22.70)	71.20 (22.00)

[1] STAI: State-Trait Anxiety Inventory, mean scores (SEM);
[2] mean rating (SEM), assessed twice, after each block (scale 1 - 10): How afraid have you been during ...;
[3] mean rating (SEM), fear = P_{CUE} - P_{ITI}, anxiety = U_{ITI} - N_{ITI}
[4] mean rating in % (SEM), assessed twice, after each block (scale 0 - 100 %): In your opinion, how likely was it that you received an electrical stimulation during … .

Table 10: Means, standard deviations, and correlations with confidence intervals

Variable	M	SD	1	1.1	1.2	2	3	4	4.1
1. Anxiety[1]	19.46	5.20							
1.1 Emotionality	9.75	2.73	.88** [.82, .93]						
1.2 Worry	9.72	3.07	.91** [.86, .94]	.61** [.43, .74]					
2. ASI-3	17.45	13.03	.21 [-.03, .43]	.20 [-.04, .42]	.18 [-.07, .40]				
3. IU-18	42.52	12.90	.68** [.53, .79]	.58** [.39, .72]	.65** [.48, .77]	.45** [.24, .62]			
4. PAF	45.39	6.19	.61** [.39, .77]	.59** [.36, .75]	.51** [.26, .70]	.25 [-.04, .51]	.44** [.18, .65]		
4.1 Emotionality	9.52	2.85	.58** [.35, .74]	.60** [.38, .76]	.44** [.17, .65]	.18 [-.12, .45]	.44** [.17, .65]	.72** [.54, .84]	
4.2 Worry	13.20	3.87	.60** [.38, .76]	.54** [.29, .71]	.54** [.29, .72]	.23 [-.06, .49]	.44** [.17, .65]	.76** [.60, .86]	.45** [.18, .65]

Note:
M and SD are used to represent mean and standard deviation, respectively. Values in square brackets indicate the 95 % confidence interval for each correlation. The confidence interval is a plausible range of population correlations that could have caused the sample correlation (Cumming, 2014).
[1] STADI: State-Trait-Anxiety-Depression Inventory;
* indicates p .05, ** indicates p .01.

D Supplementary citation report TSST

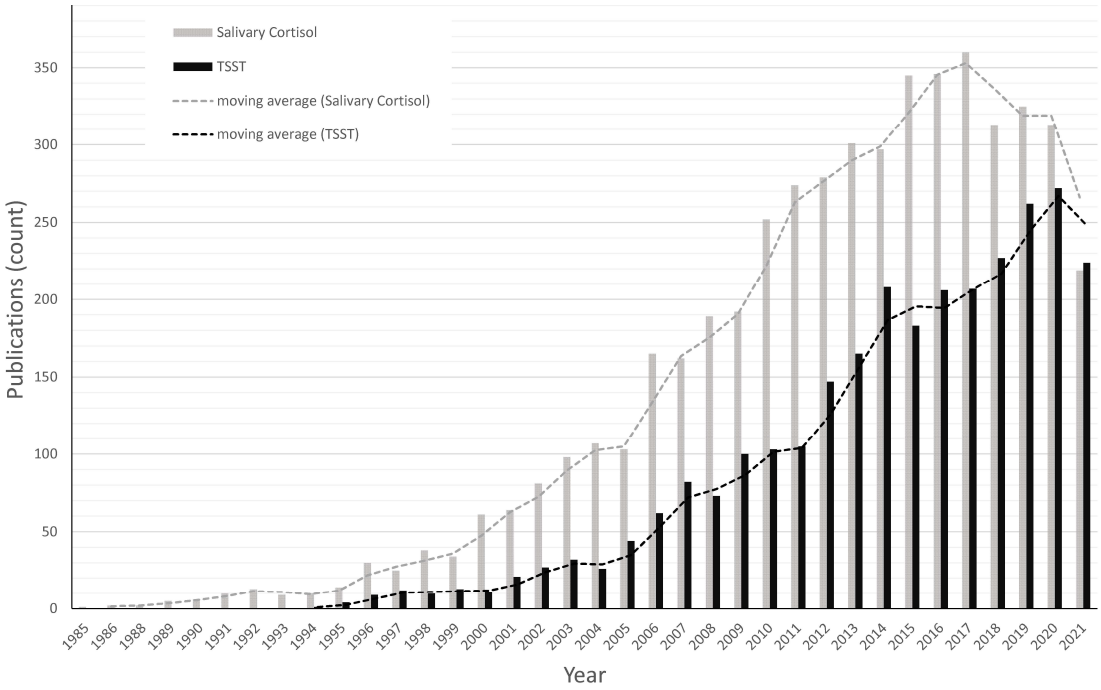

Figure 4: Count of numbers and moving average for publications citing the original protocol of the TSST (only articles, no reviews; permanent Link see below) and for the search term "salivary cortisol" (limited to categories including psychiatry, psychology or social sciences, complete list and search documentation via permanent Link, see below). Citation Report data is derived from Clarivate Web of Science, Copyright Clarivate 202_. All rights reserved.

Query links:

- TSST: `https://www.webofscience.com/wos/woscc/summary/85f65368-4f69-4e3e-9a66-9817fd1a201a-0bde8b80/relevance/1`
- Salivary cortisol: `https://www.webofscience.com/wos/woscc/summary/5e5c44a3-3ece-4578-9ced-e8528611cd94-0bdf0651/relevance/1`

Search documentation for "salivary cortisol"

All fields: salivary cortisol

Document Types: Article AND

Categories (Web of Science): Psychiatry OR Neuroscience OR Behavioral Science OR Psychology Developmental OR Psychology Biological OR Psychology Multidisciplinary OR Psychology Clinical OR Psychology Experimental OR Psychology Applied OR Psychology Social OR Psychology Educational OR Social Sciences Biomedical OR Sociology OR Neuroimaging OR Social Sciences Interdisciplinary