The balance within

THE BALANCE WITHIN

Factors influencing neurovisceral autonomic responsiveness

to endocrine and pharmacological stress challenges

DISSERTATION

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Table of Contents

Chapter 1

General Introduction

Homeostasis, stress and the stress system

Central control and assessment of autonomic activity

Aim and outline of the dissertation

HOMEOSTASIS, STRESS AND THE STRESS SYSTEM

The balance within - Defining homeostasis

Homeostasis is defined as the ideal state of a complex internal equilibrium for optimal functioning and represents a priority condition for all living organisms [1]. The central concept of homeostasis has its origins in the ancient Greek philosophers, who proclaimed a need for balance of opposing forces in nature (i.e., “harmony of the cosmos”), but also within living organisms (i.e., “isonomia”, “ataraxia”, “eustathia”) (Table 1). This concept was re-introduced by Claude Bernard in 1878 as „millieu interieur“ and later on specifically defined as homeostasis by Walter Cannon in 1935 [2, 3].

However, it was first Heracleitus and later Empedocles and Hippocrates who actually suggested that a static, unchangeable functioning state constitutes an unnatural condition [4]. On the contrary, as homeostasis is constantly challenged, it is rather the reactive capacity to maintain a complex dynamic balance state and constant fluctuation around a more ideal and less achievable homeostatic condition (non-equilibrium homeodynamic state - eustasis) that serves self-regulation and adaptability of the organism to ongoing challenges, while alterations in this reactive ability may lead to disease [5-7].

The threatened balance - Stress, stress system and allostasis

Stress is defined as a state of threatened homeodynamic balance [1, 8] by a wide range of intrinsic or extrinsic, real or perceived challenges or stimuli, defined as stressors [9, 10]. The word "stress" originates etymologically from the common proto-indo-european root "str", which has been historically associated with exertion of pressure [i.e., gr. στραγγαλίζειν, lat. strangulare (= to strangle), lat. stringere/strictus (= to tighten/tight), enm. destresse]. The first to use and popularize the term „stress“ in its current meaning was Hans Selye in 1936, who defined stress as "the non-specific response of the body to any demand placed upon it" [11, 12].

To preserve their optimal homeodynamic state, organisms have developed a highly sophisticated system, the stress system, which serves self-regulation and adaptability of the organism by energy redirection according to the current needs [1, 8, 13]. The stress system, as a homeodynamic system, normally exerts its effects in an inverted U-shaped dose-response curve with the optimal homeodynamic state achieved in the central range of the curve (Fig. 1). When stressors exceed a certain severity or temporal threshold, the so-called stress reaction is initiated (fight, flight or freeze), leading to a different homeodynamic adaptation, has been defined as allostasis (gr.: "different state"; i.e., altered homeostasis) [14, 15].

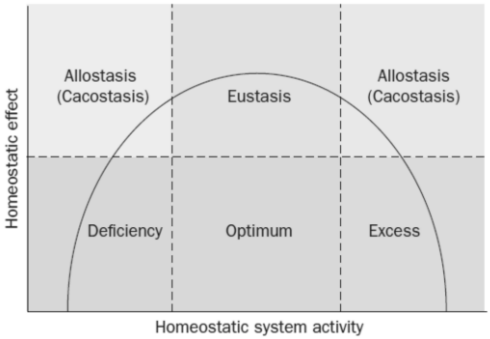
Table 1. Brief historical overview of homeostasis and stress concepts.

Historical figure		Stress concept
Pythagoras	(570–510 BC)	The ***"Harmony"*** of the cosmos
Heracleitus	(535–475 BC)	Nothing endures, but changes—***"Harmony"*** in the unity of opposites
Alcmaeon	(c. 500 BC)	The intellect is based in the brain Health is the equipoise of opposing forces: ***"Isonomia"***
Empedocles	(495–435 BC)	Matter consists of essential elements and qualities in opposition or alliance to one another. Main elements (air, fire, earth, water) kept in balance through the two main universal forces: Love and strife
Hippocrates	(460–370 BC)	A harmonious balance of the elements and qualities of life is health—disharmony is disease.
		'Νούσων φύσεις ιητροί = Vis medicatrix naturae'
		Four humor theory: blood, yellow bile, black bile and phlegm (humor balance = health)
Sceptics/Stoics Epicureans		***"Ataraxia"*** (imperturbability of mind, equanimity)
Aristotle	(385–322 BC)	Unity of mind and body, ***"Eudaimonia"***
Epicurus	(341–270 BC)	*Ataraxia* (imperturbability of mind), *Aponia* (no pain) and *Hedone* (tranquil, nonsensual pleasure) as desirable states. ***"Eustathia"*** = good balance, Carpe diem = seize the day
Thomas Sydenham	(1624–1689)	Symptoms and signs of a disease also arise from the reaction of the patients' system
Robert Hooke	(1635–1703)	Hooke's Law of elasticity—introduction of the term ***"stress"*** in mechanical physics
Charles Darwin	(1809–1882)	Survival is the interaction of internal biology with the stressful environment
Claude Bernard	(1813–1878)	Introduction of term ***"milieu interieur"***
Charles Mercier	(1851–1919)	Direct, indirect internal, indirect external stress/Mental stress
		Stress as major influence towards mental disorders
Walter Cannon	(1871–1945)	Introduction of term ***"Homeostasis"***
		Bodily responses to emotions, ***"Fight or flight"*** (and freeze) reaction
Harold G. Wolff	(1898–1962)	Stress as internal dynamic state (not stimulus)—protective adaptive response
		Stress as major influence towards disease
Hans Selye	(1907–1982)	The ***"General Adaptation Syndrome"*** —GAS (the stress syndrome)
		Diseases of adaptation, distress vs. eustress
		Definition of stress as: "Stress is the **rate of wear and tear** in the human machinery that accompanies any vital activity and, in a sense, parallels the intensity of life."
Richard Lazarus	(1922–2002)	Personal appraisal as major moderator of stress response—Dichotomy model (threat/challenge)
Peter Sterling and Joseph Eyer	1988	Introduction of term ***"Allostasis"***
Bruce S. McEwen and Eliot Stellar	1993	Introduction of term ***"Allostatic Load"***
George P. Chrousos	2009	Introduction of terms ***"Eustasis / Hyperstasis / Cacostasis", "Cacostasic Load"***,

Adopted from Agorastos & Chrousos (2021) [16].

Peter Nixon differentiated in 1979 “eustress” from “distress”, suggesting that not all stress states are harmful and that chronicity, quality, magnitude subjective appraisal and context of stressors of stressors are important moderators of the stress reaction [17]. Repeated, ephemeral and motivating stress states lead to adaptive responses and response habituation being fairly beneficial and leading to an improved homedynamic capacity (i.e., hyperstatis, gr.: “higher/better state”), while inadequate, aversive, excessive (i.e., traumatic) or prolonged stress may surpass the natural regulatory capacity and adjustive resources of the organism and result to a state of disharmony defined as cacostasis (gr.: “bad state”; i.e., a negatively altered, defective homeodynamic state, dyshomeostasis) and accumulated allostatic or - more correctly - cacostatic load (i.e., cumulative pathophysiological burden of the organism), associated with maladaptive responses [8] (Fig. 2). Therefore, latest literature suggests that the colloquial use of the term “stress“ should be rather restricted to conditions where an environmental demand exceeds the natural regulatory capacity and adjustive resources of an organism in a maladaptive manner (leading to cacostasis, i.e., reflecting only distress) [18].

Figure 1. Inverse, U-type dose-response effects of homeodynamic systems



Adopted from Chrousos (2009) [8]

Figure 2. Conceptional model of stress, homeodynamic balance and adaptive responses.

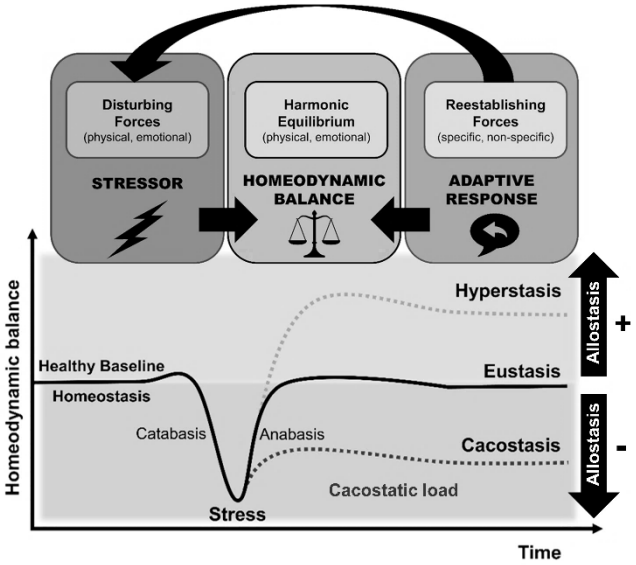


Figure Legend
The interaction between homeostasis-disturbing stressors and stressor-activated adaptive responses of the organism is dynamic and can have three potential outcomes. First, the organism can return to its basal and functional homeodynamic balance (i.e., eustasis; gr.: "good state"); second, the adaptive response may be inappropriate (for example, inadequate, excessive and/or prolonged) and the organism falls (i.e., catabasis; gr.: "descent") into a negatively altered, defective homeodynamic state (i.e., cacostasis; gr.: "bad state"), associated with cacostatic load and, third, the match may be perfect and the organism gains from the experience (i.e., anabasis; gr.: "ascent") and a new, improved homeodynamic capacity is attained (i.e., hyperstasis; gr.: "higher/better state"). Adopted from Agorastos & Chrousos (2021) [16].

The human stress system – organization and components

The human stress system includes central and peripheral components (Fig. 4). The central, greatly interconnected components of this system are located in the hypothalamus and the brainstem and interact with several other major brain nuclei and neuromodulatory systems, such as the mesocortical and the mesolimbic dopaminergic system (involved in reward and motivation), the central nucleus of the amygdalae (involved in fear, anger and arousal), the endocannabinoid system

(involved in cognitive and physiological regulation) and the central circadian system (involved in temporal organization of physiologic procedures) [1, 8, 19]. The stress system has per se a baseline, circadian activity [20], which, however, is affected by numerous cognitive, emotional, neurosensory, humoral, immune, blood-borne, digestive, thermostatic, limbic and peripheral somatic signals through different pathways. When stressors exceed a certain severity or temporal threshold, stressor-related information initiates a complex stress response (fight, flight or freeze response) to induce remarkably consistent acute, normally adaptive and time-limited micro-, meso- and macrophysiologic compensatory responses, redirecting energy according to the current needs [8, 10, 13]. Together, these responses through different stress effector tissues produce a well-orchestrated response to challenge [21].

The central stress system

In detail, the central stress system includes: a) the parvocellular corticotropine-releasing-hormone (CRH) neurons, b) the arginine-vasopressin (AVP) neurons of the hypothalamic paraventricular nuclei (PVN), c) the CRH neurons of the paragigantocellular (PGC) and parabranchial (PB) nuclei of the medulla oblongata (MO) and locus coeruleus (LC), d) the ARC peptides α-melanocyte-stimulating hormone/melanocortin (α-MSH) and β-endorphine (β-E), e) other noradrenergic (NE) cell groups in the MO and pons (LC/NE), f) central nuclei of the autonomic nervous system (ANS) and g) the central autonomic network (CAN).

The peripheral stress system

The peripheral, interrelated components of the stress system include: a) the hypothalamic-pituitary-adrenal (HPA) axis and b) the limbs of the ANS comprised of i) the sympathetic nervous and sympatho-adrenomedullary system (SNS and SAM respectively) and ii) the parasympathetic (vagal) nervous system (PNS). The principal peripheral effector molecules are the HPA axis end-hormones, the glucocorticoids (GCs; i.e., cortisol in humans), and the SAM-regulated catecholamines NE and

epinephrine/adrenaline (AD) [8]. HPA axis and ANS have largely complementary actions throughout the body and are increasingly studied together [22], as integrated and interrelated components of an internal neural regulation system (CAN). Findings suggest that the appropriate regulation of the HPA-axis depends in part on ANS, especially on vagal influences [23].

The hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis consists of the PVN, the pituitary corticotrophs and the zona fasciculata of the adrenal cortex, which, respectively, employ CRH/AVP, adrenocorticotropic hormone (ACTH) and GCs (i.e., cortisol in humans) as their signalling effector molecules [16]. CRH and AVP are released from the PVN into the hypophyseal system in response to stimulatory signals from higher regulatory centers (e.g., prefrontal cortex, PFC) and reach the pituitary gland to stimulate the secretion of ACTH. ACTH reaches the cortex of the adrenal glands through release in the systemic circulation and stimulates both production and secretion of GCs. Systemically released GCs, in turn, besides their major actions, close a negative feedback loop by suppressing the activation of the PVN and the pituitary gland. GCs exert their pleiotropic effects mainly through genomic (i.e., transactivation or transrepression of genes), nongenomic and mitochondrial actions through glucocorticoid and mineralocorticoid receptors (respectively GRs and MRs) [24, 25]. The appropriate regulation of the HPA axis depends at least in part on the ANS, especially on vagal influences [23].

The autonomic nervous system (ANS)

The ANS, although not under overt voluntary direction (*autonomous*), plays a crucial role in the maintenance of homeodynamic balance by providing a rapidly responding control system for a plethora of physiological reactions to ongoing physical, emotional and cognitive challenges [26, 27]. Since the early 20th century, pragmatic and anatomic reasons has led to a common division of the ANS into two, or sometimes three tracts: the sympathetic, parasympathetic and, the largest one, the

enteric autonomic division, although they practically mirror one larger control system [28, 29]. Especially the separation into SNS and PNS has led to enormous misconceptions, the most serious being the view that the two divisions are somehow in opposition to each other. On the contrary, SNS and the PNS are rather in a dynamic balanced interdependent state and act in concert and through numerous and multi-level, bidirectional interactions to control the abovementioned autonomic functions and secure system lability and variability [30-32]. Autonomic imbalance, on the contrary, is associated with decreased dynamic adaptability of the organism, increased morbidity and mortality [23, 27, 30, 31].

The sympathetic branch (SNS, SAM)

The sympathetic activity of the ANS originates in brainstem nuclei and gives rise to preganglionic cholinergic (ACh) efferent fibers mostly projecting to postganglionic sympathetic ganglia. The long postganglionic neurons terminate outwards on effector tissues, mostly releasing NE (i.e., SNS). Alternatively, preganglionic neurons may also directly synapse with the modified postganglionic chromaffin cells of the adrenal medulla (AM); i.e., SAM system). A sympathetic activation, thus, principally releases NE (locally and to a lesser extent systematically from the AM) or AD (systematically from the AM) together with other neuropeptides in the body [33]. Sympathetic activation generally predominates during emergency (*fight-or-flight*) situations and during exercise, preparing the body for strenuous physical activity.

The parasympathetic branch (PNS)

Whereas SNS activity depends on two peripheral branches (neural and adrenal), the PNS activity is displayed only by nerves. Accordingly, parasympathetic actions are mostly more discrete and localized compared to the sympathetic system, where a more diffuse discharge is possible. The quite long preganglionic ACh-neurons of the PNS arise from several brainstem nuclei and from the spinal sacral region (S2 - S4) and synapse with short postganglionic neurons within terminal ganglia close to or embedded to effector tissues. The sacral preganglionic neurons exit the CNS and join

together to form the pelvic nerves innervating the pelvic viscera. The preganglionic neurons that arise from the brainstem exit the CNS through the cranial nerves [N. occulomotorius (III); N. facialis (VII); N. glossopharyngeus (IX); N. vagus (X)]. The vagus nerve innervates the thoracic and abdominal viscera and has a major physiological significance, as approximately ¾ of all parasympathetic fibers originate from the vagus nerve [34]. The parasympathetic response to stress is mainly mediated by the nucleus ambiguus and the dorsal motor nucleus of the vagus nerve (DMV), possibly through input from the NTS. The PNS generally predominates during resting conditions towards conserving and storing energy or regulating basic body functions (e.g., digestion, defecation, urination). Due to its tonic function, the PNS is essential under resting conditions, and is, therefore, particularly implicated in the pathophysiology of cardiovascular diseases and other comorbidities [23, 35].

The neuroendocrine stress response

During the stress response, the ANS responds immediately via activation of the SNS/SAM system and central activation of the HPA axis [8]. SNS activation stimulates the release of NE (neural/locally) and SAM the release of NE and AD (at the end of SNS terminals and systematically from the adrenal medulla, respectively) [10]. Central HPA axis activation of the PVN stimulates the release of CRH and AVP into the hypophyseal system. CRH stimulates the subsequent adrenocorticotropin (ACTH) secretion from the pituitary corticotrophs, which then reaches the adrenal cortex through release in the systemic circulation and stimulates both production and secretion of GCs [36]. GCs have a plethora of actions representing a *second wave* of the stress response, essential to the activation, maintenance and downregulation (i.e. negative feedback of ACTH release) of the stress response through their effect at several levels of the HPA axis and the SNS [37]. GRs and MRs differentially regulate target gene expression according to transcriptional potency in response to GCs depending on receptor type, cell topology, tissue-specific expression, specific ligands (e.g., aldosterone) or relevant enzymes [24].

Figure 4. Structural components and regulation of the central and peripheral stress system.

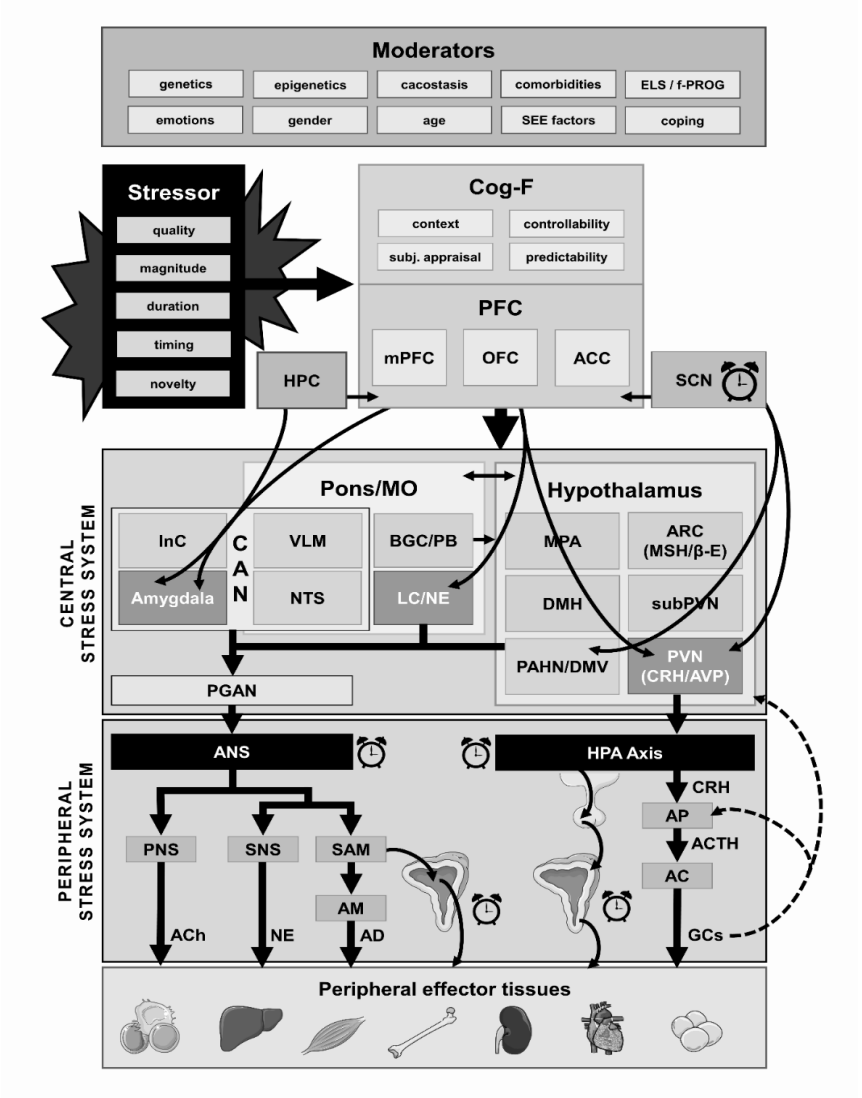


Figure Legend

AC: adrenal cortex; ACC: anterior cingulate cortex; ACh: acetylcholine; ACTH: adrenocorticotropic hormone; AD: adrenalin; AM: adrenal medulla; ANS: autonomic nervous system; AP: anterior pituitary; ARC: arcuate nucleus; AVP: arginine vasopressin; CAN: central

autonomic network; Cog-F: cognitive filter; CRH: corticotropin releasing hormone/corticoliberin; DMH: dorsomedial hypothalamus; DMV: dorsal motor nucleus of the vagus nerve; ELS: early life stress; f-PROG: fetal programming; GCs: glucocorticoids; HPA axis: hypothalamic-pituitary-adrenal axis; HPC: hippocampus; InC: insular cortex; LC: locus coeruleus; MPA: medial preoptic area; mPFC: medial prefrontal cortex; MSH: melanocyte-stimulating hormone/melanocortin; β-E: β-endorphine; NE: norepinephrine; NTS: nucleus of the solitary tract; OFC: orbitofrontal cortex; PAHN: preautonomic hypothalamic nucleus; PB: parabrachial nuclei; PFC: prefrontal cortex; PGC: paragigantocellular nuclei; PNS: parasympathetic nervous system; PGAN: preganglionic autonomic neurons; PVN: paraventricular nucleus; SCN: suprachiasmatic nucleus; SEE factors: social, economic and environmental factors; SNS: sympathetic nervous system; subPVN: subparaventricular area; VLM: ventrolateral medulla. Solid line: top-down regulation/stimulation, dashed line: bottom-up feedback regulation/inhibition; clocks: crucial circadian regulation. Adopted from Agorastos & Chrousos (2021) [16].

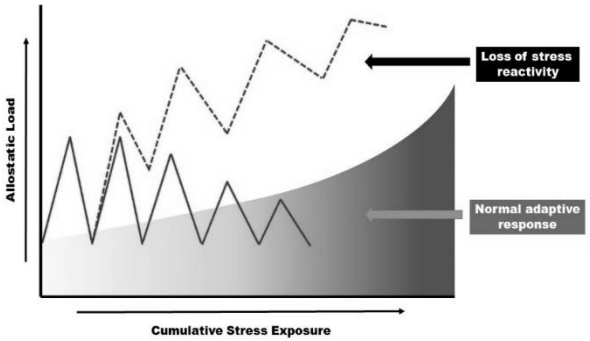
Stress system reactivity

Although the basal diurnal activity of the stress system is important for healthy functioning of the organism, it is indeed the stress system reactivity to stressors that seems to play an even more crucial role for adaptive responses, survival and overall health. The reactivity hypothesis links stress responsiveness and particularly autonomic reactivity to increased cardiovascular risk as a measure of an individual's psychobiologic response to challenges in the environment and a mediator of psychosocial and behavioral risk factors [38, 39]. In addition to the diurnal phase, many additional factors influence stress system reactivity. For example, inadequate, excessive or chronic (i.e., prolonged persistent or repeated) stress may surpass the physiological regulatory capacity of the organism and alter negatively neuroendocrine responses to stress [1, 40, 41], leading to characteristic psychophysiological alterations, such as impaired GC signaling and disrupted HPA axis and autonomic reactivity during stress [9, 42]. Thereby, factors as developmental timing, (epi)genetics, duration and nature of stressors among others play an important moderating role. Chronic dysregulation of the stress system, as in example in patients with depression or posttraumatic stress disorder (PTSD) [43, 44], can also result in disrupted stress system reactivity and further lead to numerous physical co-morbidities [44-48].

Autonomic reactivity

It is especially the precise regulation of organ and tissue functions through a fine-tuning of the two main ANS tracts (SNS, PNS) that is crucial for optimal stress reactivity, adaptive responses and health. SNS and PNS are in a dynamic interdependent state and act in concert and through numerous and multi-level, bidirectional interactions to control autonomic functions, while autonomic imbalance is associated with decreased dynamic adaptability, increased morbidity and mortality. The initial reactivity hypothesis proposed enhanced cardiovascular autonomic reactivity to be associated with increased cardiovascular risk as a mediator of psychosocial and behavioral risk factors [38, 39]. Increased autonomic reactivity responses to laboratory challenges compared with resting states have been associated with high morbidity rates and overall mortality, internalizing and externalizing behavior problems, mental health disorders and symptoms, and overall poor adjustment. However, in the last years research provided robust evidence that indeed reduced cardiovascular reactivity and slower recovery are associated with higher cardiovascular and overall morbidity and mortality risk [49, 50].

Figure 3. Disrupted stress reactivity and increased allostatic load by cumulative stress exposure.



Modified by Agorastos from McEwen (1998) [9]

CENTRAL CONTROL AND ASSESSMENT OF AUTONOMIC ACTIVITY

The multidimensional autonomic control

Berntson et al. introduced 1991 the concept of "autonomic space" [51], bounded by sympathetic and parasympathetic axes, implying that the control of the ANS rests on a multidimensional system that involves coupled and uncoupled (independant) activation modes. The exact ANS activity is fine-tuned through central and peripheral autonomic reflexes and feedback mechanisms [52]. In general, since both systems are tonically active, the PNS can both assist and antagonize SNS functions by withdrawing or increasing its activity (frequency of neuronal discharge), respectively. This ANS characteristic is of major importance and improves its ability to more precisely regulate an effector's function.

Central autonomic network (CAN)

The CAN has been proposed as the integral component of an internal central nervous system (CNS) autonomic regulation system, essential for survival. It includes the insular cortex, central nucleus of the amygdala, hypothalamus, periaqueductal gray matter, parabrachial complex, NTS, and ventrolateral medulla (VLM) [53, 54]. The insular cortex and amygdala mediate high-order autonomic control associated with cognitive perception and emotional responses through hypothalamic-brainstem pathways [27]. NTS, PVN and VLM contain a network of respiratory, cardiovagal, and vasomotor neurons, receiving afferent vagal sensory input from thoracic and abdominal viscera and other cranial nerves. These structures accordingly modulate the activity of preganglionic autonomic neurons (PGAN). However, the CAN is additionally characterized by reciprocal interconnections, parallel organization, state-dependent activity, and neurochemical complexity [26, 27, 55, 56]. Therefore, central autonomic modulation does not simply rely on a monolithic network of brain regions, but instead features certain task and division specificity [57]. In addition, the activity and functional connectivity of those brain regions involved in the CAN is majorly influenced by serotonergic signaling, with altered central serotonergic regulation being associated with autonomic

dysregulation. The central serotonergic system is also heavily involved in the regulation of stress and anxiety. Accordingly, the CAN may be critically involved in psychiatric disorders, essential hypertension, obesity, and other medical conditions [58]. CAN dysregulation [56, 59, 60] may affect downstream autonomic core centers, thereby altering peripheral ANS activity and eventually dynamics of different subordinate systems (e.g., cardiovascular system) [60-62] (Fig. 4).

Figure 4. Simplified diagram of the central autonomic nervous system network (CAN) involved in regulation of the peripheral autonomic nervous system.

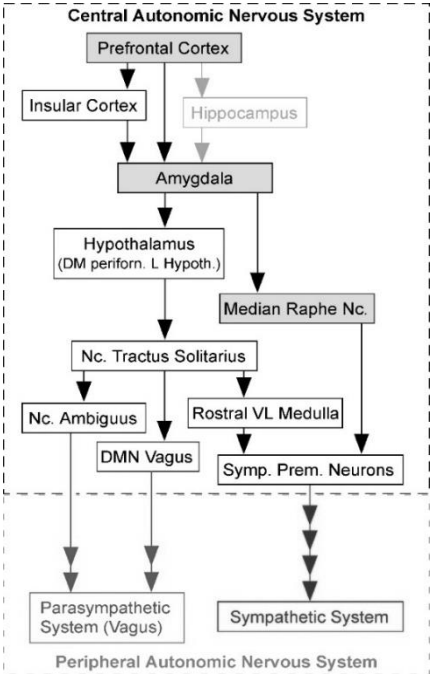


Figure Legend
DM: dorsomedial; DMN: dorsal motor nucleus (of the nervus vagus); L: lateral; Nc.: nucleus; periforn.: perifornical; Symp. Prem.: sympathetic premotor; VL: ventrolateral. Core areas involved in emotional modulation are depicted in grey. The hippocampus is only indirectly involved, e.g., in contextual fear memory-related autonomic adjustments. Autonomic function is further modulated by respiratory function, referred to as respiratory sinus arrhythmia, blood

pressure-mediated feedback systems (baroreflex circuit, through the Nc. Tractus Solitarius) as well as by endocrine systems such as the sympathetic-medullary axis and the hypothalamic pituitary-adrenal axis that are both involved in the stress response. Adopted from Agorastos et al. (2014) [63].

Central autonomic activity assessment through the cardiovascular system

One of the most appropriate systems for the functional assessment of the ANS activity is the cardiovascular system, as responses of the effector tissues are prompt, easy to access and have a high clinical and prognostic value [64, 65].

The autonomic cardiac control

The heart features a number of different effector tissues: the sinoatrial (SA) node, the atrioventricular (AV) node, the atrial and the ventricular myocardium [66]. Modulation of ion channels particularly in the primary and secondary pacemaker (SA and AV node respectively) inluence heart function in three distinct, but interdependent ways: chronotropy, inotropy and dromotropy. This modulation is predominately influenced by the ANS through tonic drives of and interactions with both sympathetic and parasympathetic cardiac nerves in an asymmetric manner depending on demand. Right-sided SNS and PNS fibers reach the SA node and modulate particularly chronotropic activity (i.e., influencing frequency of contraction; SNS: positive chronotrop; PNS: negative chronotrop), while left-sided SNS and PNS fibers innervate the AV node and myocardium and modulate dromotropic (i.e., influencing rate of electrical impulse conduction) and inotropic (i.e., influencing force of contraction) activity in the same manner [67-69] (Fig. 5).

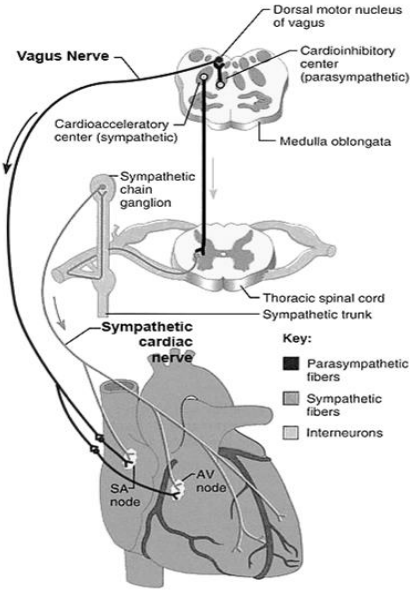
Resting heart activity is under constant tonic inhibitory control by PNS influence and dominance over SNS influence [33, 70]. In addition, heart rate (HR) is characterized by beat-to-beat variability over a wide range, implicating vagal dominance, as SNS cardiac influence is too slow and long-lasting to produce rapid beat-to-beat changes (e.g., respiratory sinus arrhythmia, RSA) [71, 72]. Thus, simultaneous PNS and SNS cardiac co-activation leads to more efficient cardiac function than SNS activation alone via permitting both longer ventricular filling and stronger myocardial contraction [73]. Autonomic imbalance of SNS and PNS cardiac

influences is, thus, associated with decreased dynamic flexibility, loss of complexity, increased vulnerability to pathologies and consequently compromised cardiac health. Due to its tonic inhibitory influence on the heart, the PNS is particularly implicated in the pathophysiology of cardiovascular diseases and other comorbidities [23].

Assessment of ANS activity through heart rate variability (HRV)

Although the cardiovascular system is particularly appropriate for the functional assessment of the ANS activity, standard cardial measures (e.g., HR) offer only limited information on ANS activity, as short- and long-term HR variations are not considered.

Figure 5. Autonomic innervation of the heart



Adopted from Marieb (2003) [68]

One of the best established and widely used non-invasive methods for the quantitative assessment of ANS activity is the computerized analysis of heart rate

variability (HRV) [74, 75]. HRV results from HR oscillations within its physiological range (beat-to-beat variability), controlled by parasympathetic and sympathetic modulation of intrinsic cardiac pacemakers and reflects heart-brain-interactions and ANS dynamics. Higher HRV implicates parasympathetic dominance favoring energy conservation, while low HRV suggests enhanced sympathetic and/or attenuated parasympathetic cardiac modulation for mobilization of energy resources particular during high attention, arousal and stress. Low HRV is associated with higher overall mortality, specifically heart mortality (e.g.,, heart infraction), and is considered a valid marker of heart disease [23, 54, 76]. Thus, HRV reflects the capacity of the organism for regulated physical and emotional responding. However, SNS and PNS are interdependent systems that act on different time scales [77].

HRV measures

A simplified overview of common and important main HR measures in the different analytical domains is depicted in Table 2. A diagram depicting the individual steps from ECG recording to HR data analysis is shown in Figure 6.

Linear measures

- *Time domain measures:* Instantaneous HR is calculated on the basis of the RR interval. HRV in the time domain is based on the normalized time between two detected heartbeat QRS events (NN interval, excluding unreliable intervals) and normally calculated by the standard deviation of the N-N intervals (SDNN), the root-mean-square of subsequent interval differences (RMSSD), and the percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms (NN50%). Low SDNN, NN50 and RMSSD denote low vagal tone [74].

- *Frequency domain measures:* Frequency measures represent the power (energy) as a function of frequency with regard to specific frequency bands commonly based on fast Fourier transformation [74]. HRV in the frequency domain is normally calculated by analysis of two frequency components [low frequency (LF) 0.04-0.15 Hz and high frequency (HF) 0.15-0.4 Hz]. Results are often presented as power

percentage (%) of each frequency component from the total power or in normalized units and the LF/HF ration as a potential measure of sympatho-vagal balance. Calculation of percentage (%) of frequency components may prevent the appreciation of the fractional distribution of the energy by the expression of the spectral components in absolute units and minimize the effect of the changes in total power on the LF and HF component [74]. Clinical and experimental studies evaluate congruently efferent vagal activity as the major contributor to the HF component, which however may be also affected by sympathetic nerve activation by perhaps as much as 10% [74, 78]. The interpretation of the LF component is more controversial. Earlier studies considered the LF component as a marker of sympathetic modulation, while it recently has been postulated that LF rather reflects a complex mix of sympathetic, vagal, and other unidentified factors, with vagal factors accounting for the largest portion of the variability in this frequency range [78, 79]. Consequently, the hypothesis that the LF/HF ratio can accurately quantify sympatho-vagal balance is questioned [78, 79]. Another disadvantage is that the frequency bands used in animal models are different from those in humans and that these frequency bands represent only a fraction of the whole energy spectrum of the power analysis.

Nonlinear measures

Two major functional properties of HR dynamics, non-stationarity (drift-like behaviour of HR) and interdependence (correlation of heartbeat intervals in its temporal sequence under physiological conditions), formally require the additional assessment of HR dynamics by nonlinear methods that determine the organization of the 'noisy' HR signal with intrinsic long-range correlation and high level of complexity [77]. One unifractal approach, the detrended fluctuation analysis (DFA) [80], derived from the random walk theory, uses scaling coefficients (α) as a measure of the correlation of heartbeats in its temporal sequence, thereby indicating ANS dysregulation. A scaling coefficient $\alpha = 1.5$ indicates 'Brownian noise' in which there only is short-term correlation, i.e., one heartbeat interval is correlated to the next

one only. This is observed when the tonic parasympathetic outflow to the heart is blocked, through atropine treatment or as a consequence of heart transplantation [81]. A value of $\alpha = 1.0$ indicates persistent long-range correlation of successive heartbeat intervals, as observed in physiological HR dynamics of mammalian species including mice and man. A scaling coefficient of $\alpha = 0.5$ reflects 'white noise' and indicates the lack of any correlation between heartbeat intervals (randomness). The straight-line relationship is separated by a breakpoint indicating different slopes, α_{fast} and α_{slow}, which are interpreted to reflect the coefficients of distinct ranges, short-range and long-range scaling, respectively [77]**.**Pathological states are characterized by a breakdown of long-range correlations, i.e., α strongly deviating from 1.

Table 2. Important heart rate measures in the three different analytical domains

Domain	Measure	Units	Definition
Time Domain	**SDNN**	ms	Standard deviation of the NN intervals
	NN50	-	Number of adjacent NN intervals differing by >50 ms
	pNN50%	%	Percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms
	RMSSD	ms	Root-mean-square of subsequent NN interval differences
Frequency Domain	**ULF**	ms^2	Ultra low frequency (≤ 0.003 Hz) power
	VLF	ms^2	Very low frequency (0.003 – 0.04 Hz) power
	LF	ms^2	Low frequency (0.04 – 0.15 Hz) power
	HF	ms^2	High frequency (0.15 – 0.4 Hz) power
	LF/HF ratio	-	(Inaccurate) Index of sympatho-vagal balance
Nonlinear Domain	**α_{fast}, α_{slow}**	-	Scaling coefficients of the detrended fluctuation analysis to determine internal correlations

Table Legend
NN intervals: normalized time between two heartbeat QRS events after excluding unreliable QRS intervals. Adopted from Agorastos et al. (2014) [63].

Figure 6. Schematic overview of the steps and processes involved from ECG recording to heart rate variability analyses.

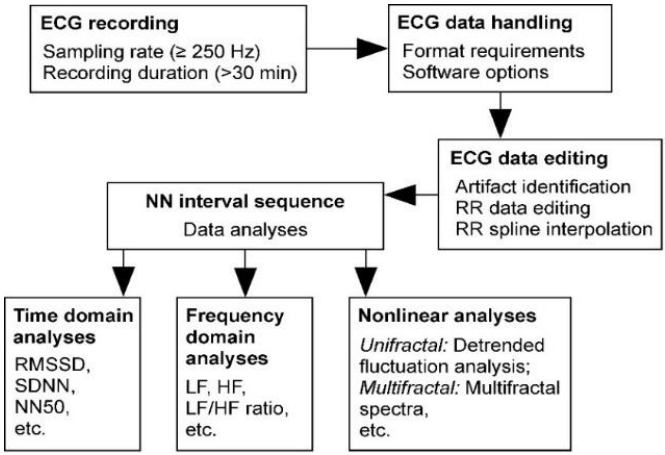


Figure Legend
The abbreviations of the different measures are provided in Table 2. Adopted from Agorastos et al. (2014) [63].

One general benefit is that the DFA scaling coefficient is resistant to HR changes due to altered physical activity [80], as tonic parasympathetic activity underlies long-range correlation of heartbeat interval fluctuations in the healthy state [81], and is therefore considered a sensitive readout to assess functional alterations in disorders indicative of reduced vagal tone. Another benefit of nonlinear analyses is that the obtained scale-invariant measures provide functional information about the dynamical properties of heartbeat interval fluctuations that afford a full translational aspect between animal models and man irrespective of species-specific HR difference [82]. In addition, it has been repeatedly shown that nonlinear methods are highly sensitive predictors of cardiac dysfunction attributed to autonomic dysregulation in the absence of cardiac disease, suggesting a primary role of central nervous system dysfunction for elevated cardiac risk in affective disorders [77, 83]. Nevertheless, to date, this method is still applied only in a relatively limited number of publications due to its complexity [84]. Nonlinear

methods require much longer ECG/HR recordings, while ECG/HR signals with transient data loss need to be interpolated when only few beats are missing and heavily confounded ECG by artifacts needs manual editing for nonlinear analysis, if usable at all. Thus, nonlinear analysis depends on high-quality long-term ECG/HR data.

Stress challenges for objective assessment of autonomic reactivity

Autonomic reactivity is moderated by a large number of factors. In particular, besides biological (genetic, functional) vulnerability, additional individual factors (e.g., context sensitivity to stressors, memory cues, coping mechanisms) modulate the complex CAN activity and are related to psychological, behavioral, and health processes [85]. For example, individuals differ substantially in the extent to which they ascribe threat-related and psychological meaning to events, contexts, and other stimuli, but also in how and to what extend they construe their available coping resources. Such individual differences arise from psychological appraisal processes that are instantiated in forebrain neural circuits that also involve or influence central CAN brain regions (i.e., amygdala) and can, thus, moderate peripheral physiology and reactivity [86]. These circuits can generate anticipatory visceromotor commands ('visceral predictions') to alter parameters of cardiovascular physiology that prepare individuals to behaviorally cope with appraised threats (psychological stressors) [87].

Accordingly, in order to objectively assess individual (biological) autonomic reactivity to stress without cognitive or memory influences, forebrain circuits should be bypassed. Thus, psychological/mental stress paradigms (e.g., Trier Social Stress Test) are not appropriate, but instead experimental endocrine or neurochemical stress provocation challenges could be a proper alternative. Objective stress challenges using endocrine or pharmacological stress system activation have already considerably expanded our knowledge of the pathophysiology and psychopharmacology of anxiety and stress-related disorders. The combination of such objective stress challenges with neurophysiological

autonomic assessment methods (e.g., HRV), could offer an objective readout of central autonomic reactivity to stress activation and help us identify biological factors in the CNS that may modulate the autonomic reactivity.

Pharmacologic stress challenge

In the following studies (Chapter 2-4), we have used intravenous administration of cholecystokinin tetrapeptide (CCK-4) for pharmacological stress provocation in healthy humans. Intravenous administration of CCK-4 reliably reproduces consistent, dose-dependent, short-lasting anxiety paroxysms via CCK B receptors in the CNS and constitutes a well-established model to investigate autonomic and neuroendocrine panic reactions in healthy volunteers [88-93].

Endocrine challenges

Overnight Metyrapone Stimulation Test (MST)

Matyrapone blocks the enzymatic conversion of 11-deoxycortisol to cortisol and leads to a rapid cortisol fall and decreased cortisol-mediated negative feedback at hypothalamic and pituitary levels, increasing CRH and ACTH secretion. The MST test is considered to be a simple and sensitive alternative test to evaluate the ACTH reserve and it is useful to evaluate the response of the HPA axis [94, 95]. Subjects receive 1 g of Metyrapone orally at 11.00 p.m., in order to assess neuroendocrine function the next day.

Overnight Low-Dose Dexamethasone Suppression Test (DST)

Dexamethason is a synthetic glucocorticoid, that imitates the effects of cortisol on the HPA axis. The low-dose DST is one of the most commonly used tests to assess HPA axis reactivity by measuring the change in peripheral cortisol levels in response to externally administered dexamethason [96]. Subjects receive 1 mg of dexamethason orally at 11.00 p.m., in order to assess neuroendocrine function the next day.

AIM AND OUTLINE OF THE DISSERTATION

Aim of the dissertation

The overall aim of this dissertation is to explore biological factors that objectively modulate central autonomic reactivity to stress in humans using stress provocation challenges. As stress reactivity is often influenced by subjective/cognitive factors, the following studies employed only objective stress challenges using endocrine and pharmacological stress provocation. Heart rate variability analyses were applied as a readout of central autonomic activity. In order to increase the translational comparability of the findings, both linear and non-linear heart rate variability measures were included, when possible.

Outline of the dissertation

In the first three studies, the cholecystokinin tetrapeptide (CCK-4) paradigm was utilized as a pharmacological stress/panic challenge to investigate autonomic reactivity in healthy humans. The two first studies investigated the influence of serotonergic signaling on autonomic reactivity.

In **Chapter 2**, using the pharmacological CCK-4 challenge, we investigated the modulation of autonomic reactivity by the 5-HTT-linked polymorphic region (5-HTTLPR) genotype in a group of 30 healthy young men, 15 of each with the long/long (l/l) or short/short (s/s) genotype for the 5-HTTLPR.

In **Chapter 3**, using the same stress challenge, we assessed the effects of long-term selective serotonin reuptake inhibitor (SSRI) application on autonomic activity in 30 healthy young men in a double-blind, placebo (PLA)-controlled, randomized, within-subject cross-over design.

Chapter 4 presents the results of a study assessing the effect of group II metabotropic glutamate receptors ($mGluR_{2/3}$) on autonomic reactivity, using the CCK-4 stress provocation challenge in a double-blind, randomized placebo-controlled, cross-over study with the $mGluR_{2/3}$ agonist LY544344 in healthy humans.

In the two final studies, we utilized overnight pharmacoendocrine HPA axis challenges with dexamethasone (suppression) and metyrapone (stimulation) on two

consecutive days to investigate the influence of the HPA axis on autonomic activity the following day using linear and non-linear HRV measures.

In **Chapter 5**, we assessed the direct effects of HPA axis suppression and stimulation on ANS activity at rest in 39 young healthy individuals.

In **Chapter 6**, using also an endocrine challenge paradigm, we investigated the influence of prior depression history on the modulation of resting autonomic activity by HPA axis stimulation in a group of 14 physically healthy, antidepressant-free patients with a current episode of clinical, non-psychotic major depression.

In **Chapter 7**, the main results of the work presented in this thesis are integrated and discussed. In addition, clinical implications and future perspectives are addressed.

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Chapter 2

The 5-HTTLPR genotype modulates heart rate variability and its adjustment by pharmacological panic challenge in healthy men.

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Abstract

Background: Abnormal serotonin transporter (5-HTT) function and autonomic nervous system (ANS) dysregulation has been proposed in panic disorder. However, in contrast to hypothalamo-pituitary-adrenocortical (HPA) functioning, ANS reactivity during panic response has yet not been investigated in humans with respect to the 5-HTT genotype. The present study assessed the influence of challenging by cholecystokinin tetrapeptide (CCK-4) on heart rate variability (HRV) measures, to monitor autonomic reactivity and its relationship to 5-HTT-linked polymorphic region (5-HTTLPR) genotypes. We hypothesized substantial effects of the 5-HTTLPR genotype on autonomic reactivity.

Methods: We studied 30 healthy young men, 15 of each with the long/long (l/l) or short/short (s/s) genotype for the 5-HTTLPR. All participants received an intravenous application of 50 µg CCK-4. HRV measures were assessed in both groups at baseline and immediately after CCK-4 application.

Results: Our results indicated lower parasympathetic activity in s/s carriers during baseline, time and frequency domain measures. CCK-4 application significantly enhanced the sympathetic tone in both groups, leading to diminished group differences. A significant treatment by genotype effect indicated reduced autonomic reactivity to CCK-4 challenge in the s/s compared to l/l carriers. Our findings show enhanced sympathetic and/or diminished cardiac vagal activity under basal conditions and blunted autonomic reactivity in s/s versus l/l carriers.

Conclusions: Our study provides novel data supporting claims that the s/s genotype represents a genetic vulnerability factor associated with inadequate hyporeactivity to stress and extends current knowledge on the impact of the central serotonergic activity on the sympathoadrenal pathway.

Introduction

The autonomic nervous system (ANS) serves self-regulation and adaptability of the organism and consists of two branches, the sympathetic (SNS) and the parasympathetic (PNS) system, normally being in an optimal dynamic balance state (Peng et al. 1994; Thayer and Lane 2000). Autonomic imbalance is associated with decreased dynamic flexibility, increased vulnerability to pathologies and consequently compromised health (Thayer and Sternberg 2006). One of the best established methods to assess ANS activity is the analysis of heart rate variability (HRV) (Camm et al. 1996). Higher HRV implicates parasympathetic dominance of intrinsic cardiac pacemakers, while low HRV suggests enhanced sympathetic and/or attenuated parasympathetic cardiac modulation, is associated with higher overall mortality and considered a valid marker of heart disease (Thayer and Sternberg 2006; Thayer et al. 2012). Mental health research has therefore used HRV as a marker of physical and emotional responding to investigate physiological changes in psychiatric disorders, including depression and anxiety disorders (Gorman and Sloan 2000). Most studies suggest an association between psychopathology and persistent ANS imbalance with sympathetic hyperactivation (Thayer and Sternberg 2006; Kemp et al. 2010).

Elevated autonomic responsiveness to stressors is also associated with reduced serotonergic activity of the central nervous system (CNS) (Audero et al. 2008; Hildreth et al. 2008). Serotonin (5-hydroxytryptamine, 5-HT) is involved in the modulation of cognitive and emotional behavior (Ogren et al. 2008). Its activity is partly regulated by the serotonin transporter (5-HTT) that lowers serotonin action through its uptake from the synaptic cleft. The 44-base pair insertion/deletion length polymorphism in the 5' regulatory region of the human 5-HTT gene is here of specific interest (Lesch and Gutknecht 2005). The common variation in the 5-HTT gene linked polymorphic region (5-HTTLPR) consists of two alleles, labeled “s” (short) and “l” (long), and the short variant is associated with reduced transcriptional efficiency of the 5-HTT gene (Heils et al. 1996).

The 5-HTTLPR is strongly related to engagement of neuronal systems subserving fear and anxiety, and has been repeatedly suggested as an important modulator of the individual susceptibility to stressful life events (Wankerl et al. 2010; Karg et al. 2011), through alterations of neuroendocrine and autonomic stress responsiveness (Gotlib et al. 2008; Caspi et al. 2010; Mueller, A. et al. 2012; Miller et al. 2013). Specifically, short allele carriers have been associated with an increased stress sensitivity (Gotlib et al. 2008; Wankerl et al. 2010; Miller et al. 2013) and amygdala and cortisol reactivity (Heinz et al. 2007; Miller et al. 2013), risk of major depressive disorder (MDD) (Caspi et al. 2003; Jacobs et al. 2006; Clarke et al. 2010; Karg et al. 2011), anxiety and anxiety-related personality traits like neuroticism (Lesch et al. 1996; Osher et al. 2000) and PTSD (Xie et al. 2009). However, to date, only relatively few studies have investigated the association of 5-HTTLPR genotype to autonomic responses to stressors (Wu et al. 2010). Among them, most studies assessed only heart rate (HR) and blood pressure, while results on the impact of the long or short allele on cardiovascular reactivity were generally inconsistent, suggesting a potential mediation by sex or other confounders. In addition, all prior studies have exclusively used a psychological stress model to investigate stress responsiveness.

Thus, the main objective of the present study is to compare several autonomic measures of HR dynamics in response to a panic challenge with cholecystokinin tetrapeptide (CCK-4) in a homogenous group of young healthy men. Intravenous administration of CCK-4 reliably reproduces consistent, dose-dependent, short-lasting anxiety paroxysms via CCK B receptors in the CNS resembling panic attacks. CCK-4 activates both pituitary-adrenocortical hormone release and sympathetic activity and constitutes a well-established model to investigate autonomic and neuroendocrine panic reactions in patients with panic disorder, but also in healthy volunteers (Bradwejn et al. 1991; Wiedemann et al. 2001; Kellner et al. 2002; Eser et al. 2007; Kellner 2011; Demiralay et al. 2012). In order to determine differences resulting from altered neuroautonomic control depending on the 5-HTT genotype, we compared the HRV effects of CCK-4 on young

healthy men, 15 each with the homozygous short or long variation allele genotype of the 5-HTTLPR. We hypothesized baseline differences between s/s and l/l carriers, reduced HRV after CCK-4 administration and an influence of the 5-HTTLPR genotype on the autonomic response.

Methods and Materials

Subjects

We collected data from 30 healthy young Caucasian male study volunteers (all 15 eligible subjects with the s/s genotype of the 5-HTTLPR and 15 randomly chosen eligible l/l genotype subjects from the screening sample), who participated in an experimental anxiety provocation study approved by the Ethics Committee of the Hamburg Medical Board. Screening procedure and study protocol have been described in detail elsewhere (Kellner et al. 2009). This study analyzed only data of participants receiving placebo pretreatment. Exclusion criteria were: presence or history of any physical and Axes I and II mental co-morbidities, history of sporadic panic attacks, family history of Axis I mental disorders, frequent usage of any either illicit or prescribed drugs or over the counter medications, current use of any medication, consumption of more four cigarettes per day, drinking of more than four cups of coffee a day and more than 100 g of alcohol per week, current adverse life events, night shifts or transcontinental flights across more than four time zones during the past four weeks, abnormal physical and neurological examinations, basic blood laboratory tests deviating from normal range, positive urine toxicology screen, and pathological chest x-ray or initial electrocardiogram (ECG). After full oral and written explanation of the purpose and procedures of the investigation, written informed consent was obtained from each subject.

Procedures

Venous blood samples were obtained for DNA extraction and determination of 5-HTTLPR genotype as described before (Maron et al. 2004; Kellner et al. 2009). After standardized lunch at 12:00 am, subjects were studied from 1:00 pm to 5:00 pm in

a supine position in a soundproof experimental room. CCK-4 (Clinalfa, Läufelfingen, Switzerland) was stored at –80°C and freshly prepared for each injection. A total of 50 µg of CCK-4 were dissolved in 10 ml sterile saline. At 3:00 pm, subjects received 50 µg CCK-4 as intravenous bolus injection within 20 s. Subjects were closely monitored after the injection of CCK-4. The Acute Panic Inventory (API) (Dillon et al. 1987) was administered before CCK-4 injection at 2:45 pm and again at 3:05 pm to determine post-hoc the peak level of provoked panic and anxiety. The rater was unaware of the genotype of the subjects. ECG recordings were obtained throughout using a 5-lead holter recording system (Schillller medilog® AR12, Schiller Medizintechnik GmbH, Ottobrunn, Germany). Data were recorded at 4096 Hz sampling rate in 16-bit resolution and stored digitally on the recorder. ECG recording was performed by specially trained study staff. ECG analyses were performed using specific software (Schiller medilog® DARWIN, Schiller Medizintechnik GmbH, Ottobrunn, Germany).

ECG analysis

Data from two 5-min segments (baseline and at CCK-4 application) were used to determine differences between groups. Recording of the CCK-4 time segment was initiated 45-60 s after the bolus injection of CCK-4 at 3:01 p.m., while baseline recording started 60 min before CCK-4 injection at 14:00. Instantaneous HR was calculated on the basis of the RR interval. HRV in the time domain was calculated by taking the standard deviation of the N-N intervals (SDNN), by calculating the root mean square of subsequent differences (RMSSD) and, by the percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms (NN50%). HRV in the frequency domain was calculated by analysis of two frequency components [low frequency (LF) 0.04-0.15 Hz and high frequency (HF) 0.15-0.4 Hz]. Results are presented as percentage (%) of each frequency component from the total power and a LF/HF ratio.

Statistical analyses

Preliminary analyses were performed to ensure no violation of the assumptions of normality, linearity and homoscedasticity. Differences between the two groups were analyzed by Mann Whitney *U*-tests for non-parametric and ANOVA for parametric variables. To test for correlations, a two-tailed Pearson correlation coefficient *r* was calculated. Additionally, repeated-measures ANOVA and mixed between-within subjects ANOVA were used to assess the impact of CCK-4 application on HR dynamics and the differences within groups. An error probability of $p < 0.05$ was accepted as statistically significant. Effect size is reported as eta squared ($\eta^2 = 0.01$: small effect size, $\eta^2 = 0.06$: medium effect size, $\eta^2 = 0.14$: large effect size). To correct for potentially inflated type I error because of multiple comparisons we used the false discovery rate (FDR) approach (Benjamini and Hochberg 1995), as in our previous study (Agorastos et al. 2013). Following a previously reported procedure (Verhoeven et al. 2005) *p*-values were corrected by the minimum positive FDR with a threshold set at 5%. Statistical analyses were conducted using the Statistical Package for Social Sciences Version 20 (SPSS, Chicago, IL).

Results

All 30 volunteers completed the study. Enrolled subjects had a median age of 27 years (range 19 – 36) and BMI of 21.9 kg/m^2 (range 18.0 – 27.1). Age and BMI did not differ significantly between genotype groups (age: $Z = -1.805$, $p = .074$; BMI: $Z = -.457$, $p = .653$).

Acute panic inventory ratings

Median API scores at baseline were 1 (range 0 – 5) and differed significantly from scores after CCK-4 application of 13.5 (range 6 – 29; $p < .001$). Baseline and post CCK-4 API scores also did not differ significantly between genotype groups (pre: $Z = 1.920$, $p = .055$; post: $Z = 1.015$, $p = .310$). The median change in API scores (baseline to

CCK-4) was 13 (range 4 – 28), and also did not differ between genotype groups (pre: $Z = .104$, $p = .919$).

Baseline heart rate measures

A group comparison revealed several differences with moderate to large effect sizes between the two genotypes (*cf*. Table 1). Subjects with the s/s genotype showed overall lower values for RMSSD, NN50(%), HF(%), while a higher LF/HF ratio emerged. There were no statistically relevant differences with respect to the other investigated parameters.

Heart rate measures after CCK-4 application

In the 5-min interval after CCK-4 application, no differences in any heart rate measure between the two groups could be detected (*cf*. Table 1).

Differences between baseline and post CCK-4 heart rate measures

When comparing baseline and CCK-4 data in the total population, repeated-measures ANOVA indicated significant to highly significant differences with very large effect sizes in all assessed measures. CCK-4 application led to a significant increase in HR ($F_{1, 29} = 40.056$; $p < 0.001$; ${}_p\eta^2 = .589$), SDNN ($F_{1, 29} = 97.792$; $p < 0.001$; ${}_p\eta^2 = .777$) and LF/HF ratio ($F_{1, 29} = 12.636$; $p = 0.001$; ${}_p\eta^2 = .311$) and a significant decrease in RMSSD ($F_{1, 29} = 9.753$; $p = 0.004$; ${}_p\eta^2 = .258$), NN50(%) ($F_{1, 29} = 13.521$; $p = 0.001$; ${}_p\eta^2 = .325$), LF(%) ($F_{1, 29} = 36.888$; $p < 0.001$; ${}_p\eta^2 = .568$) and HF(%) ($F_{1, 29} = 66.784$; $p < 0.001$; ${}_p\eta^2 = .705$).

When investigating differences in the two groups separately, only subjects with an l/l genotype had a significantly higher RMSSD score, NN50(%) and LF/HF ratio (*cf*. Table 1), while in all other measures both groups showed highly significant differences between baseline and CCK-4.

A mixed between-within subject ANOVA was conducted to assess the impact of 5-HTTLPR genotype (s/s vs. l/l) on dependent variables across the two time periods (baseline, CCK-4 application). The results of the mixed ANOVA showed

substantial effects for the group x treatment interaction with respect to HR, RMSSD, NN50(%) and HF(%). The l/l group showed an higher increase in HR and a higher decrease in RMSSD, NN50(%) and HF(%) than the s/s group (*cf*. Table 2).

Table 1. Heart rate measures across different analysis domains and differences between baseline and CCK-4 provocation according to 5-HTTLPR genotype.

		Baseline				CCK-4				Baseline vs. CCK-4	
Domain	**Measure**	**s/s**	**l/l**	***p***	***η²***	**s/s**	**l/l**	***p***	***η²***	**s/s (*p*)**	**l/l (*p*)**
Time	HR (bpm)	67.27 ± 8.95	66.44 ± 10.86	.821	.002	74.54 ± 8.34	80.36 ± 16.44	.232	.051	**.001***	**<.001****
	SDNN (ms)	70.45 ± 30.12	78.81 ± 28.98	.445	.021	137.61 ± 37.78	142.03 ± 54.53	.798	.002	**<.001****	**<.001****
	RMSSD (ms)	39.65 ± 17.16	58.39 ± 33.64	**.068#**	**.116**	38.15 ± 11.41	42.77 ± 23.14	.496	.017	.597	**.005***
	NN50(%)	18.79 ± 14.78	32.94 ± 25.88	**.079#**	**.108**	15.75 ± 8.93	18.88 ± 15.49	.502	.016	.248	**.003***
Frequency											
	LF (%)	.31 ± .12	.31 ± .11	.903	.000	.14 ± .12	.15 ± .08	.826	.003	**.002***	**<.001****
	HF (%)	.11 ± .05	.17 ± .08	**.025***	**.167**	.04 ± .03	.05 ± .03	.720	.004	**<.001****	**<.001****
	LF/HF_{log}	.47 ± .18	.30 ± .31	**.066#**	**.118**	.55 ± .23	.51 ± .24	.600	.010	.183	**.003***

Table Legend

Values are means ± standard deviation. SDNN: standard deviation of the N-N intervals; RMSSD: root mean square of subsequent differences; NN50%: percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms; LF: low frequency 0.04-0.15 Hz; HF: high frequency0.15-0.4 Hz; LF(%) and HF(%):.percentage of each frequency component from the total power. *P*-values denoting statistically significant differences or a trend are shown in bold. FDR analysis revealed no potential Type I errors.

***p* < 0.00.1; **p* < 0.05; #trend (0.1 > *p* > 0.05); +moderate effect size; ++large effect size.

Correlation analyses

Baseline and post CCK-4 API scores, as well as the change in API scores (baseline to CCK-4) did not correlate with any of the heart dynamic measures, neither to their change after CCK-4 application (data not shown). Similarly, there were no statistically significant correlations of any heart dynamic measures with both age and BMI (data not shown).

Table 2. Changes in heart rate measures after CCK-4 application in the two tested 5-HTTLPR genotypes.

		Change (Δ) in time		Mixed ANOVA Group x Treatment	
Domain	**Measure**	**s/s**	**l/l**	***p***	$_{p}\eta^2$
Time	HR (bpm)	7.27 ± 6.74	13.92 ± 11.08	**.057#**	**.123+**
	SDNN (ms)	67.15 ± 40.91	63.22 ± 30.55	.768	.003
	RMSSD (ms)	-1.49 ± 10.69	-15.62 ± 18.33	**.015***	**.192++**
	NN50 (%)	-3.05 ± 9.78	-14.06 ± 15.14	**.026***	**.167++**
Frequency	LF (%)	-.17 ± .17	-.16 ± .12	.952	.000
	HF (%)	-.06 ± .05	-.12 ± .07	**.019***	**.180++**
	LF/HF_{log}	.08 ± .22	.21 ± .23	.120	.084+

Table Legend
Values are means ± standard deviation. HR: heart rate; SDNN: standard deviation of the N-N intervals; RMSSD: root mean square of subsequent differences; NN50%: percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms; LF: low frequency 0.04-0.15 Hz; HF: high frequency0.15-0.4 Hz; LF(%) and HF(%): percentage of each frequency component from the total power. *P*-values denoting statistically significant differences or a trend are shown in bold. FDR analysis revealed no potential Type I errors.
***p* < 0.00.1; **p* < 0.05; #trend (0.1 > *p* > 0.05); +moderate effect size; ++large effect size.

Discussion

This study assessed the autonomic response to a panic challenge with CCK-4 in healthy men to determine the effects of CCK-4 on autonomic measures of HRV and to investigate potential differences between serotonin transporter genotypes, as potential indicators of altered neuroautonomic control. To the best of our knowledge, our study is the first one analyzing autonomic responses by HRV after panic challenges in association to 5-HTTLPR genotypes in healthy young men.

The main findings of this study include (1) enhanced sympathetic and/or reduced vagal baseline activity in the s/s group, (2) distinctive impact of CCK-4 on HRV leading to an enhanced sympathetic and/or attenuated cardiac vagal modulation, (3) blunted autonomic reactivity to CCK-4 challenge in the s/s group compared to l/l, and (4) diminished differences between the two genotype groups after CCK-4 application. Jointly, these findings indicate enhanced sympathetic and/or diminished cardiac vagal activity and blunted autonomic reactivity in subjects with the s/s in comparison to the l/l genotype for the 5-HTTLPR.

The HRV variables used in this analysis have been selected according to the guidelines for short-term recordings of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (Camm et al. 1996), which are also supported by new studies (Meyer and Stiedl 2003; Jarrin et al. 2012). We used total power percentage (%) of each frequency component because the expression of the spectral components in absolute units may prevent the appreciation of the fractional distribution of the energy (Camm et al. 1996). The representation of LF and HF in percentage of total power or in normalized units tends to minimize the effect of the changes in total power on the LF and HF component. Repeating our analyses by using the relative normalized units of LF and HF after subtracting the VLF component (LFN, HFN) instead of the percentage of each frequency component from the total power did not affect our results.

Clinical and experimental studies evaluate congruently efferent vagal activity as the major contributor to the HF component, which however may be also affected by sympathetic nerve activation by perhaps as much as 10% (Akselrod et al. 1981; Pomeranz et al. 1985; Camm et al. 1996; Billman 2011; 2013). The interpretation of the LF component is more controversial. Earlier studies considered the LF component as a marker of sympathetic modulation, while it recently has been postulated that LF rather reflects a complex mix of sympathetic, vagal, and other unidentified factors, with vagal factors accounting for the largest portion of the variability in this frequency range (Billman 2013; Reyes del Paso et al. 2013).

Consequently, the hypothesis that the LF/HF ratio can accurately quantify sympatho-vagal balance is questioned (Stauss and Persson 2006; Billman 2013; Reyes del Paso et al. 2013). Nevertheless, higher HR, LF(%) and LF/HF ratio together with lower RMSSD, NN50% and HF(%) can be jointly accurately interpreted as enhanced sympathetic cardiac modulation and/or attenuated cardiac vagal modulation, as in our study (Lombardi and Stein 2011). The physiological significance of the ULF and VLF components are still by far not well understood (Camm et al. 1996), which is the reason for not including them in our study.

The CCK-4 paradigm constitutes a well-established model to investigate pathophysiological autonomic and neuroendocrine panic reactions (Bradwejn et al. 1991; Eser et al. 2007). CCK-4 elicits a marked anxiogenic response, reflected by robust increases in anxiety ratings (Jerabek et al. 1999; Eser et al. 2007), as also confirmed by our study. Prior studies indicate bilateral increases in extracerebral blood flow in the anterior cingulate cortex (ACC), the claustrum-insular-amygdala region, and the cerebellar vermis (Benkelfat et al. 1995). Moreover, increase of glutamate concentration in the bilateral ACC (Zwanzger et al. 2013), as well as increased CCK-4-mediated ACTH/cortisol plasma levels were observed (Eser et al. 2007; Zwanzger et al. 2013). Cardiovascular effects of CCK-4 application have been repeatedly investigated in previous studies, indicating a moderate but robust increase of heart rate and systolic blood pressure (Benkelfat et al. 1995; Jerabek et al. 1999; Wiedemann et al. 2001; Eser et al. 2007; Eser et al. 2011; Zwanzger et al. 2013). However, to the best of our knowledge, only one earlier study from our group reported sympathetic activation by CCK-4 using heart rate variability (Wiedemann et al. 2001). Thus, our findings of enhanced sympathetic and/or attenuated vagal cardiac modulation of HRV by CCK-4 replicate and extend our previous findings.

The present study investigated baseline autonomic measures as a function of the 5-HTTLPR genotype. Since serotonin pathways influence brain areas involved in vagal cardiovascular regulation and thereby modulate sympathetic efferent activity (Youn et al. 2013), genetic variation affecting serotonergic transmission may also partly account for heritable individual differences in behaviorally-induced

cardiovascular reactivity (Ramage 2001; Jordan 2005; Youn et al. 2013). Human and animal studies have provided evidence for a potential influence of 5-HTTLPR polymorphisms on the brain-heart interaction (Kauppila et al. 2013; Mueller, E. M. et al. 2013). However, to date, no study has investigated the association of 5-HTTLPR with ANS function using HRV in healthy individuals. Our study, thus, provides first evidence in healthy men for enhanced sympathetic and/or reduced vagal baseline activity in homozygous short allele carriers in comparison with homozygous long allele carriers. This finding is in accordance to the study by Ellis et al. (2011), reporting reduced resting respiratory sinus arrhythmia as indirect measure of ANS activity in s/s allele carriers.

Furthermore, our study included the investigation of ANS changes by a panic challenge with respect to the 5-HTTLPR genotype. Short allele genotypes are associated with reduced heart rate reactivity to emotional stressors (Williams et al. 2008; Brummett et al. 2011), although also contradictory results or no differences between genotypes have been reported, partly suggesting a potential mediation by sex or other confounders (McCaffery et al. 2003; Mitro et al. 2008; Murakami et al. 2009; Way and Taylor 2011). However, most studies assessed only heart rate and/or blood pressure as autonomic measures, and all prior studies investigated exclusively the effects of psychological stressors. Our findings replicate prior findings of reduced cardiac reactivity in short allele carriers and extend recent literature through the assessment of autonomous reactivity using HRV measures and the use of an experimental panic challenge.

The initial reactivity hypothesis proposed enhanced cardiovascular autonomic reactivity to be associated with increased cardiovascular risk as a mediator of psychosocial and behavioral risk factors (Phillips 2011; Phillips and Hughes 2011). Following this hypothesis, our data suggests that the enhanced autonomic reactivity to CCK-4 challenge observed in the l/l group may represent a marker of increased cardiovascular risk, as similarly postulated in prior studies (Brummett et al. 2011). Support for this hypothesis comes from studies reporting an association between the long allele and higher risk for cardiovascular diseases

(Arinami et al. 1999; Fumeron et al. 2002; Coto et al. 2003; Ni and Watts 2006), but also other somatic illnesses (Bozzini et al. 2009; Zhang et al. 2013). Contrary to the generally driven assumption, the long allele of the 5-HTTLPR has been also associated with psychiatric diseases, especially anxiety disorders (Grabe et al. 2009; Long et al. 2013; Pinheiro et al. 2013; Reinelt et al. 2013; Shinozaki et al. 2013), while there is supporting evidence for beneficial roles of the short allele particularly in cognitive functions as a consequence of pleiotropy (Homberg and Lesch 2011). Accordingly, the enhanced autonomic reactivity in l/l carriers could also be interpreted as hyper-responsiveness, thus indicating risk factor for anxiety and panic.

However, in the last years research provided robust evidence that indeed reduced cardiovascular reactivity and slower recovery are associated with overall cardiovascular risk (Heponiemi et al. 2007; Salomon et al. 2009). Our study supports this hypothesis because the s/s carriers show both enhanced sympathetic and/or reduced vagal baseline activity and blunted autonomic reactivity to CCK-4 challenge. Lower stress-related heart rate reactivity to psychological stressors has been linked to sleep deprivation (Yang et al. 2012), atherosclerosis and history of cardiovascular disease (Weidner et al. 2001; Heponiemi et al. 2007), obesity (Carroll et al. 2008), smoking (Phillips et al. 2009), depression (Carroll et al. 2007; York et al. 2007; Salomon et al. 2009; Phillips 2011; Schwerdtfeger and Rosenkaimer 2011), psychiatric symptom severity (McTeague et al. 2010), childhood adversities or other trauma (McTeague et al. 2010; Lovallo et al. 2012), reduced cortisol reactivity (Ginty et al. 2012), poor cognitive ability (Ginty et al. 2012), higher perceived stress (Ginty and Conklin 2011) and poor general self-reported health (Phillips 2011), possibly indicating a corresponding under-recruitment of brain systems during prolonged mental stress (Ginty et al. 2013). The s/s genotype has also been associated with these states (Caspi et al. 2003; Kremer et al. 2005; Jacobs et al. 2006; Sookoian et al. 2007; Clarke et al. 2010; Deuschle et al. 2010; Iordanidou et al. 2010; Karg et al. 2011; Beaver et al. 2012; Miller et al. 2013). Thus, our findings are in accordance with a prior study also reporting higher cardiovascular reactivity in l/l carriers, but

we disagree with the interpretation of increased cardiovascular risk in the l/l genotype (Brummett et al. 2011) and conclude that the s/s genotype contributes to increased cardiovascular risk through autonomic hyporesponsiveness (Otte et al. 2007).

Although the precise functional mechanisms remain unclear, our study supports an important role of 5-HTTLPR genotype in the modulation of the magnitude of acute cardiovascular responsiveness, which may affect susceptibility to stress-related disorders. Dysregulation of the central autonomic network may affect downstream autonomic core centers, thereby altering peripheral ANS activity and eventually the dynamics of heartbeat interval fluctuations (Thayer and Lane 2009; Stiedl et al. 2010).

Limitations

Some limitations have to be taken into account for the presented results. Our study analyzed data from a small sample group. It is important to note that, across all parameters investigated, no subject had a cardiovascular history and deviating laboratory or physical tests. We particularly accounted for several laboratory markers (e.g. fasting glucose, hemoglobin A1c levels, cholesterol/lipoproteins, pro-inflammatory cytokines acute-phase proteins) and certain lifestyle habits (e.g. drug, alcohol or tobacco intake) that have been shown to be associated with ANS dysregulation due to HRV alterations (Thayer and Sternberg 2006; Dinas et al. 2013). Because of the relatively small sample size, some distinctive group differences represent large effect sizes, but are not statistically significant. However, as significance depends on effect size and sample size, these differences might reflect true and significant differences in a larger-scale study (Fritz et al. 2012).

In addition, our study did not include subjects with the s/l genotype, in order to compare only putatively extreme groups. Since some studies suggest a non-linear gene dose effect of the 5-HTTLPR (Neumeister et al. 2006), inclusion of s/l subjects in further studies with a considerably larger sample size is necessary to correctly explore genotype differences. Regarding functional variants of the long

allele, posterior analyses of our DNA specimens revealed that all long alleles in this study were of the LA subtype (Lesch, personal communication).

In order to avoid putative confounding effects of the menstrual cycle phase on response to CCK-4 (Le Melledo et al. 1999) and to avoid problems associated with contraception, only males were studied. Thus, the effects still need to be determined in women as gender differences cannot be ruled out. Furthermore, as disturbed sleep is also associated with autonomic alterations (Nielsen et al. 2010), it is important to note that we unfortunately did not include sleep quality in our initial assessments and can herewith not exclude sleep quality-related bias. Finally, our study did not assess data on cardiac recovery after the stress challenge, which is also a major indicator of cardiac reactivity (Salomon et al. 2009). In the future we aim at including nonlinear measures of heart rate dynamics to better identify the role of sympathetic and parasympathetic changes that was not possible here because of the interdependence of heartbeat interval fluctuations far beyond the 5-min interval for ECG assessment (Meyer and Stiedl, 2003).

Conclusions

Identification of genetic factors that influence stress reactivity is of major importance for the linkage of psychosocial and environmental stress factors to disease outcome (Wu et al. 2010). By assessing autonomic responses to a defined pharmacological stress challenge in association with variation of the serotonin transporter genotype, the present study indicates enhanced sympathetic and/or diminished cardiac vagal activity and blunted autonomic reactivity in subjects with the 5-HTTLPR s/s genotype. Our study a. confirms prior findings suggesting the short allele of the 5-HTTLPR as genetic vulnerability factor associated with altered reactivity to environmental influences (Kuepper et al. 2012) and b. extends current knowledge on the impact of central serotonergic neurotransmission for autonomic regulation. Future studies are needed to replicate this finding and further explore the role of autonomic stress reactivity as a potential biological mechanism conveying an elevated risk for the development of stress-related disorders in short allele

carriers (Lang and McTeague 2009). Nevertheless, the existence of functional subpopulations of serotonergic neurons acting at numerous sites of the CNS and the evidence for their tight control by stress hormones (Chaouloff 1993; Johnson et al. 2004) suggest a complex interplay of central serotonergic activity with ANS and hypothalamic-pituitary-adrenal axis function towards maintenance of homeodynamics during stressful events and adaptation processes impacting on cardiac stress responsiveness.

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Chapter 3

Blunted autonomic reactivity to pharmacological panic challenge under long-term escitalopram treatment in healthy men.

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Abstract

Background: Central serotonergic pathways influence brain areas involved in vagal cardiovascular regulation and thereby influence sympathetic efferent activity. Selective serotonin reuptake inhibitors (SSRIs) affect multiple serotonergic pathways including central autonomic pathways. However, only few studies assessed SSRI-mediated effects on autonomic reactivity in healthy individuals using heart rate variability (HRV).

Methods: The present study assessed the influence of long-term treatment with escitalopram (ESC) on autonomic reactivity to an intravenous application of 50 µg cholecystokinin tetrapeptide (CCK-4) in 30 healthy young men using a double-blind, placebo (PLA)-controlled, randomized, within-subject cross-over design. Main outcome measures were time and frequency domain HRV parameters assessed at both baseline and immediately after CCK-4 application.

Results: Results showed substantial effects for the treatment × CCK-4 challenge interaction with respect to HR ($p < .001$; ${}_p\eta^2 = .499$), SDNN ($p < .001$; ${}_p\eta^2 = 576$), RMSSD ($p = .015$; ${}_p\eta^2 = 194$), NN50(%) ($p = .008$; ${}_p\eta^2 = .224$), LF(%) ($p = .014$; ${}_p\eta^2 = .196$) and moderate effects with respect to HF(%) ($p = .099$; ${}_p\eta^2 = .094$), with PLA subjects showing a higher increase in HR and SDNN and a higher decrease in RMSSD, NN50(%), LF(%) and HF (%) than in the ESC condition. Thus, ESC treatment significantly blunted the autonomic reactivity to CCK-4 challenge compared to PLA. Secondary analysis indicated no effect of 5-HTTLPR polymorphism on CCK-4-induced autonomic response.

Conclusions: Our results support findings suggesting an effect of SSRI treatment on autonomic regulation and provide evidence that ESC treatment is associated with blunted autonomic reactivity in healthy men.

Introduction

The autonomic nervous system (ANS) regulates a plethora of physiological reactions and serves self-regulation and adaptability of the organism towards meeting the metabolic demands of ongoing physical, emotional and cognitive challenges (Thayer and Lane, 2000; Critchley, 2005). On the other hand, autonomic imbalance is associated with decreased dynamic adaptability of the organism, increased morbidity and mortality (Thayer and Lane, 2000; Thayer and Sternberg, 2006).

Central autonomic control underlies the task- and division-specific influence of the brainstem and other cerebral and cerebellar structures of the central autonomic network (Beissner et al., 2013). The activity and functional connectivity of these brain regions is partly influenced by serotonergic signaling (Strawn et al., 2012; Fisher and Hariri, 2013). Central serotonin (5-hydroxytryptamine, 5-HT) transmission is, thus, not only involved in the modulation of emotional and cognitive behavior, but also in autonomic regulation (Ramage, 2001; Jordan, 2005; Youn et al., 2013). Consequently, altered serotonin regulation in the central nervous system (CNS) is associated with autonomic dysregulation, while reduced CNS serotonergic activity is linked to elevated autonomic responsiveness to stressors (Audero et al., 2008; Hildreth et al., 2008; Cummings et al., 2011).

The serotonergic activity of the CNS can be pharmacologically modulated. Selective serotonin reuptake inhibitors (SSRIs) have emerged as a major therapeutic advance in psychopharmacology and are currently the most frequently prescribed and best characterized therapeutic compounds affecting multiple central projection pathways in the topography of serotonin function (Vaswani et al., 2003; Sghendo and Mifsud, 2012). Binding of these drugs to the presynaptic serotonin transporter leads to its negative allosteric modulation which effectively inhibits its ability to reuptake serotonin from the synaptic cleft (Goodnick and Goldstein, 1998). However, the acute SSRI action is associated with rather modest synaptic serotonin increase due to negative feedback loops through somatodendritic 5-HT_{1A} autoreceptors. However, chronic SSRI administration leads to desensitization of 5-

HT_{1A} autoreceptors and downregulation of the negative feedback inhibition and, thus, results in increased serotonin release at postsynaptic heteroreceptor sites (Sghendo and Mifsud, 2012; Walker, 2013).

Since serotonergic pathways influence brain areas involved in vagal cardiovascular regulation and thereby modulate sympathetic efferent activity (Ramage, 2001; Jordan, 2005; Youn et al., 2013), SSRI administration affecting serotonergic transmission may also modulate autonomic reactivity. For example, there is an FDA safety warning on the SSRI Celexa (citalopram hydrobromide) for causing pathological cardiovascular symptoms at high doses (FDA, 2012; Castro et al., 2013). These symptoms can be mimicked in mice by strong activation of postsynaptic 5-HT_{1A} receptors to which serotonin has a high affinity (Youn et al., 2013). On the other hand, SSRI demonstrate the safest cardiovascular profile of all antidepressants and are considered drugs of first choice in cardiovascular risk patients (Roose and Miyazaki, 2005; Hamer et al., 2011; Chittaranjan et al., 2013; Hare et al., 2014).

Nevertheless, only relatively few studies have assessed the acute and long-term effects of SSRIs on autonomic function. Most studies investigated autonomic effects in either psychiatric patients or patients with cardiovascular disease. In healthy adults, the few studies suggest absent clinically significant effects on baseline autonomic measures through single-dose (Ahrens et al., 2007) short-term (Penttila et al., 2001; Siepmann et al., 2003; Chappell et al., 2013) or long-term treatment with SSRIs (Pohl et al., 2003). Only two studies investigated the effects of acute SSRI treatment on autonomic stress reactivity in healthy adults, reporting beneficial effects of a single dose of escitalopram through attenuated autonomic responses to social and physiological stress tasks in healthy females (Hanson et al., 2013; Kemp et al., 2014). However, to date, no study has assessed the effects of long-term SSRI treatment on ANS reactivity in healthy individuals using autonomic measures in the clinical dose range.

Thus, the main objective of our study was to assess the effects of long-term SSRI treatment on autonomic reactivity to pharmacological panic challenge in

healthy individuals. One of the best-established non-invasive methods to assess parasympathetic activity is the analysis of heart rate variability (HRV) (Camm et al., 1996; Reyes del Paso et al., 2013). HRV results from heart rate (HR) oscillations within its physiological range (beat-to-beat variability), controlled by parasympathetic and sympathetic modulation of intrinsic cardiac pacemakers (Akselrod et al., 1981) and reflects the capacity of the organism for regulated physical and emotional responding. Higher HRV implicates parasympathetic dominance favoring energy conservation, while low HRV suggests attenuated cardiac parasympathetic modulation (Reyes del Paso et al., 2013). Low HRV is associated with higher overall mortality, specifically heart mortality, and is considered a valid marker of heart disease (Thayer and Sternberg, 2006; Thayer et al., 2012). Psychiatric research has repeatedly used HRV to investigate physiological alterations in psychiatric disorders (Gorman and Sloan, 2000; Kemp and Quintana, 2013), suggesting an association between psychopathology and reduced parasympathetic activity (Thayer and Sternberg, 2006; Kemp et al., 2010).

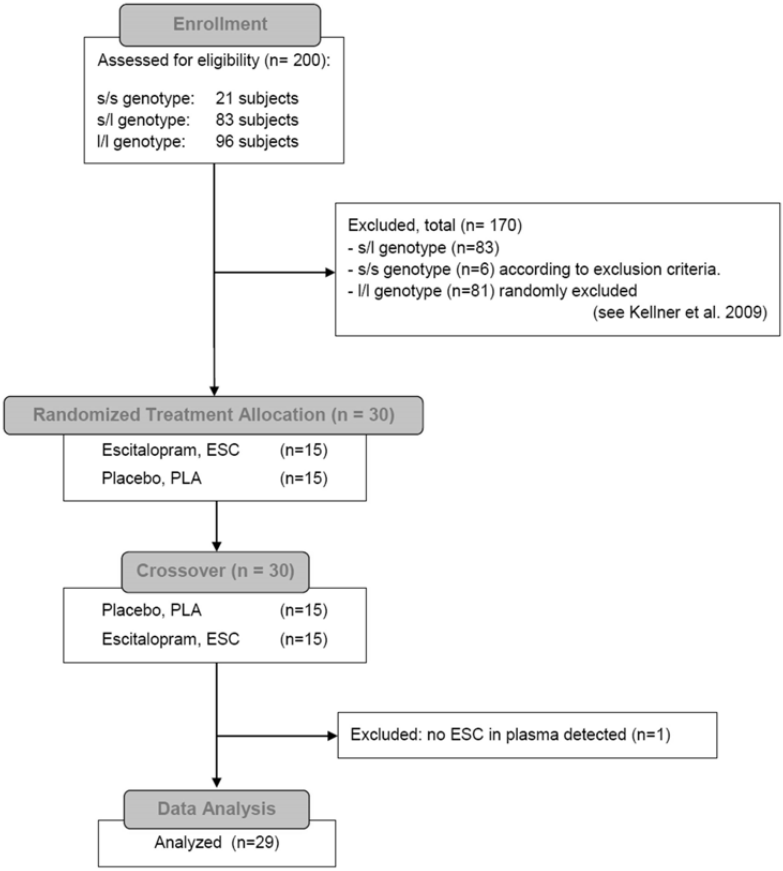
We therefore investigated autonomic reactivity measured by HRV to the pharmacological panic challenge by the cholecystokinin tetrapeptide (CCK-4) in a homogenous group of young healthy men. Intravenous administration of CCK-4 reliably reproduces consistent, dose-dependent, short-lasting anxiety paroxysms via CCK B receptors in the CNS and constitutes a well-established model to investigate autonomic and neuroendocrine panic reactions in healthy volunteers (Bradwejn et al., 1991; Eser et al., 2007; Kellner, 2011). Based on previous findings (Golding et al., 2002; Hanson et al., 2013; Kemp et al., 2014) we hypothesized that chronic SSRI treatment would lower autonomic reactivity elicited by CCK-4. To determine differences potentially resulting from altered neuroautonomic control depending on the serotonin transporter gene-linked polymorphic region (5-HTTLPR) genotype, as previously reported by assessing only the non-medicated subjects of our sample (Agorastos et al., 2014), we additionally analyzed differences between short/short (s/s) and long/long (l/l) carriers of the 5-HTTLPR genotype.

Methods and Materials

Subjects

We collected data from 30 healthy young Caucasian male study volunteers (all 15 eligible subjects with the s/s genotype of the 5-HTTLPR and 15 randomly chosen eligible l/l genotype subjects from the screening sample), who participated in an experimental panic provocation study approved by the Ethics Committee of the Hamburg Medical Board. Screening procedure and study protocol have been described in detail elsewhere (Kellner et al., 2009; Hinkelmann et al., 2010b). Participant selection and attrition across the experiment, e.g., based on specific exclusion criteria, are provided in Figure 1. Exclusion criteria were: presence or history of any physical and Axes I and II mental co-morbidities, history of sporadic panic attacks, family history of Axis I mental disorders, frequent usage of any either illicit or prescribed drugs or over the counter medications, current use of any medication, consumption of more four cigarettes per day, drinking of more than four cups of coffee a day and more than 100 g of alcohol per week, current adverse life events, night shifts or transcontinental flights across more than four time zones during the past four weeks, abnormal physical and neurological examinations, basic blood laboratory test values deviating from the normal range (including thyroid function tests, transaminases, electrolytes, CO_2 anion gap, fasting glucose, basic blood and coagulation tests, blood lipids, hemoglobin A1c, C-reactive protein, creatinine, folic acid, vitamin B12), positive urine toxicology screen, and pathological chest x-ray or initial electrocardiogram (ECG). Current or lifetime psychiatric disorders were excluded using the Structured Clinical Interview for the DSM-IV, axes I and II, assessed by a trained physician. All other exclusion criteria were assessed in a clinical interview setting through study questionnaires. After full oral and written explanation of the purpose and procedures of the investigation, written informed consent was obtained from each subject.

Figure 1. CONSORT Flow Diagram displaying the progress of all participants through the trial.



Procedures

Panic challenges were performed after 42 days of daily treatment with 10 mg escitalopram in a double-blind, placebo-controlled, randomized, within-subject crossover-design with a wash-out phase of at least three weeks in-between pre-treatment periods. To check for compliance of intake of study medication,

escitalopram (ESC) was measured in plasma specimens of the study day after the study had been finished as described in Greiner et al. (2007).

Venous blood samples were obtained for DNA extraction and the 5-HTTLPR genotype was determined as described before (Maron et al., 2004; Kellner et al., 2009). After standardized lunch at 12:00 am, subjects were studied from 1:00 pm to 5:00 pm exclusively in supine position in a soundproof experimental room. CCK-4 (Clinalfa, Läufelfingen, Switzerland) was stored at –80°C and freshly prepared for each injection. A total of 50 µg of CCK-4 were dissolved in 10 ml sterile saline. At 3:00 pm, subjects received 50 µg CCK-4 as intravenous bolus injection within 20 s. Subjects were closely monitored after the injection of CCK-4. ECG recordings were obtained throughout using a 5-lead holter recording system (Schiller medilog® AR12, Schiller Medizintechnik GmbH, Ottobrunn, Germany). Data were recorded at 4096 Hz sampling rate in 16-bit resolution and stored digitally on the recorder. ECG recording was performed by specially trained study staff. ECG analyses were performed using specific software (Schiller medilog® DARWIN, Schiller Medizintechnik GmbH, Ottobrunn, Germany). Adverse side effects were assessed through a German version of the UKU side effects rating scale (Lingjaerde et al., 1987).

ECG analysis

Data from two 5-min segments (baseline and immediately after CCK-4 application) were used to determine differences between treatment conditions. Recording of CCK-4-mediated effects was initiated 45-60 s after the bolus injection at 3:01 p.m., while baseline recording started 60 min before CCK-4 injection at 2:00 p.m. Instantaneous HR was calculated on the basis of the RR interval. HRV in the time domain was calculated by taking the standard deviation of the N-N intervals (SDNN), by calculating the root-mean-square of subsequent interval differences (RMSSD), and by the percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms (NN50%). HRV in the frequency domain was calculated by analysis of two frequency components [low frequency (LF): 0.04-0.15 Hz; high frequency

(HF): 0.15-0.4 Hz]. Results are presented as percentage (%) of each frequency component from the total power and the LF/HF ratio. The assessed HRV variables have been selected according to the guidelines for short-term recordings of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (Camm et al., 1996), which are also supported by new studies (Meyer and Stiedl, 2003; Jarrin et al., 2012). We used the percentage (%) of the total power of each frequency component to compare the fractional energy (Camm et al., 1996) rather than the absolute energy per frequency band.

Statistical analyses

Preliminary analyses were performed to ensure no violation of the assumptions of normality, linearity and homoscedasticity. Parameters with skewed distribution, i.e. the LF/HF ratio, were $\log_{10}$ transformed for further parametric analysis. All data are given in mean values (SEM). CCK-4-induced changes have been calculated as $\Delta_M = M_{CCK\text{-}4} - M_{baseline}$. Differences between treatment conditions were tested for significance by analyses of variance (ANOVAs). Additionally, mixed between-within subjects ANOVAs were used to assess the impact of CCK-4 application (baseline vs. CCK-4), (CCK-4 $\times$ treatment) and then additionally 5-HTTLPR genotype (s/s vs. l/l) (CCK-4 $\times$ treatment $\times$ genotype) on HR dynamics and the differences within groups (PLA vs. ESC). An error probability of $p < 0.05$ was accepted as statistically significant. Effect size is reported as eta squared ($\eta^2 = 0.01$: small effect size, $\eta^2 = 0.06$: medium effect size, $\eta^2 = 0.14$: large effect size). To correct for potentially inflated type I error because of multiple comparisons we used the false discovery rate (FDR) approach (Benjamini and Hochberg, 1995), as in our previous studies (Agorastos et al., 2013; Agorastos et al., 2014). Following a previously reported procedure (Verhoeven et al., 2005) *p*-values were corrected by the minimum positive FDR with a threshold set at 5%. Statistical analyses were conducted using the Statistical Package for Social Sciences Version 20 (SPSS, Chicago, IL).

Results

All 30 volunteers completed the study. Enrolled subjects had a median age of 27 years (range 19 - 36) and BMI of 21.9 kg/m^2 (range 18.0 - 27.1) and there were no differences in these measures between the ESC and PLA conditions (data not shown). In one subject, no ESC could be detected in plasma after 42 days of verum treatment. Therefore, this subject was excluded from further analyses. Mean plasma ESC concentration was 15.5 ng/ml on day 42, mean plasma desmethyl-ESC level was 6.6 ng/ml. No active drug was detected in any subject during placebo (PLA) intake. Side effects as per UKU ratings did not differ significantly between treatments.

CCK-4 challenge x treatment interaction effects

Mixed ANOVAs were conducted to assess the impact of treatment (ESC vs. PLA) on dependent variables across the two time periods (baseline, post-CCK-4). The results showed substantial effects for the treatment $\times$ CCK-4 challenge interaction with respect to HR (Wilks λ = .501; $F_{(1,28)}$ = 27.853; $p < .001$, $_p\eta^2$ = .499), SDNN (Wilks λ = .424; $F_{(1,28)}$ = 38.051; $p < .001$; $_p\eta^2$ = 576), RMSSD (Wilks λ = .806; $F_{(1,28)}$ = 6.723; p = .015, $_p\eta^2$ = 194), NN50(%) (Wilks λ = .776; $F_{(1,28)}$ = 8.066; p = .008, $_p\eta^2$ = .224), LF(%) (Wilks λ = .804; $F_{(1,28)}$ = 6.845; p = .014, $_p\eta^2$ = .196) and moderate, but not statistically significant effects with respect to HF(%) (Wilks λ = .906; $F_{(1,28)}$ = 2.908; p = .099, $_p\eta^2$ = .094). Subjects showed an significantly higher increase in HR and SDNN and a higher decrease in RMSSD, NN50(%) and LF(%) in the PLA than in the ESC condition (Figure 2; Table 1).

Figure 2. Effects of placebo and long-term escitalopram treatment on a range of cardiovascular time domain (A-D) and frequency domain measures (E-G).

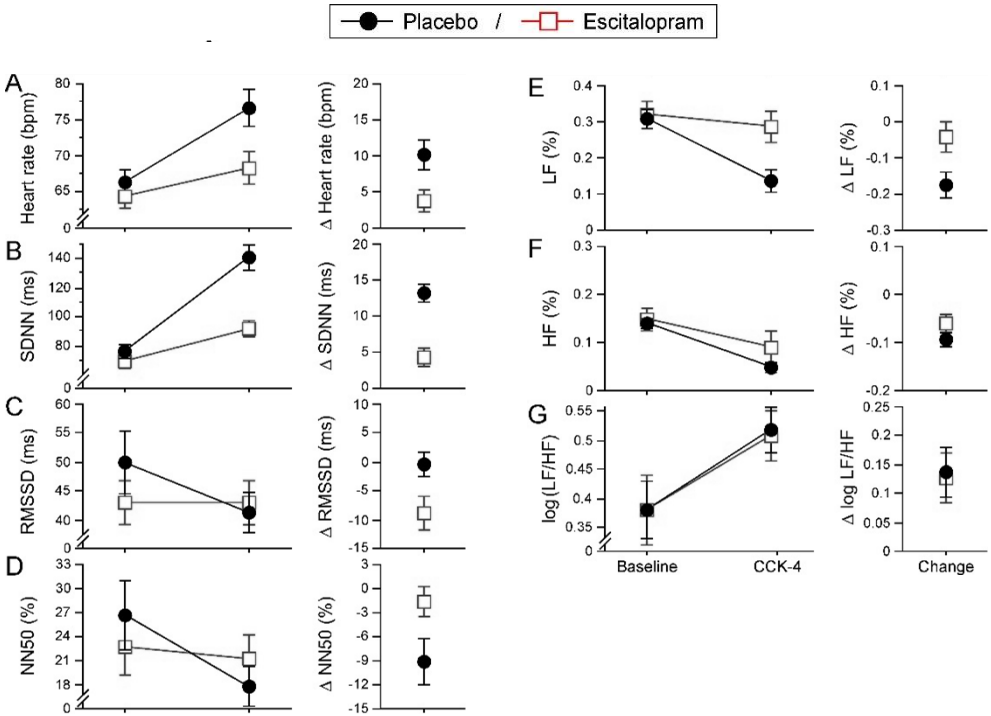


Figure Legend

Values are presented as means ± SEM. Measures were determined at baseline and after pharmacological panic challenge by CCK-4 (left panels). CCK-4-induced changes (right panels) have been calculated as $\Delta_M = M_{post\ CCK\text{-}4} - M_{baseline}$. SDNN: standard deviation of the N-N intervals; RMSSD: root mean square of subsequent differences; NN50%: percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms; LF: low frequency 0.04-0.15 Hz; HF: high frequency 0.15-0.4 Hz; LF (%) and HF (%): percentage of each frequency component from the total power.

Table 1. Effects of CCK-4 challenge and treatment x CCK-4 challenge on heart dynamics.

		rmANOVA		Mixed ANOVA	
		CCK-4 challenge		Treatment x CCK-4 challenge	
Domain	**Measure**	***p***	***$_p\eta^2$***	***p***	***$_p\eta^2$***
Time	HR (bpm)	**<.001**	**.548^{++}**	**<.001**	**.499^{++}**
	SDNN (ms)	**<.001**	**.753^{++}**	**<.001**	**.576^{++}**
	RMSSD (ms)	**<.001**	**.303^{++}**	**.015**	**.194^{++}**
	NN50 (%)	**<.001**	**.349^{++}**	**.008**	**.224^{++}**
Frequency	LF (%)	**<.001**	**.672^{++}**	**.014**	**.196^{++}**
	HF (%)	**<.001**	**.651^{++}**	.099	.094^{+}
	LF/HF_{log}	**<.001**	**.240^{++}**	.888	.001

Table Legend

Repeated-measures ANOVAs and between-within subjects ANOVAs were used to assess the impact of CCK-4 challenge and the differences within treatment groups (Placebo vs. ESC). HR: heart rate; SDNN: standard deviation of the N-N intervals; RMSSD: root mean square of subsequent differences; NN50%: percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms; LF: low frequency 0.04-0.15 Hz; HF: high frequency 0.15-0.4 Hz; LF(%) and HF(%): percentage of each frequency component from the total power. *P*-values denoting statistically significant differences are shown in bold. FDR analysis revealed no potential type I errors.

$^{+}$moderate effect size; $^{++}$large effect size.

Table 2. Differences between treatment conditions in HRV measures.

		PLA vs. ESC				Baseline vs. CCK-4			
		Baseline		CCK-4		PLA		ESC	
Domain	Measure	*p*	${}_p\eta^2$	*p*	${}_p\eta^2$	*p*	${}_p\eta^2$	*p*	${}_p\eta^2$
Time									
	HR (bpm)	.129	.080	**<.001**	**.591**$^{++}$	**<.001**	**.541**$^{++}$	**.007**	**.233**$^{++}$
	SDNN (ms)	.119	.085	**<.001**	**.609**$^{++}$	**<.001**	**.780**$^{++}$	**<.001**	**.438**$^{++}$
	RMSSD (ms)	**.044**§	**.137**$^{+}$	.483	.018	**.007**	**.230**$^{++}$	.886	.001
	NN50 (%)	.072	**.111**$^{+}$	.088	**.100**$^{+}$	**.002**	**.301**$^{++}$	.369	.029
Frequency	LF (%)	.690	.006	**<.001**	**.435**$^{++}$	**<.001**	**.591**$^{++}$	.372	.029
	HF (%)	.557	.012	**<.001**	**.472**$^{++}$	**<.001**	**.670**$^{++}$	**.001**	**.307**$^{++}$
	LF/HF_{log}	.930	.000	.930	.000	**.003**	**.271**$^{++}$	**.001**	**.318**$^{++}$

Table Legend

HR: heart rate; SDNN: standard deviation of the N-N intervals; RMSSD: root mean square of subsequent differences; NN50%: percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms; LF: low frequency 0.04-0.15 Hz; HF: high frequency 0.15-0.4 Hz; LF(%) and HF(%): percentage of each frequency component from the total power. *P*-values denoting statistically significant differences or a trend are shown in bold.

$^{+}$moderate effect size; $^{++}$large effect size; § potential type I error based on FDR analysis.

Main effects of CCK-4 challenge

When investigating the effect of CCK-4 challenge in the total population, repeated-measures ANOVAs indicated significant to highly significant differences between baseline and post-CCK-4 measures with very large effect sizes in all assessed measures leading to higher HR (Wilks $\lambda = .453$; $F_{(1,58)} = 69.081$; $p < .001$; ${}_p\eta^2 = .548$), SDNN (Wilks $\lambda = .247$; $F_{(1,58)} = 173.605$; $p < .001$; ${}_p\eta^2 = .753$) and LF/HF_{log} (Wilks $\lambda = .760$; $F_{(1,58)} = 17.976$; $p < .001$; ${}_p\eta^2 = .240$) and lower RMSSD (Wilks $\lambda = .697$; $F_{(1,58)} =$

24.808; $p < .001$; $_p\eta^2 = .303$), NN50% (Wilks $\lambda = .651$; $F_{(1,58)} = 30.578$; $p < .001$; $_p\eta^2 = .349$), LF(%) (Wilks $\lambda = .328$; $F_{(1,58)} = 116.745$; $p < .001$; $_p\eta^2 = .672$), HF(%) (Wilks $\lambda = .349$; $F_{(1,58)} = 106.489$; $p < .001$; $_p\eta^2 = .651$) values (Figure 2; Table 1).

Treatment differences

- Baseline: A group comparison revealed only differences with moderate effect sizes but no statistical significance between the two groups with respect to baseline HR measures (Figure 2; Table 2).

- CCK-4 challenge: Post-CCK-4-injection HR measures indicated statistically significant differences with large effect sizes between the two treatment conditions (Figure 2; Table 2). Subjects in the ESC condition showed overall significantly lower HR increase (Wilks $\lambda = .409$, $F_{(1,28)} = 40.450$, $p < .001$, $_p\eta^2 = .591$) and SDNN (Wilks $\lambda = .391$, $F_{(1,28)} = 43.642$; $p < .001$, $_p\eta^2 = .609$) and higher NN50% (Wilks $\lambda = .900$, $F_{(1,28)} = 3.119$; $p = .088$, $_p\eta^2 = .100$), LF(%) (Wilks $\lambda = .565$, $F_{(1,28)} = 21.537$; $p < .001$, $_p\eta^2 = .435$) and HF(%) (Wilks $\lambda = .528$, $F_{(1,28)} = 25.026$; $p < .001$, $_p\eta^2 = .472$) values.

Differences depending on the 5-HTTLPR genotype

Since we previously reported significantly lower autonomic reactivity to CCK-4 in s/s versus l/l 5-HTTLPR carriers without treatment (Agorastos et al., 2014) we also conducted an additional analysis to investigate the genotype effects. Mixed models using repeated measures ANOVA were used to assess the impact of the 5-HTTLPR genotype, i.e. s/s vs. l/l, on HRV measures including both treatment conditions (CCK-4 $\times$ treatment $\times$ genotype). There was no statistically significant effect of genotype and no significant interaction between genotype and treatment condition (data not shown). When subjects in the ESC condition were analyzed separately, s/s carriers displayed reduced autonomic reactivity in comparison to the l/l carriers, with a similar trend to our previous findings (Agorastos et al., 2014). However, these differences were not statistically significant.

Discussion

This study assessed the effects of long-term SSRI treatment on acute CCK-4-mediated effects on HRV in healthy man, using the most selective SSRI available (Burke, 2002; Sanchez et al., 2003) as a potential modulator of autonomic control. Although not the first study to examine the impact of SSRI treatment on stress responsiveness, to the best of our knowledge, our study is the first one analyzing autonomic responses following long-term SSRI treatment related to pharmacological panic challenge by CCK-4 based on HR measures in healthy young men.

The main findings of this study include (1) no statistically significant evidence for ESC-associated effects on baseline vagal activity, (2) attenuated cardiac vagal modulation in both treatment conditions by CCK-4, (3) significantly lower vagal tone and increased autonomic reactivity upon CCK-4 challenge in the PLA vs. ESC group, and (4) no 5-HTTLPR genotype effect on HRV measures and their changes after CCK-4 application over both treatment conditions. Jointly, these findings indicate blunted autonomic reactivity to CCK-4 in the ESC treatment condition in comparison to PLA.

CCK-4 application has been repeatedly shown to lead to a robust increase of HR and systolic blood pressure (Benkelfat et al., 1995; Jerabek et al., 1999; Eser et al., 2007). However, so far only two previous studies from our group reported autonomic effects of CCK-4 on HRV (Wiedemann et al., 2001; Agorastos et al., 2014). Thus, our findings of enhanced sympathetic and/or attenuated vagal cardiac modulation of HRV by CCK-4 in both PLA and ESC treatment conditions replicate and extend our previous findings.

The present study investigated resting autonomic measures as a function of central serotonergic activity in both treatment conditions. HRV effects of long-term SSRI administration have been investigated in various categories of patients. In depression, and in contrast to other antidepressant categories (e.g., tricyclic antidepressants, serotonin-norepinephrine reuptake inhibitors) (Siepmann et al., 2007; Kemp et al., 2010; Chang et al., 2012), SSRI treatment has been reported not

to have a significant impact on resting HRV (Rechlin, 1994; Straneva-Meuse et al., 2004; Koschke et al., 2009; Kemp et al., 2010; Kemp et al., 2011; Brunoni et al., 2013; Hanson et al., 2013; Kemp et al., 2014), although contradictory results have also been reported (Licht et al., 2010). In patients with panic disorder or posttraumatic stress disorder, SSRI treatment has been mainly associated with positive effects on HRV measures, i.e. reduced sympathetic activity/normalized ANS activity and baroreflex response, (Tucker et al., 1997; Cohen et al., 2000). SSRI treatment in patients with cardiovascular diseases has been also associated with an improvement of HRV indices independent of the improvement of depressive symptoms, suggesting a clear benefit for the prognosis of the cardiovascular disease (Gorman and Sloan, 2000; Leftheriotis et al., 2010; Mazza et al., 2010; Pizzi et al., 2011). However, only few studies have investigated single-dose or long-term SSRI treatment effects on HRV or other autonomic measures at rest in healthy adults. These studies suggest no clinically significant autonomic SSRI effects in both males and females in resting state (Penttila et al., 2001; Pohl et al., 2003; Siepmann et al., 2003; Ahrens et al., 2007; Chappell et al., 2013; Hanson et al., 2013; Kemp et al., 2014). Thus, our results in males support these previous studies.

Our study also assessed the modulating effect of long-term ESC treatment on autonomic reactivity in response to a pharmacological panic challenge in healthy subjects. To date, effects of SSRI treatment on autonomic reactivity have been only sparsely investigated and predominantly in psychiatric patients using various stressor models. Effects of SSRI treatment on autonomic reactivity have been reported in patients with panic disorder and PTSD, suggesting a positive effect of SSRIs on HRV reactivity to physiological (orthostatic) or psychological challenges, towards normalizing autonomic indices (Tucker et al., 1997; Tucker et al., 2000).

In healthy mixed-gender individuals, Takata et al. (Takata et al., 2002) reported decreased orthostatic baroreflex sensitivity after long-term paroxetine treatment, while Golding et al. (Golding et al., 2002) observed decreased autonomic reactivity to a psychological stressor, which is also supported by our results. However, both studies used only HR and blood pressure measures to assess

autonomic activity. Two recent studies investigated ESC treatment effects on autonomic stress reactivity in healthy females, reporting attenuated autonomic responses in social and physiological stress tasks (Hanson et al., 2013; Kemp et al., 2014). Both studies, however, only investigated effects of a single-dose of ESC. Besides a previous report from our group (Kellner et al., 2009) on the same sample group, only one additional study investigated long-term effects of ESC on CCK-4 challenge in mixed-gender healthy volunteers (Toru et al., 2013). In contrast to results in patients with panic disorder (Shlik et al., 1997; van Megen et al., 1997), no inhibitory effect of ESC was observed upon panic symptoms elicited by CCK-4 in healthy subjects. However, both studies also did not report any ESC effects on autonomic measures. Thus, this study is the first one to report long-term effects of the SSRI ESC on ANS reactivity to a pharmacological panic challenge by CCK-4 in healthy subjects indicating reduced autonomic reactivity following long-term treatment by ESC in comparison to the PLA control in healthy subjects.

The CCK-4-induced increase in HR, together with a decrease in SDNN, RMSSD, NN50%, LF(%) and HF(%), is interpreted as increased sympathetic and/or attenuated parasympathetic (vagal) cardiac modulation consistent with previous claims (Camm et al., 1996; Lombardi and Stein, 2011). The initial reactivity hypothesis proposed enhanced cardiovascular autonomic reactivity to be associated with increased cardiovascular risk as a mediator of psychosocial and behavioral risk factors (Phillips, 2011; Phillips and Hughes, 2011). Following this hypothesis, our data suggest that the reduced autonomic reactivity to CCK-4 challenge observed in the ESC group may represent a marker of reduced cardiovascular risk, similarly as postulated in a prior study (Brummett et al., 2011). This is also supported by the statistically absent differences in baseline HR between the two treatment conditions, as resting HR is considered an independent predictor of cardiovascular risk (Fox et al., 2007). SSRI treatment has shown beneficial cardiovascular effects in cardiovascular disease, which are associated with reduced autonomic reactivity. Similarly, advantageous cardiovascular effects of SSRIs leading to normalization of autonomic measures have been also reported in psychiatric disorders (see above),

which have been associated with increased (Monk et al., 2001; Blechert et al., 2010; Felmingham et al., 2012) or reduced (Kikuchi et al., 2009; McTeague et al., 2010; Shinba, 2013) autonomic reactivity. In healthy females, comparable moderating effects on HR and HRV have been observed by acute SSRI treatment and regular high-intensity exercise to a physical stress challenge (Hanson et al., 2013). On the contrary, robust evidence was provided that reduced cardiovascular reactivity and slower recovery are associated with overall cardiovascular risk (Heponiemi et al., 2007; Salomon et al., 2009), which should be discussed here. Unfortunately, our study did not assess data on cardiac recovery after the stress challenge, which is also a major indicator of cardiac reactivity. Thus, it cannot be unambiguously resolved on the basis of the currently used measures, whether the reduced stress responsiveness reflects a beneficial ('anxiolytic-like') or a maladaptive state ('hypo-responsiveness'). Nonlinear measures (Meyer and Stiedl, 2003) may provide for an unambiguous interpretation of physiological versus pathological change but cannot be applied with short ECG recordings as used here.

The existence of functional subpopulations of serotonergic neurons acting at numerous sites of the CNS and the evidence for their tight control by stress hormones (Chaouloff, 1993; Johnson et al., 2004) suggest a complex interplay of central serotonergic activity with ANS and cardiac stress responsiveness. SSRI treatment may, thus, both influence supra-ordinate mechanisms (e.g., central autonomic network) and/or affect hormone secretion patterns (Shores et al., 2001; Agelink et al., 2004). With respect to cardiovascular disease patients specifically, additional pleiotropic SSRI effects may also be responsible for the beneficial effects reported (Escolar et al., 2005; Paraskevaidis et al., 2006). Our study also investigated the potential contribution of the 5-HTTLPR polymorphism on PLA versus ESC treatment affecting CCK-4-mediated HR responses. Prior studies have reported a moderating role of 5-HTTLPR polymorphisms on the brain-heart interaction (Kauppila et al., 2013; Mueller et al., 2013). However, to date, our prior study assessing 5-HTTLPR genotype effects exclusively in healthy subjects receiving PLA was the first one investigating the association of 5-HTTLPR with ANS function using

HRV in healthy individuals. We reported enhanced sympathetic and/or diminished baseline cardiac vagal activity and blunted autonomic reactivity in s/s versus l/l carriers without drug treatment (Agorastos et al., 2014). Our current analyses revealed no overall effect of the 5-HTTLPR polymorphism on autonomic responses to CCK-4 challenge. When ESC condition was investigated separately, analyses suggested a similar 5-HTTLPR polymorphism effect as to our previous study on PLA condition, but without statistical significance.

Limitations

Some limitations have to be taken into account for the presented study. To avoid putative confounding effects of the menstrual cycle phase on response to CCK-4 (Le Melledo et al., 1999) and to avoid problems associated with contraception, only male subjects were studied. Thus, long-term HRV effects still need to be investigated in women, as gender differences cannot be ruled out. Similarly, our results should be replicated in individuals of older age, since age-related changes in 5-HT transmission and SSRI effects have been reported (Olivier et al., 2011; Kemp et al., 2014). Effects of SSRIs after longer treatment (>4 weeks, as commonly used in psychiatric practice) still need to be investigated.

Since ESC is considered the most selective SSRI (Burke, 2002; Sanchez et al., 2003), our results may not be generalizable to other substances of the SSRI group. Despite sharing the same principal mechanism of action, recent reviews investigating SSRIs effects in healthy persons suggest various inconsistencies dues to differences in their pharmacodynamic and pharmacokinetic profiles (Goodnick and Goldstein, 1998; Knorr and Kessing, 2010). In addition, several long-term SSRI effects (e.g., neuroplastic and neurotrophic changes, effects on gene expression, anti-inflammatory properties) have been postulated, opposing the initially assumed simplistic monoaminergic pharmacological properties of these drugs (Kroeze et al., 2012; Walker, 2013), so that different pathways of action may be responsible for the observed effects. Another limitation is the use of one standardized dosage of ESC

only. However, the ESC plasma levels measured were within the range recommended for treatment of affective disorders.

Furthermore, as disturbed sleep is also associated with autonomic alterations (Nielsen et al., 2010), it is important to note that we did not determine sleep quality in our initial assessments and can herewith not exclude sleep quality-related bias, although we did not encounter complains about altered sleep patterns. In order to rule out conditioning effects of repeated CCK-4 administration (Hinkelmann et al., 2010a), we controlled for the randomized treatment order, which did not affect our results. In addition, our study did not include subjects with the s/l genotype. Since some studies suggest a nonlinear gene dose effect of the 5-HTTLPR (Neumeister et al., 2006), inclusion of s/l subjects in further studies with a considerably larger sample size is necessary to precisely characterize genotype differences. Regarding functional variants of the long allele, posterior analyses of our DNA specimens revealed that all long alleles in this study were of the LA subtype (Lesch, personal communication).

Finally, it is important to note that across all parameters investigated, no subject had a cardiovascular history and deviating laboratory or physical tests. We particularly accounted for several laboratory markers (e.g., fasting glucose, hemoglobin A1c levels, cholesterol/lipoproteins, pro-inflammatory cytokines, acute-phase proteins) and certain lifestyle habits (e.g., drug, alcohol or tobacco intake) that have been shown to be associated with ANS dysregulation altering cardiovascular measures including HRV (Thayer and Sternberg, 2006; Dinas et al., 2013).

Conclusions

Studies about differential long-term effects of SSRIs on HRV reactivity using pharmacological stress challenges have not been reported so far in healthy subjects. By assessing autonomic responses to a defined pharmacological panic challenge, we provide first data that chronic SSRI treatment is associated with reduced autonomic reactivity in healthy subjects. Our study supports an important role of central serotonergic activity in the modulation of the magnitude of acute cardiovascular

responsiveness, which may affect susceptibility to stress-related disorders. Future studies are needed to replicate this finding and to further explore the functional contribution of effects through and regulation of different receptor subtypes of the serotonergic system to mechanistically understand the role of SSRI on ANS function in both health and disease.

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Chapter 4

Metabotropic glutamate2/3 receptor agonism facilitates autonomic recovery after pharmacological panic challenge in healthy humans.

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Abstract

Background: Group II metabotropic glutamate receptors ($mGluR_{2/3}$) are suggested to modulate anxiety, arousal and stress including autonomic control. However, no study has investigated $mGluR_{2/3}$-related effects on baseline autonomic activity and reactivity to emotional challenge in humans yet.

Methods: Using a double-blind, randomized placebo-controlled, cross-over study design, we investigated the influence of 1-week treatment with the $mGluR_{2/3}$ agonist LY544344, prodrug of LY354740, on autonomic reactivity to a cholecystokinin tetrapeptide (CCK-4) panic challenge in 8 healthy young men. Main outcome measures were time and frequency domain heart rate variability parameters during baseline, CCK-4 challenge and recovery.

Results: There was no evidence for LY544344-mediated effects on baseline and CCK-4 challenge vagal activity, but significantly lower recovery LF(%) and LF/HF ratio in the LY544344 group suggesting enhanced autonomic recovery.

Conclusions: This pilot study provides first human data that $mGluR_{2/3}$ agonism is involved in autonomic responsiveness suggesting an important role of $mGluR_{2/3}$ in central autonomic regulation.

Introduction

Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system (CNS). There are three families of ionotropic and three groups of metabotropic, G protein-coupled glutamate receptors (mGluR) (Meldrum, 2000; Vandenberg and Ryan, 2013). Group II mGluRs ($mGluR_{2/3}$), in particular, are predominantly found in CNS regions involved in arousal and emotion and modulate synaptic neurotransmission (Schoepp, 1994; Kuzmiski and Bains, 2010). Accordingly, $mGluR_{2/3}$ agonists have been investigated in experimental conditions of fear, anxiety and stress sensitization, and suggested as a new pharmacotherapeutic option in the treatment of anxiety disorders (Swanson et al., 2005). Previously, we have reported a significant reduction of the number of pharmacologically-induced panic symptoms and subjective anxiety ratings after treatment with the $mGluR_{2/3}$ agonist LY544344 in healthy samples (Kellner et al., 2005).

However, $mGluR_{2/3}$ also modulate excitability of neurons of the central autonomic network (CAN) and, thus, participate in the fine-tuning of the cardiovascular autonomic control through glutamatergic inhibition (Simms et al., 2006; Browning and Travagli, 2007; Li and Pan, 2007). Nevertheless, to date, no study has reported on autonomic $mGluR_{2/3}$ effects in humans. Here, we present first data on $mGluR_{2/3}$ agonism effects on autonomic reactivity in healthy individuals, using heart rate variability (HRV) data from the same study sample of our previous report (Kellner et al., 2005), as one of the best-established non-invasive methods to assess parasympathetic activity (Camm et al., 1996).

Methods

The study assessed the effects of 1-week treatment with the $mGluR_{2/3}$ agonist LY544344, the peptidyl prodrug of LY354740, on autonomic reactivity to a pharmacological panic challenge by the cholecystokinin tetrapeptide (CCK-4) using HRV in a homogenous group of young healthy men in a double-blind, randomized placebo (PLA)-controlled, within-subject cross-over study with a 2-week drug-free

phase between treatments. We collected data from 12 healthy and medication-free young Caucasian male study volunteers (age 24–35 years, mean age 27.0 years). Study protocol, screening procedures and exclusion criteria have been described in detail previously (Kellner et al., 2005).

Drug intake occurred at 8 a.m. and 8 p.m. on days 1–7 and at 8 a.m. on day 8. LY54344 dosage was titrated in order to decrease potential initial nausea (day 1: 20 mg bid; day 2: 40 mg bid; days 3-7: 80 mg bid). On day 8 of each treatment period, and after standardized breakfast at 8 a.m., a total of 50 µg of CCK-4 (Clinalfa, Läufelfingen, Switzerland) dissolved in 5 ml sterile saline was administered at 11 a.m. as intravenous bolus injection within 20 s. ECG recordings were obtained throughout using a 5-lead holter recording system and analyzed using specific software (Schiller medilog® AR12, Schiller medilog® DARWIN, Schiller Medizintechnik GmbH, Ottobrunn, Germany). Baseline, CCK-4 challenge and recovery ECG data recordings were used to determine differences between treatment conditions. Baseline and recovery recordings started at 10.30 a.m. and 11.30 a.m. for a 5-min heart period respectively. Recording of CCK-4-mediated effects was initiated 45-60 s after the bolus injection at 11:01 a.m. for a 2-min period. HRV measures were calculated in both time and frequency domain according to the guidelines for short-term recordings of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (Camm et al., 1996), as in our previous studies (Agorastos et al., 2014; Agorastos et al., 2015). Mixed between-within subject analyses of variance (ANOVAs) were conducted to assess the impact of treatment (LY544344 vs. PLA) on dependent variables across the three time periods (baseline; CCK-4 challenge; recovery) and post-hoc ANOVAs to assess differences between treatment conditions. To correct for multiple comparisons we used the false discovery rate (FDR) approach (threshold: 5%) (Benjamini and Hochberg, 1995). Consistent with our initial report (Kellner et al., 2005), two individuals with absent adrenocorticotropin (ACTH) decrease after LY544344 compared to PLA were excluded from data analysis. Another two individuals were

not included to the analysis due to incomplete ECG data. Complete data from eight individuals were analyzed.

Results

Our results showed significant to highly significant time effects with very large effect sizes in all assessed HRV measures, indicative of profound effects of CCK-4 challenge on autonomic responsiveness (data not shown). When investigating the treatment × time interaction, substantial effects with respect to LF(%) (Wilks λ = .445; $F_{(2,15)}$ = 8.119; p = .005, ${}_p\eta^2$ = .555) and LF/HF ratio (Wilks λ = .578; $F_{(2,15)}$ = 4.740; p = .028; ${}_p\eta^2$ = .422) became apparent, while all other measures showed no statistically significant effects.

Figure 1. Effects of placebo and LY544344 on LF (%) (A) and LF/HF ratio (B) through baseline, CCK-4 challenge and recovery.

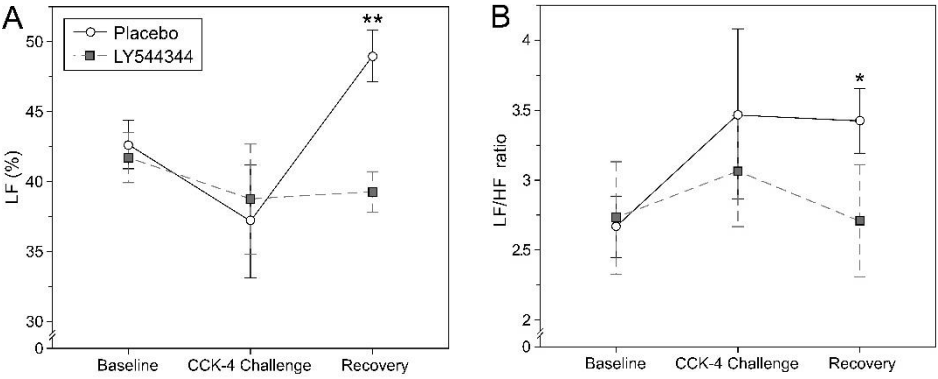

Figure Legend

Values are means ± SEM. LF: low frequency 0.04-0.15 Hz; HF: high frequency 0.15-0.4 Hz; LF (%): percentage of LF frequency component from the total power. *p < 0.05, **p < 0.01.

A post-hoc group comparison for LF(%) and LF/HF ratio for each time point separately revealed differences with large effect sizes between the two groups only with respect to recovery LF(%) and LF/HF ratio HR measures, with lower recovery LF(%) and LF/HF ratio in the LY544344 condition (p = .003, ${}_p\eta^2$ = .728; p = .047, ${}_p\eta^2$ = .452 respectively, Figure 1). FDR indicated no potential type I errors.

Discussion

Summarizing, the main findings of this study include (1) attenuated cardiac vagal modulation in both treatment conditions by CCK-4 as shown in previous studies (Agorastos et al., 2014; Agorastos et al., 2015), (2) no statistically significant evidence for LY544344-associated effects on baseline and CCK-4 challenge autonomic activity, but (3) enhanced recovery with significantly lower recovery LF(%) and LF/HF ratio 30 min after CCK-4 challenge in the LY544344 group. Given that slower autonomic recovery is associated with overall cardiovascular risk (Heponiemi et al., 2007), LY544344-associated enhancement of autonomic recovery after CCK-4 challenge can be considered a beneficial effect. This finding could suggest favorable effects of $mGluR_{2/3}$ agonism on stress-related autonomic responses in accordance with animal data (Iijima et al., 2007; Ye et al., 2013). These effects of $mGluR_{2/3}$ agonism on cardiac autonomic control could be mediated directly, i.e. through modulation of the CAN (Simms et al., 2006; Browning and Travagli, 2007; Li and Pan, 2007) or indirectly, i.e. through modulation of baroreceptor signal transmission (Simms et al., 2006; Sekizawa et al., 2009; Goldstein et al., 2011). Either way, our study supports an important role of central $mGluR_{2/3}$ in the modulation of neuroautonomic regulation underlying cardiovascular responsiveness, which may affect susceptibility to anxiety and stress-related disorders.

Our pilot study reports findings on a strictly investigational use of a non-FDA approved product. The major limitation is the small subject number included and the investigation of only male subjects in order to avoid confounding menstrual cycle phase effects on CCK-4 response or problems associated with contraception.

Thus, our results are not applicable in female population. In addition, these experimental sample effects may not reflect clinical population and have to be validated in patients with anxiety, affective and/or stress-related disorders. However, to the best of our knowledge, this study is the first one assessing potential modulating effects of $mGluR_{2/3}$ agonism on neuroautonomic control in humans. Future studies are needed to replicate this finding in larger samples and to further explore $mGluR_{2/3}$-associated effects on autonomic regulation across genders and clinical samples.

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Chapter 5

Vagal effects of endocrine HPA axis challenges on resting autonomic activity assessed by heart rate variability measures in healthy humans.

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Abstract

Background: The hypothalamic-pituitary-adrenal axis (HPA axis) and the autonomic nervous system (ANS) are considered to play the most crucial role in the pathophysiology of stress responsiveness and are increasingly studied together. However, only few studies have simultaneously assessed HPA axis and ANS activity to investigate their direct interaction in pathophysiology, while no study so far has assessed the dynamic interplay between the two systems in healthy subjects through endocrine challenges.

Methods: The present study assessed the direct effects of overnight pharmacoendocrine HPA axis challenges with dexamethasone (suppression) and metyrapone (stimulation) on ANS activity at rest as determined by linear and nonlinear measures of heart rate variability (HRV) in 39 young healthy individuals.

Results: Findings indicated significant effects of metyrapone, but not dexamethasone on autonomic activity at rest based on HRV measures. HRV after metyrapone was overall significantly reduced in comparison to baseline or post-dexamethasone conditions, while the combined metyrapone-related reduction of HRV measures RMSSD, NN50(%) and HF(%) with concomitant increase of the unifractal scaling coefficient α_{fast} value jointly indicated a specifically diminished vagal activity.

Conclusions: We provide first data that HPA axis stimulation (metyrapone) is associated with reduced vagal tone, while HPA axis suppression (dexamethasone) has no effect on autonomic modulation of heart function. Our results support a vital role of the parasympathetic nervous system in the interplay between ANS and HPA axis and, thus, in the modulation of stress-related cardiovascular responsiveness and the susceptibility to stress-related disorders.

Introduction

Stress is defined as the state of threatened homeodynamic balance [non-equilibrium state as opposed to homeostasis; (Ikegami and Suzuki, 2008)] of an organism (Chrousos, 2009; Ikegami and Suzuki, 2008; Koolhaas et al., 2011). Excessive stress exposure may oversensitize neuroendocrine responses to stress, leading to an altered dynamic state associated with decreased adaptability and profound effects on physiological development leading to chronic physical and mental morbidity (Chrousos, 2009; de Kloet et al., 2005; Koolhaas et al., 2011; Lupien et al., 2009; McEwen, 1998). The human 'stress' system constitutes the major dynamic regulatory domain of the organism as it orchestrates a plethora of physiological reactions to acute or chronic challenges.

The human 'stress' system includes central and peripheral components. The highly interconnected central components of the stress system are mainly located in the hypothalamus and the brainstem but are driven by inputs mainly from the prefrontal cortex and the amygdala besides the direct baroreflex-mediated feedback regulation through brainstem circuits via the nucleus tractus solitarii. The central response facilitates neural pathways enhancing adapting functions such as arousal, vigilance and focused attention, while inhibiting adaptive functions irrelevant in this emotional state such as eating, growth and reproduction. The peripheral components of the stress system include the hypothalamic-pituitary-adrenal (HPA) axis and the limbs of the autonomic nervous system (ANS), i.e. the sympathetic (SNS) and sympatho-adrenomedullary (SAM) system and the parasympathetic system (PNS). The principal peripheral effector molecules are the HPA axis-regulated glucocorticoids (GCs), involved also in the negative feedback mechanism centrally inhibiting corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) secretion, and the SAM-regulated catecholamines nor-epinephrine and epinephrine (Chrousos, 2009; Elenkov and Chrousos, 2006; Tsigos and Chrousos, 2002; Ulrich-Lai and Herman, 2009). The HPA axis and the ANS are considered to play the most crucial role in stress-related

maladaptive consequences leading to pathophysiology (Chrousos, 2009; de Kloet et al., 2005; Stratakis and Chrousos, 1995).

The HPA axis and ANS are increasingly studied together (Licht et al., 2010), as their activity normally shows a certain degree of analogy and complementarity at several neuroendocrine levels. Thereby, the physiological functions of the HPA axis depend at least in part on the ANS (Thayer and Sternberg, 2006), while on the other hand HPA axis-related GC signaling may have an important role in the regulation of ANS activity (Raison and Miller, 2003). However, only relatively few studies have simultaneously assessed HPA axis and ANS activity to investigate their direct association (Adlan et al., 2018; Wiedemann et al., 2001), while most of these studies are conducted in patients (Casement et al., 2018) or through subjective stress exposure paradigms (Ali et al., 2017; Pulopulos et al., 2018). Until now, only very few studies have assessed the direct physiological dynamic interplay between the two systems in healthy subjects through pharmacological challenges. Nonell et al. (2005), for example, report a shift of the autonomic response to an orthostatic challenge towards parasympathetic activity by prolonged but not acute hydrocortisone administration. However, the effects on unchallenged autonomic function are essentially unclear.

Thus, the main objective of our study was to assess the effects of HPA axis manipulation (i.e. stimulation and suppression of the HPA axis) on baseline ANS activity in healthy individuals. We therefore investigated autonomic activity measured by heart rate variability (HRV) at rest to overnight pharmacoendocrine HPA axis challenges with metyrapone (stimulation) and dexamethasone (suppression) in a group of healthy subjects. Metyrapone (MET) crosses the blood-brain barrier and reduces, not only at the adrenal glands but also within the brain, cortisol levels by blocking the enzymatic conversion of 11-deoxycortisol to cortisol by CYP11B1 (11-beta-hydroxylase, P-450c11), the last step in the synthesis of cortisol. This leads to a rapid fall of cortisol and an accumulation of 11-deoxycortisol, which does not inhibit ACTH secretion. This results in decreased cortisol-mediated negative feedback at hypothalamic and pituitary levels, which increases CRH and

ACTH secretion. Dexamethasone (DEX) is an exogenous steroid that binds mainly to glucocorticoid receptors in the anterior pituitary gland. This results in regulatory modulation through negative feedback and suppression of ACTH and consequently lowers cortisol secretion (Cole et al., 2000). We hypothesized that stimulation of the HPA axis would be associated with reduced, while suppression with enhanced HRV.

Materials and Methods

Study participants and inclusion criteria

The study was approved by the Ethics Review Committee (ERC) of the Hamburg Medical Board and conducted at the Department of Psychiatry and Psychotherapy, University Medical Center Hamburg-Eppendorf, Hamburg, Germany (file Nr. PV4161). Healthy subjects between 18-65 years of age were recruited by printed/electronical public advertisement and invited to an initial appointment in our department. After full oral and written explanation of the purpose and procedures of the investigation, written informed consent was obtained from each subject before initiating the screening procedure. Screening included a thorough physical and neurological examination, routine blood laboratory tests, urine toxicology screen, electrocardiogram (ECG) recording at rest and a structured face-to-face interview.

Exclusion criteria included: presence or history of any physical (e.g., history of any chronic or acute inflammatory, metabolic, neurological or immune system-associated medical conditions) and Axes I and II mental co-morbidities, body mass index (BMI) values beyond 18-30 kg/m^2, frequent usage of any either illicit or prescribed drugs or over the counter medications, current use of any psychiatric medication for at least 8 weeks, drinking of more than 100 g of alcohol per week, current adverse life events, night shifts or transcontinental flights across more than four time zones during the past four weeks, abnormal physical and neurological examinations, basic blood laboratory test values deviating from the normal range (incl. thyroid function tests, transaminases, electrolytes, CO_2 anion gap, fasting glucose, basic blood and coagulation tests, blood lipids, haemoglobin A1c, C-reactive

protein, creatinine, folic acid, vitamin B12), positive urine toxicology screen, actual menstruation, pregnancy, nursing, or not using a reliable method of birth control, any contraindication for dexamethasone or metyrapone and pathological initial ECG. Hypothyroidism in the euthyroid state through hormonal substitution, as well as hypertension in normotensive state through antihypertensive medication, did not serve as exclusion criteria. Current or lifetime psychiatric disorders were excluded using the Structured Clinical Interview for the DSM-IV, axes I and II, assessed by a trained physician, as well as through a self-rating by the Beck's Depression Inventory (BDI-II, cut-off of 9 points). All other exclusion criteria were assessed in a clinical interview setting through study questionnaires. Study completers received a reimbursement of 50 €.

Study procedures

Volunteers who met study inclusion and exclusion criteria were scheduled for study initiation within 1 week of final laboratory results. Additional physical examination in the first morning before study initiation also provided assurance that no subject had any acute clinical manifestations or febrile body temperature. The study assessed autonomic nervous system activity through various HRV measures at the same time on three continuous days: Day 1 (baseline), Day 2 (post-metyrapone) and Day 3 (post-dexamethasone). All subjects were encouraged to maintain a regular sleep time starting at around 11.00 p.m. for all three nights before HRV assessment, with wake-up at 7.00 a.m. and avoidance of physical strain (e.g. physical exercise, sexual activity, etc.) on all three mornings during the study. Furthermore, participants were encouraged to use public transportation or private motor vehicles (i.e. no bicycles or walking > 500 m to exclude to potential effects of elevated physical activity on HR measures) for reaching the study facility (at 8.30 a.m.) and to avoid any intake of food or beverages (water was allowed) until completion of the assessment (10.30 a.m.). Adverse side effects were assessed through a German version of the UKU side effects rating scale (Lingjaerde et al., 1987).

Endocrine challenges

- Overnight Metyrapone Stimulation Test (MST): The MST test is considered to be a simple and sensitive alternative test to evaluate the ACTH reserve and it is useful to evaluate the response of the HPA axis (Avgerinos et al., 1996; Fiad et al., 1994). Subjects received 1 g of MET (Metopiron®, Novartis, Arnhem, Netherlands) orally at 11.00 p.m. on Day 1, to assess its effects on HRV the next morning (Day 2, approx. 9 h after metyrapone intake).
- Overnight Low-Dose Dexamethasone Suppression Test (DST): The low-dose DST is one of the commonly used tests to assess HPA axis reactivity by measuring the change in peripheral cortisol levels in response to externally administered DEX (Gwirtsman et al., 1982). Subjects received 1 mg of DEX (Fortecortin®, Merck, Darmstadt, Germany) orally at 11.00 p.m. on Day 2, to assess its effects on HRV the next morning (Day 3, approx. 9 h after DEX intake).

The temporal order of the two endocrine challenges was chosen to avoid any interference between the two interventions, as half-life times significantly differ between metyrapone (approx. 2 h) and dexamethasone (35 - 54 h). Subjects were blinded with respect to the specific order of endocrine challenges.

Assessment of heart rate variability

- ECG recordings: After an initial blood draw (8.45 a.m. of each day), all subjects were given 15 min in sitting and 15 min in supine position in a single bedded room before the ECG recording was initiated. The ECG holter recording was initiated at 9.15 a.m. of each day. All subjects stayed in bed for 75 min with at least 60-min continuous ECG recording. The 60-min ECG recording allowed to generate a recording interval both long enough for nonlinear analysis based on long-lasting heartbeat interval correlations (see Meyer and Stiedl, 2003) without transient data loss. During this time, there was no presence of study personnel in the study room (subjects were monitored through a one-way mirror window and a room-installed microphone), while relative silence (no radio, electronic media, or disturbing conversation outside the study room) was maintained. ECG recordings were obtained throughout using a

5-lead holter recording system (Schiller medilog® AR12, Schiller Medizintechnik GmbH, Ottobrunn, Germany). Data were recorded at 4096 Hz sampling rate with 16-bit resolution and stored digitally on the recorder. ECG recording was performed by specially trained study staff.

- ECG editing: ECG data were exported as text files with time and amplitude information. These were imported into LabChart (v. 7.1, ADInstruments, Spechbach, Germany) to calculate the tachograms, while the import into LabChart allowed for manual data editing, i.e., removal of artifacts and insertion of unrecognized beats. ECG data editing was a laborious process supervised by an experienced scientist (Dr. Oliver Stiedl). All data pre-processing followed the recommendations of the HRV Task Force (Camm et al., 1996), as well as recently revised guidelines (Laborde et al., 2017) before any analysis was performed. Subsequent data analysis occurred on the basis of the edited LabChart data.

- HRV analyses: The assessed HRV variables have been selected according to the guidelines for short-term recordings of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (Camm et al., 1996), which are also supported by later studies (Jarrin et al., 2012; Meyer and Stiedl, 2003). We used the percentage (%) of the total power of each frequency band to compare the fractional energy (Camm et al., 1996) rather than the absolute energy.

a) Linear analyses: The HR patterns obtained from LabChart were analyzed using linear time domain and frequency domain using the LabChart software extension HRV. Instantaneous HR was calculated on the basis of the RR interval of the ECG signal. HRV in the time domain was calculated by taking the standard deviation of the N-N intervals (SDNN), by calculating the root mean square of subsequent N-N interval differences (RMSSD) and, by the percentage of adjacent NN intervals that differ in length by > 50 ms [NN50(%)]. HRV in the frequency domain was calculated by analysis of several frequency components (low frequency 0.04-0.15 Hz [LF] and high frequency 0.15-0.4 Hz [HF]) (Camm et al., 1996).

b) Nonlinear analyses: Unifractal nonlinear analysis of HR dynamics was performed using the detrended fluctuation analysis (DFA) (Peng et al., 1995) using customized MATLAB (MathWorks, Natick, MA, USA) scripts as described in prior studies (Meyer and Stiedl, 2003; Peng et al., 1995). DFA is derived from the random walk theory, and uses scaling coefficients (α) as measure of the correlation of heartbeats in its temporal sequence, thereby indicating ANS dysregulation. A scaling coefficient α = 1.5 indicates 'Brownian noise' in which there only is short-term correlation, i.e., one heartbeat interval is correlated with the previous interval only. This is observed when the tonic parasympathetic outflow to the heart is blocked by atropine treatment (Meyer, 2002; Yamamoto et al., 1995) or as a consequence of heart transplantation (Meyer, 2002). A value of α = 1.0 indicates 'pink noise' with persistent long-range correlation of successive heartbeat intervals, as observed in physiological HR dynamics of mammalian species including mice and man. A scaling coefficient of α = 0.5 reflects 'white noise' and indicates the lack of any correlation between heartbeat intervals (randomness). The straight-line relationship is separated by a breakpoint indicating different slopes, α_{fast} and α_{slow}, which are interpreted to reflect the coefficients of distinct ranges, short-range and long-range scaling, respectively (Meyer and Stiedl, 2003). Pathological states are characterized by a breakdown of long-range correlations, i.e., α strongly deviating from 1.0.

Laboratory assays

We determined cortisol (CORT, ng/ml), desoxy-cortisol (D-CORT, ng/ml) and adrenocorticotropic hormone (ACTH, pg/ml) plasma levels using commercially available immunoradiometric assays and radio-immunoassays (BRAHMS, Berlin, Germany; Nichols Institute, San Juan Capistrano, CA, USA; ICN Biomedicals, Carson, CA, USA). Intra- and inter-assay coefficients of variation were below 8%.

Statistical analyses

Preliminary analyses were performed to ensure no violation of the assumptions of normality, linearity and homoscedasticity. Because of several parameters showing

skewed distribution, all parameters were ln-transformed for further parametric analysis. All data are given in mean values (SEM) adjusted for age, sex, waist-hip-ratio and smoking. For presentation purposes, Tab. 1 and Tab. S1 include sample means in terms of geometric means on the original scale through back-transformation through exponentiation of ln-transformed data. Differences between treatment conditions (Day 1, Day 2: Post-MET, Day 3: Post-DEX; time effect) were tested for significance by one-way repeated measures analysis of variance (rmANOVA) for correlated samples controlling for age, sex, waist-hip-ratio and smoking. Pairwise comparisons between the three days were conducted by the Bonferroni post-hoc test. An error probability of $p < 0.05$ was accepted as statistically significant. Effect size is reported as partial eta squared ($_p\eta^2 = 0.01$: small effect size, $_p\eta^2 = 0.06$: medium effect size, $_p\eta^2 = 0.14$: large effect size). To correct for potentially inflated type I error because of multiple comparisons we used the false discovery rate (FDR) approach (Benjamini and Hochberg, 1995), as in our previous studies (Agorastos et al., 2013; Agorastos et al., 2016; Agorastos et al., 2014; Agorastos et al., 2015). Following a previously reported procedure (Verhoeven et al., 2005) *p*-values were corrected by the minimum positive FDR with a threshold set at 5%. Statistical analyses were conducted using the Statistical Package for Social Sciences Version 20 (SPSS, Chicago, IL).

Results

72 healthy subjects were screened. 43 participants were found eligible for participation in the study. Of those, 2 declined participation at the time of the scheduling phone call, 1 did not attend at scheduled appointment and 1 dropped out due to mild gastrointestinal side effects on Day 1 by metyrapone. We collected data from 39 healthy Caucasian study completers, found eligible for participation in the study. The final sample included 12 males (30.8%), 7 smokers (17.9%) and had a mean age of 35.7 ± 13.6 (19 - 65) years and a mean waist-hip-ratio of 0.81 ± 0.07 (0.69 - 0.94). None of the subjects of the final sample was receiving additional medication (i.e. antihypertensive medication or thyroid hormone substitution). Side

effects as per UKU ratings did not differ significantly between days indicating no significant adverse effects (data not shown). Endocrine analyses of CORT, D-CORT and ACTH indicated a highly significant effect of time (i.e. treatment condition) in all three measures with very large effect sizes, which confirmed the expected effects of each treatment condition (CORT: Wilks $\lambda = .009$; $p < .001$, ${}_p\eta^2 = 0.991$; D-CORT: Wilks $\lambda = .015$; $p < .001$, ${}_p\eta^2 = 0.985$; ACTH: Wilks $\lambda = .159$; $p < .001$, ${}_p\eta^2 = 0.841$, *cf.* Table 2 and Figure 2).

The results of rmANOVAs indicated a significant effect of time (i.e. treatment condition) on several HRV variables including RMSSD, NN50(%), HF(%) and α_{fast} across the three conditions with very large effect sizes (*cf*. Tab. 1). Post-hoc pairwise comparison revealed significant and highly significant differences between either Day 1 / Day 2 or Day 2 / Day 3. Day 2 values had significantly lower RMSSD, NN50(%), HF(%) and higher α_{fast} values than Day 1, while higher α_{fast} and lower HF(%) values than Day 3 (*cf.* Figure 1). Day 1 and Day 3 values showed only a significant difference with respect to NN50(%) (Day 1 vs. Day 3). There were no statistically significant differences with respect to NN50(%), SDNN, LF(%) and α_{slow} between the three experimental conditions.

FDR analysis indicated no potential type I errors. There was no statistically significant correlation between HRV measures and endocrine levels whatsoever (data not shown).

Table 1. Adjusted geometric means of linear and nonlinear HRV measures across the three conditions and time effects of endocrine challenges

Domain	Measures	Adjusted geometric means			rmANOVA (time effect)			
		Day 1 Baseline	Day 2 Post-MET	Day 3 Post-DEX	Wilks' λ	*F*	*p*	$_p\eta^2$
Time domain	HR (bpm)	56.94 (1.22)	58.32 (1.02)	58.09 (1.02)	.872	1.981	.157	.128^{+}
	RMSSD (ms)	62.36 (1.07)	55.20 (1.07)	59.03 (1.08)	.763	4.188	**.026**	**.237**$^{++}$
	SDNN (ms)	98.00 (1.05)	94.82 (1.05)	93.13 (1.05)	.934	.946	.401	.066^{+}
	NN50 (%)	29.05 (1.08)	24.56 (1.10)	24.00 (1.12)	.764	4.173	**.026**	**.236**$^{++}$
Frequency Domain	HF (%)	16.20 (1.10)	13.67 (1.10)	17.74 (1.11)	.594	9.208	**.001**	**.406**$^{++}$
	LF (%)	20.61 (1.08)	18.99 (1.08)	21.24 (1.09)	.909	1.349	.276	.091^{+}
Non-linear	α_{fast}	.98 (1.03)	1.04 (1.03)	.97 (1.04)	.675	6.496	**.005**	**.325**$^{++}$
	α_{slow}	.92 (1.02)	.91 (1.02)	.89 (1.02)	.903	1.453	.252	.097^{+}

Table Legend

Values are presented as geometric mean values (SEM) adjusted for age, sex, waist-hip-ratio and smoking. HR: heart rate; SDNN: standard deviation of the N-N intervals; RMSSD: root mean square of subsequent N-N interval differences; NN50(%): percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms; LF: low frequency 0.04-0.15 Hz; HF: high frequency 0.15-0.4 Hz; LF (%) and HF (%): percentage of each frequency component from the total power; α: scaling coefficient alpha of nonlinear analysis. Differences between treatment conditions (i.e. Day 1: baseline; Day 2: post-dexamethasone; Day 3: post-metyrapone; time effect) were tested for significance by one-way repeated measures analysis of variance (rmANOVA) for correlated samples controlling for age, sex, waist-hip-ratio and smoking. p-values denoting statistically significant differences ($p < .05$) are shown in bold. FDR analysis revealed no potential type I errors.

$^{+}$moderate effect size; $^{++}$large effect size.

Figure 1. Effects of metyrapone (MET) and dexamethasone (DEX) on HRV measures.

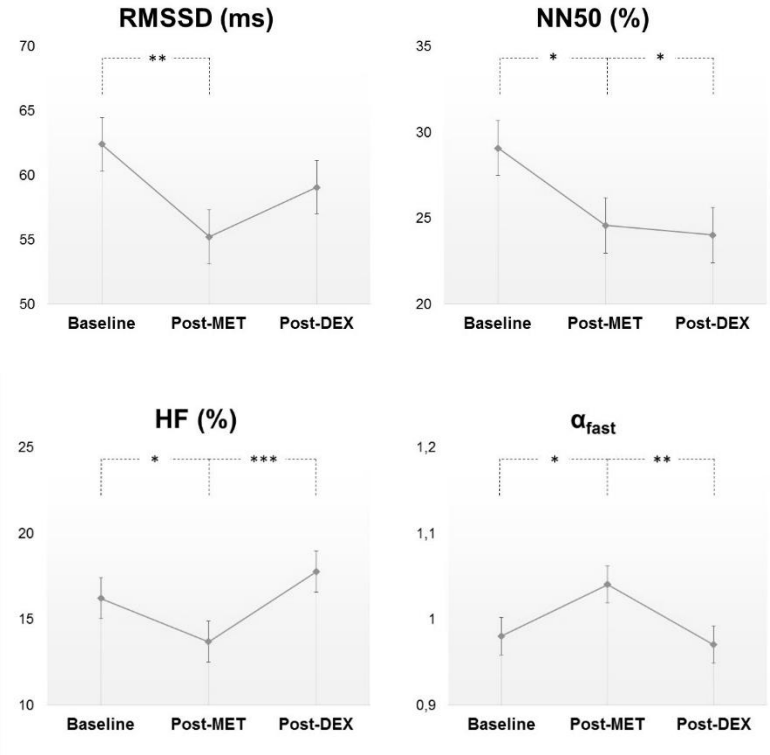

Figure Legend

Pairwise comparisons between the three experimental conditions (i.e. Day 1: baseline; Day 2: post-metyrapone, Post-MET; Day 3: post-dexamethasone, Post-DEX) were conducted by the Bonferroni post-hoc test controlling for age, sex, waist-hip-ratio and smoking. Values are presented as geometric mean values (±SEM) adjusted for age, sex, waist-hip-ratio and smoking. RMSSD: root-mean-square of subsequent NN interval differences; NN50(%): percentage of the number of pairs of adjacent NN intervals differing in length by >50 ms; HF (%): percentage of the high frequency (0.15-0.4 Hz) power from the total power; α: scaling coefficient alpha of nonlinear unifractal analysis. FDR analysis revealed no potential type I errors.

***** .01 ≤ *p* < .05**, **** .001 ≤ *p* < .01**, *****$p < .001$.

Table 2. Adjusted geometric means of endocrine measures across the three conditions and time effects of endocrine challenges

Measures	Adjusted geometric means			rmANOVA (time effect)			
	D1 Baseline	D2 Post-MET	D3 Post-DEX	Wilks' λ	F	p	$_{p}\eta^2$
Desoxy-cortisol (ng/ml)	2.77 (1.63)	35.66 (1.09)	1.99 (2.46)	.015	885.494	**< .001**	**.985**$^{++}$
Cortisol (ng/ml)	280.90 (1.05)	291.78 (1.05)	23.41 (1.05)	.009	1725.158	**< .001**	**.991**$^{++}$
ACTH (pg/ml)	29.11 (1.08)	98.99 (1.19)	11.35 (1.08)	.159	60.860	**< .001**	**.841**$^{++}$

Table Legend

Values are presented as geometric mean values (SEM) adjusted for age, sex, waist-hip-ratio and smoking. Differences between treatment conditions (i.e. Day 1: baseline; Day 2: post-metyrapone, Post-MET; Day 3: post-dexamethasone, Post-DEX; time effect) were tested for significance by one-way repeated measures analysis of variance (rmANOVA) for correlated samples controlling for age, sex, waist-hip-ratio and smoking. ACTH: Adrenocorticotropic hormone (pg/ml). *p*-values indicating statistically significant differences ($p < .001$) are shown in bold. FDR analysis revealed no potential type I errors.

$^{+}$moderate effect size; $^{++}$large effect size.

Figure 2. Effects of dexamethasone (DEX) and metyrapone (MET) on endocrine measures approximately 9 h after administration.

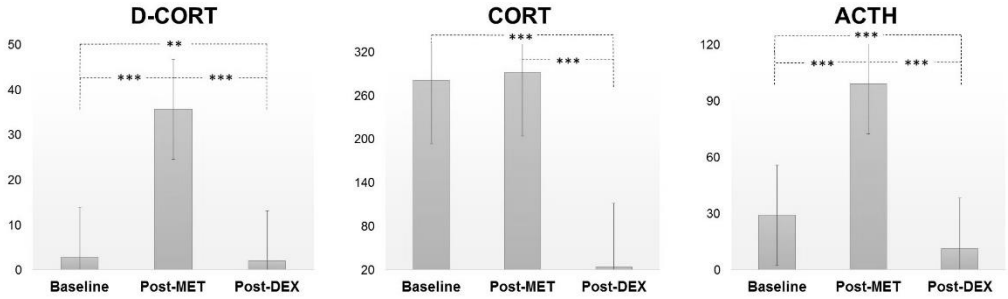

Figure Legend

MET and DEX were administrated at approximately 11.00 p.m. of day 1 and day 2 respectively. Endocrine measurements were conducted in plasma acquired through blood draw at approximately 08.45 a.m. of the following day (day 2 and day 3 respectively), 30 min before initiation of ECG recording. The DEX-induced D-CORT increase was indicated (left panel), the MET-induced CORT suppression was confirmed (middle panel) and the DEX-induced ACTH increase was verified. Pairwise comparisons between the three conditions (i.e. Day 1: baseline; Day 2: post-metyrapone, Post-MET; Day 3: post-dexamathasone, Post-DEX) were conducted using the Bonferroni post-hoc test to control for age, sex, waist-hip-ratio and smoking. Values are presented as geometric mean values (SEM) adjusted for age, sex, waist-hip-ratio and smoking. D-CORT: Desoxy-cortisol (ng/ml); CORT: cortisol (ng/ml); ACTH: Adrenocorticotropic hormone (pg/ml). FDR analysis revealed no potential type I errors.

*****.01 ≤ p < .05**, ****.001 ≤ p < .01**, *****p < .001.

Discussion

This study assessed the effects of pharmacoendocrine HPA axis manipulation through dexamethasone and metyrapone challenges (i.e. suppression and stimulation of the HPA axis, respectively) on baseline autonomic activity through assessment of HRV measures in healthy individuals. Therefore, commonly used linear and nonlinear (unifractal) methods for analysis of HRV and its dynamics were combined to examine autonomic effects at rest by the two pharmacological interventions. To the best of our knowledge, this is the first study investigating the direct interplay between the HPA axis and the resting ANS activity in healthy subjects through objective endocrine challenges and also the only study so far assessing effects of metyrapone on HRV.

The main findings of this study include i) significant differences of HRV variables with lower values of RMSSD, NN50(%), HF(%) and higher values of α_{fast} after metyrapone in comparison to baseline and post-dexamethasone conditions, ii) negligible HRV differences between baseline and post-dexamethasone conditions and iii) no statistically significant differences with respect to NN50(%), SDNN, LF(%) and α_{slow} between the three conditions. Taken together, these findings indicate significant effects of HPA axis stimulation by metyrapone, but not suppression by dexamethasone on autonomic activity at rest as measured by HRV. HPA axis stimulation with metyrapone was associated with overall significantly reduced HRV. In particular, given that increased α_{fast} values reflect reduced PNS activity (Meyer, 2002; Meyer and Stiedl, 2003), the metyrapone-related combined reduction of RMSSD, NN50(%), HF(%) with an increase of α_{fast} values and no difference in NN50(%), SDNN, LF(%) values jointly reflects a diminished tonic parasympathetic activity (Lombardi and Stein, 2011; Meyer, 2002), in comparison to baseline or post-dexamethasone measures at rest.

HRV results from heart rate (HR) oscillations within its physiological range (beat-to-beat variability), controlled by parasympathetic and sympathetic modulation of intrinsic cardiac pacemakers (Akselrod et al., 1981) and constitutes the best-established, non-invasive method of analysis of autonomic activity (Camm

et al., 1996; Reyes del Paso et al., 2013). Heart activity under resting conditions experiences a constant tonic inhibitory control by PNS and dominance over SNS influence (Jose and Collison, 1970; Schmidt et al., 2000), as SNS cardiac influence is too slow and long-lasting to produce rapid beat-to-beat changes (Baumert et al., 1995; Kleiger et al., 1992; Meyer and Stiedl, 2003). Reduced HRV is considered a valid marker of heart disease (Thayer et al., 2012; Thayer and Sternberg, 2006), while the PNS is particularly implicated in the pathophysiology of cardiovascular diseases and other comorbidities and associated with decreased dynamic flexibility, loss of complexity and increased overall vulnerability (Thayer and Sternberg, 2006).

Since tonic parasympathetic activity underlies long-range correlation of heartbeat interval fluctuations in the healthy state (Meyer, 2002), we also included nonlinear (unifractal) HRV analysis as an important and highly sensitive readout to particularly assess vagal activity changes after challenge. Nonlinear analysis is a physiologically relevant and statistically appropriate method when analysing physiological data, because two major properties, non-stationarity (i.e. drift-like fluctuations of heartbeat intervals) and interdependence (i.e. temporal long-range correlation of heartbeat intervals), formally confound the use of linear analyses for HR data (Meyer and Stiedl, 2003). Nonlinear analysis thus provides a very sensitive measure that improves HRV assessment and is highly predictive of cardiac dysfunction attributed to autonomic dysregulation in the absence of cardiac disease (Baumert et al., 2004; Meyer and Stiedl, 2003). Unfortunately, to date, this method is still only applied in a relatively limited number of related publications on human data (Agorastos et al., 2013; Aubert et al., 2009) due to its complexity and availability of only short ECG recording intervals (commonly 5 min).

This study showed that HPA axis stimulation with metyrapone was associated with overall significantly reduced HRV due to diminished parasympathetic activity, while HPA axis suppression with dexamethasone had no effect on autonomic activity. The mechanistic understanding of ANS and HPA axis coupling could be of major importance for the understanding of the pathophysiology of stress-related disorders. Accordingly, a progressive divergence of the HPA axis

and the ANS activity following stress, has been proposed as a vital pathophysiological trajectory leading to the long-term impact of excessive stress on the stress system and the chronic preservation of symptoms (Pervanidou, 2008).

HPA axis and ANS are interrelated components of an internal neural regulation system (central autonomic network, CAN) (Benarroch, 1993; Thayer and Lane, 2000). The CAN integrates high-order autonomic control associated with cognitive perception, while mediating emotional responses through hypothalamic-brainstem pathways to preganglionic autonomic neurons (Thayer and Lane, 2000). For example, amygdala and brain stem neurons of the nucleus of the solitary tract are critical for both vagal activity and regulation of HPA stress responses, as parasympathetic NTS neurons send direct projections to hypophysiotropic CRH neurons in the amygdala and herewith also modulate HPA activity and its feedback control (Herman, 2017). Dysregulation of the CAN (Davis and Natelson, 1993; Loewy and Spyer, 1990; Saper, 2004) may thus affect downstream autonomic core centers and alter both peripheral ANS and HPA axis activity and responsivity (Davis and Natelson, 1993; Stiedl et al., 2010; Thayer and Lane, 2009). HRV reflects the activity of the CAN (Stiedl et al., 2010; Thayer et al., 2012) and represents a marker for regulated physical and emotional responding capacity (Thayer and Lane, 2000).

Although the precise brain processes associated with CAN and stress system dysregulation remain unclear, a central role of the hypothalamic regulatory level could be presumed. For example, CRH receptors are favorably positioned in the CNS to modulate the sympathetic and parasympathetic branches of the cardiac autonomic nervous system according to their distribution (Oliveira et al., 2015; Tran et al., 2014), including core PNS output areas (Stiedl et al., 2005). CRH has been suggested to inhibit vagal or activate sympathetic outflow at least in part via the CRH subtype 1 (CRH_1) receptor (Arlt et al., 2003; Nijsen et al., 2000a; Nijsen et al., 2000b), while CRH_1 receptor antagonists increase cardiac vagal and decrease sympathetic activity in rats (Wood and Woods, 2007). Central CRH may thus lead to altered dynamical properties with a significant loss of intrinsic structural complexity of cardiac control due to central neuroautonomic hyperexcitation, i.e., enhanced

sympatho-vagal antagonism as indicated in mice (Meyer and Stiedl, 2006; Stiedl et al., 2005). On the other hand, CRH released in the bed nucleus of the stria terminalis (BNST) during stress, contributes to cardiac stress responses, particularly by activating vagal outflow in rats (Nijsen et al., 2001). However, CRH_1 receptor deletion in mice has been shown to not affect heart rate adjustment and behavioural responses to acute fearful stimuli despite absent HPA axis activation (Tovote et al., 2005), which favours a central role of the suprahypothalamic rather than the hypothalamic circuitry.

Evidence suggests that the medial prefrontal cortex (mPFC) and anterior cingulate (ACC) modulate amygdala activity and that HRV reflects the strength of mPFC-amygdala interaction (Goldstein, 2012). mPFC hypofunction underlies the deficient rational control of emotional responses resulting in exaggerated amygdala activity (Falconer et al., 2008). Higher HRV was associated with stronger resting state functional connectivity (rsFC) between the amygdala and the mPFC (Sakaki et al., 2016). Interestingly, rsFC between mPFC and amydgala is majorly regulated by stress-related neuroendocrine levels (Kiem et al., 2013; Quaedflieg et al., 2015), with HPA axis stimulation (i.e. higher cortisol levels) being associated with stronger negative rsFC between these areas at rest (Veer et al., 2012). Given this, MET may lead to reduced rsFC between mPFC and amygdala (i.e., the inhibitory control of mPFC on the amygdala) and thus reduced HRV, as shown in our study (Thayer, 2006). This inhibitory control appears to be largely vagally mediated (Smeets, 2010; Thayer and Sternberg, 2006; Weber et al., 2010). This conclusion is consistent with the neurovisceral integration model, which underlines the importance of the PNS in providing negative feedback on sympathoexcitatory stress responses (Thayer and Sternberg, 2006). Furthermore, our interpretation is consistent with the finding that tonic PNS function is crucial for the dynamical properties of heartbeat interval fluctuations (Stiedl et al., 2009; Stiedl et al., 2010), as determined by nonlinear analysis.

Considering the very short half-life of metyrapone (approx. 2 h), the profound effects of metyrapone challenge on ANS activity 8-9 h after administration

clearly suggest indirect effects of HPA axis stimulation. Given the association of higher cortisol levels with enhanced serotonin reuptake (i.e. lower levels of brain serotonin) (Tafet et al., 2001a; Tafet et al., 2001b), as well as the association of lower levels of brain serotonin with reduced HRV (Booij et al., 2006), higher cortisol levels (i.e. HPA axis stimulation) may act indirectly via altered serotonergic signalling to influence the functional connectivity between PFC and the amygdala (Pezawas et al., 2005). Thereby, autonomic and particularly vagal activity (Jordan, 2005; Thayer et al., 2006) is altered via 5-HT_{1A} receptor involvement (Youn et al., 2013). This is also supported by two prior studies of our group, reporting significant effects of both the serotonin transporter-linked polymorphic region (5-HTTLPR) genotype (Agorastos et al., 2014) and long-term treatment with the selective serotonin reuptake inhibitor (SSRI) escitalopram (Agorastos et al., 2015) on stress-related autonomic reactivity, as measured by HRV measures.

It is very important to note that this study is measuring differences at specific time points of the day after nocturnal HPA axis stimulation (i.e. single-time point measurements), which can only reflect indirect effects of the experimental treatment conditions 9 hours later. The complex pharmacokinetics of DEX and MET and their yet not fully understood interrelated pharmacodynamics, in addition to the nocturnal circadian phase with evolving different sleep stages and huge changes of HPA axis activity (e.g., nocturnal cortisol rise) and circadian gene expression, represent vital limitations. Our results, thus, represent only a small piece of evidence in the highly complex HPA/ANS interplay, which remains to be further investigated. Future studies could also shed more light to the large number of pathways possibly responsible for the negligible HRV differences between baseline and post-DEX conditions reported here, in contrast to our initial hypothesis.

Finally, some additional limitations of our study merit discussion. Because of rigid exclusion criteria, the combined endocrine challenge and its time intensive nature, our study investigated only a relatively small number of healthy volunteers. Our findings should, thus, be replicated in larger study populations. On the other hand, all subjects were extremely carefully selected to minimize the probability of

medical (e.g., medication use, depression) and behavioural (e.g., substance, tobacco and alcohol use) confounders. It is particularly important to note that across all parameters investigated, no subject had a cardiovascular history and deviating laboratory or physical tests. We particularly accounted for several laboratory markers (e.g., fasting glucose, hemoglobin A1c levels, cholesterol/lipoproteins, pro-inflammatory cytokines, acute-phase proteins) and certain lifestyle habits (e.g., drug, alcohol or tobacco intake) that have been shown to be associated with ANS dysregulation altering cardiovascular measures including HRV (Dinas et al., 2013; Thayer and Sternberg, 2006). With respect to our baseline HRV values, mean RR (NN) and RMSSD were found in the physiological range for healthy humans (Nunan et al., 2010) and only the SDNN values in our study (93-98 ms) slightly exceed the upper range of 93 ms listed by Nunan et al. (2010). This is probably attributed to the supine position of our study volunteers during the ECG recordings, as supine positioning is known to increase HRV when compared to other postures such as sitting or standing (Young and Leicht, 2011). However, as disturbed sleep is also associated with autonomic alterations (Nielsen et al., 2010), it is important to note that we did not objectively measure sleep quality in our initial assessments and can herewith not exclude sleep quality-related bias. In addition, although having excluded actual menstruation, we have not controlled for the specific menstrual phase of women participants. Secondary analyses investigating the impact of peri- and postmenopausal status (2 and 4 women, respectively) on our results, did not alter our findings. Respiration should be added as additional measure in future studies since particularly deep breathing profoundly increases HRV, which is referred to as respiratory sinus arrhythmia. Finally, overall the dynamic ANS and endocrine changes along the time scale from pharmacological intervention to the measurement interval (her after 9 h) need to be checked in future studies to develop a mechanistic understanding of the reported long-term effects.

Conclusions

Variation in the activity of the stress-responsive ANS and HPA axis is particularly important for adaptive stress responses and may thus give rise to individual differences in resilience as ability to cope with stressful events. Studies about effects of HPA axis activity on baseline autonomic activity have not been reported so far in healthy subjects. By assessing autonomic responses through linear and nonlinear analyses of HRV to endocrine HPA axis challenges, we provide first data that metyrapone treatment is associated with reduced vagal tone, potentially via elevated long-lasting CRH action, while HPA axis suppression has no distinct effect on autonomic activity. Our study supports a vital role of the PNS in the interplay between ANS and HPA axis, as well as in the modulation of stress-related cardiovascular responsiveness. PNS activity may, thus, particularly affect susceptibility to stress-related disorders and represent an important pathway towards the higher cardiac mortality seen in these disorders. In addition, our results underline the utility of HRV as potential biomarker for stress system sensitivity and vulnerability to stress-related disorders. Future studies are needed to replicate this finding and to further explore the functional contribution of PNS on stress responsiveness in order to mechanistically understand its role over SNS in the pathophysiology of stress-related disorders.

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Chapter 6

Inverse autonomic stress reactivity in depressed patients with and without prior history of depression.

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Abstract

Background: There is a considerable association between major depressive disorder (MDD) and cardiovascular disease, most possibly relying on abnormalities in the autonomic nervous system (ANS)-related cardiac reactivity, although the exact underlying pathophysiological pathway is unclear. This study tends to shed some additional light on this background by investigating ANS reactivity in MDD with respect to previous depression history through an objective stress challenge paradigm.

Methods: The study assessed the effects of an overnight hypothalamus-pituitary-adrenal (HPA) axis stimulation with metyrapone (MET) on baseline ANS activity through linear and non-linear heart rate variability (HRV) measures in the morning of two continuous days in a group of 14 physically healthy, antidepressant-free patients with clinical, non-psychotic MDD, to investigate differences in autonomic reactivity with respect to prior MDD history.

Results: The main findings of this study include statistically significant time x group interactions with respect to several HRV measures, suggesting substantial differences on autonomic reactivity between patients with and without depression history. Hereby, recurrent-episode MDD patients showed lower vagal activity, while first-episode MDD patients increased vagal activity after HPA axis stimulation.

Conclusions: These findings indicate that HPA axis stimulation in MDD patients leads to inverse vagal response according to MDD history. We suggest that chronic stress system overactivation, as found in MDD, might lead to a progressive inversion of the original stress response through HPA axis and ANS divergence over the course of a recurrent illness. HRV could, thus, represent a significant biomarker in MDD with temporal sensitivity.

Introduction

Major depressive disorder (MDD) is a disease of high lifetime prevalence and constitutes worldwide the leading cause of disability, showing a broad range of physical co-morbidities and higher overall mortality (Otte et al., 2016). In particular, there is a considerable comorbidity between MDD and cardiovascular disease, most possibly relying on abnormalities in the autonomic nervous system (ANS)-related cardiac reactivity (Grippo and Johnson, 2009), although the exact underlying pathway is still unclear. ANS-related cardiac reactivity, as measured by heart rate variability (HRV) analysis has, thus, received considerable attention in depression research. A recent metanalytic review confirm lower HRV due to reduced vagal but not increased sympathetic activity in MDD (Kemp et al., 2010).

Along with ANS dysregulation, MDD is characterized by a distinct neuroendocrine profile with hypothalamic-pituitary-adrenal (HPA) axis hyperactivity (Otte et al., 2016). The HPA axis and ANS are both integrated components of an internal neuroregulatory system (central autonomic network) and are increasingly studied together, as their activity normally shows a certain degree of analogy and complementarity at several levels. However, up to date, only very few studies (Licht et al., 2010) have simultaneously assessed this dynamic interplay between the two systems in general, but also in MDD in particular. In addition, there are no reports on ANS reactivity to objective stress challenges in patients with a current MDD with respect to depression history.

Thus, the main objective of our study was to assess ANS reactivity in MDD patients according to their MDD history status (i.e., first vs. recurrent episode) using an objective stress challenge of overnight pharmacoendocrine HPA axis stimulation. Given the blunted HPA axis feedback circuits with altered glucocorticoid and corticotropin-releasing hormone signaling found in chronic MDD (Otte et al., 2016), we hypothesized that recurrent MDD would impact on ANS regulation, leading to blunted effects in ANS stress reactivity with disease progression.

Methods

The study assessed the effects of HPA axis stimulation with metyrapone (MET) on baseline ANS activity through linear and non-linear HRV measures in the morning of two continuous days (Day 1: baseline and Day 2: post-MET) in a group of antidepressant-free patients with a clinical, non-psychotic MDD and without other physical and psychiatric comorbidities, in order to investigate differences in autonomic reactivity with respect to prior MDD history. The study was conducted at the Department of Psychiatry and Psychotherapy, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. All patients were recruited through our specialized depression outpatient unit and provided written informed consent. Inclusion criteria included age of 18 - 65 years, a DSM-IV-TR diagnosis of non-psychotic MDD, single or recurrent, through a structured face-to-face clinical interview by specially-trained psychiatrists and an additional confirmatory minimum cut-off score for at least moderate depression on both the German version of the clinician-rated Inventory of Depressive Symptomatology (IDS_{30}-C) and the Hamilton Depression Rating Scale (HDRS) (cut-off scores 23/24 and 17/18, respectively). Exclusion criteria included presence or self-reported history of any physical and Axis I mental co-morbidities, history of psychotic MDD, in addition to all other criteria described in detail in healthy samples previously (Agorastos et al., 2019). History of childhood maltreatment and sleep quality were assessed by the Childhood Trauma Questionnaire (CTQ) and the Pittsburgh Sleep Quality Index (PSQI), respectively, and are presented as a total score. Adverse side effects were assessed through a German version of the UKU side effects rating scale.

All subjects were encouraged to maintain a regular sleep time around 11.00 p.m. for both nights before study days 1-2, with wake-up at 7.00 a.m. and avoidance of physical strain and any intake of food or beverages on both study mornings. Subjects received 1 g of MET (Metopiron®, Novartis, Arnhem, Netherlands) orally at 11.00 p.m. on Day 1, to assess its effects on HRV the next morning (Day 2, ~ 9 h after metyrapone intake). MET blocks the enzymatic conversion of 11-deoxycortisol to cortisol and leads to a rapid cortisol fall and a

decreased cortisol-mediated negative feedback at hypothalamic and pituitary levels, increasing CRH and ACTH secretion. The MET overnight stimulation test (MST) test is considered to be a sensitive test to evaluate the ACTH reserve and it is useful to evaluate the response of the HPA axis (Fiad et al., 1994). After an initial blood draw (8.45 a.m. of each day), all subjects were given 15 min in sitting and 15 min in supine position in a single bedded room before the ECG recording was initiated (9.15 a.m. of each day). All subjects stayed in bed for 75 min with at least 60-min continuous ECG recording, allowing to generate a recording interval of sufficient duration for nonlinear analysis based on long-lasting heartbeat interval correlations without transient data loss (Meyer and Stiedl, 2003). The assessed HRV variables have been selected according to the guidelines for short-term recordings of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (Camm et al., 1996). Detailed description of ECG recording, data export and editing and linear and non-linear HRV analysis can be found elsewhere (Agorastos et al., 2013; Agorastos et al., 2019). A detailed description of further screening and study procedures is presented in our prior publication (Agorastos et al., 2019).

Results

We collected and analysed data from 14 Caucasian study completers with MDD. Nine patients (64.3%) reported previous depressive episodes. Patients did not differ according to depression history (first *vs*. recurrent episode) with respect to age, BMI, and baseline depression, childhood trauma exposure and sleep scores, gender distribution or smoking status, nor did they differ with respect to baseline endocrine parameters (Table 1). Side effects as per UKU ratings did not differ significantly between days indicating no significant adverse effects (*data not shown*). Adjusted means (SEM) of HRV measures at Day 1 (Baseline) and Day 2 (post-MET) are presented at Figure 1.

Table 1. Demographic, physical and psychometric measures in MDD patients and group differences between patients according to history of prior depressive episodes.

Value	Total Sample	First MDD Episode	Recurrent MDD Episode	*Group differences*		
		N (%)		χ^2	*p*	
Participants	14 (100.0%)	5 (35.7%)	9 (64.3%)			
Males	5 (35.7%)	2 (40.0%)	3 (50.0%)	.062	.803	
Smokers	8 (57.1%)	3 (60.0%)	3 (33.3%)	.933	.334	
		Mean (SEM)		*F*	*t*	*p*
Age (yrs)	37.3 (2.4)	36.7 (3.9)	37.7 (3.2)	.005	-.184	.857
BMI (kg/m²)	25.1 (1.2)	24.1 (1.8)	25.6 (1.7)	1.107	-.545	.595
		Mean (SEM)		*F*	*t*	*p*
PSQI	12.7 (1.3)	15.0 (2.3)	11.5 (1.7)	.341	1.230	.247
CTQ	37.4 (3.7)	38.4 (3.7)	36.8 (5.5)	.501	.203	.843
HDRS	20.9 (.9)	20.6 (1.1)	21.1 (1.3)	.384	-.270	.792
IDS-C	41.0 (1.6)	38.0 (3.5)	42.5 (1.6)	.497	-1.369	.201
		Mean (SEM)		*F*	ηp^2	*p*
CORT - Day 1	347.1 (44.5)	356.7 (62.3)	341.7 (45.4)	.035	.004	.856
ACTH - Day 1	50.5 (8.2)	44.4 (13.6)	54.0 (9.9)	.303	.037	.597
CoP - Day 1	5.1 (.8)	6.3 (1.4)	4.4 (1.1)	1.010	.112	.344
CORT - Day 2	333.9 (30.6)	387.1 (59.9)	304.4 (43.6)	1.157	.126	.313
ACTH - Day 2	107.5 (17.6)	107.3 (32.6)	107.6 (23.8)	.000	.996	.000
CoP - Day 2	5.6 (.8)	6.6 (1.5)	5.0 (1.1)	.752	.411	.086

Table Legend

175 depressed patients were screened. 61 patients were found eligible for participation in the study. Of those, 42 declined participation at the time of the scheduling phone call, 4 did not attend at scheduled appointment and 1 dropped out due to mild gastrointestinal side effects on Day 1 by MET. We collected and analysed data from 14 Caucasian study completers with MDD. Values are presented as total numbers (percent of total) and means (SEM). Psychometric scores report total scores. BMI: Body Mass Index; PSQI: Pittsburgh Sleep Quality Index; CTQ: Childhood Trauma Questionnaire; IDS-C: Inventory of Depressive Symptomatology

– Clinician; HDRS: Hamilton Depression Rating Scale. Group differences were assessed through linear analysis of *t*-test (parametric after ln-transformation), *chi*-squared test (nominal) or ANCOVA controlling for age, gender, BMI and smoking after ln transformation. None of the patients of the final sample had received additional medication (i.e., antihypertensive medication or thyroid hormone substitution). Plasma levels of cortisol (CORT, ng/ml), copeptin (CoP, pmol/l) and adrenocorticotropic hormone (ACTH, pg/ml) at the morning of the two consecutive days were determined using commercially available immunoradiometric assays and radio-immunoassays (DRG International, USA; MP Biomedicals, Solon, USA; BRAHMS Kryptor, Berlin, Germany; respectively). The lowest detection limits for cortisol and for ACTH were 0.9 ng/ml and 5.7 pg/ml, respectively. Intra- and inter-assay coefficients of variation were below 8% for all assays.

A mixed between-within subject analysis of variance (ANOVA) controlling for age, gender, BMI and smoking status was conducted to assess differences of treatment (i.e., MET) effects on HRV measures across the two time periods (Day 1, Day 2) between patients with and without depression history. Our results showed statistically significant time effects (i.e. treatment condition) with very large effect sizes on several HRV variables including RMSSD, NN50(%), HF(%) and LF/HF (Figure 1, legend), indicative of profound effects of MET stimulation challenge on autonomic activity, similarly to previous studies (Agorastos et al., 2019). There was a statistically significant group $\times$ time interaction with substantial effects with respect to NN50(%), HF(%), LF/HF and α_{fast} (Figure 1, legend), suggesting a statistically significant difference on reactivity of these HRV measures to HPA axis stimulation with MET between patients with and without depression history, while all other measures showed no statistically significant effects. Univariate ANCOVAs controlling for age, gender, BMI and smoking, revealed no statistically significant differences in CORT, ACTH and CoP plasma levels of both Days 1-2 between the two groups (*data not shown*).

Figure 1. Linear and nonlinear HRV measures at baseline and after MET challenge in patients with (recurrent episode) and without (first episode) depression history.

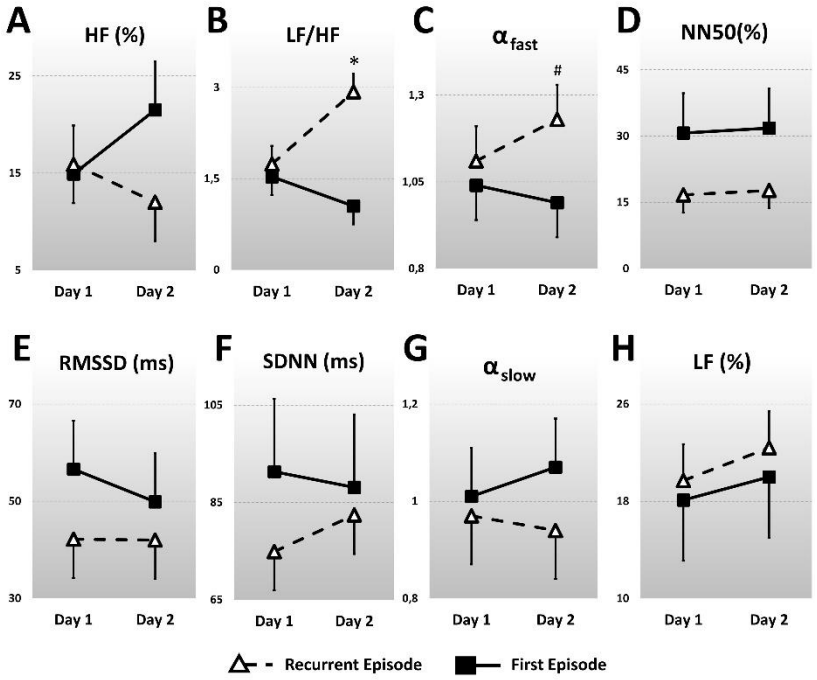

Figure Legend

Day 1: Baseline, Day 2: Post-MET. Panels A-D: Statistically significant time x group interactions. Panels E-H: Statistically non-significant time x group interactions. Values are presented as mean values (SEM) adjusted for age, gender, BMI and smoking. Mean heart rate is not presented in the figure because of negligible interaction (Wilks λ = 1.000; F = .000; p = .999, ${}_p\eta^2$ = .000). SDNN: standard deviation of the N-N intervals; RMSSD: root mean square of subsequent N-N interval differences; NN50(%): percentage of the number of pairs of adjacent NN intervals that differ in length by >50 ms; LF: low frequency 0.04-0.15 Hz; HF: high frequency 0.15-0.4 Hz; LF (%) and HF (%): percentage of each frequency component from the total power; α: scaling coefficient alpha of nonlinear analysis. Mixed between-within subject ANOVAs were conducted to assess the impact of treatment (MET) on dependent variables across the two time periods (Day 1, Day 2) controlling for age, gender, BMI and smoking after ln transformation to assess differences between

treatment conditions. To correct for potentially inflated type I error because of multiple comparisons we used the false discovery rate (FDR) approach with a 5% threshold. Our results showed statistically significant time effects (i.e. treatment condition) with very large effect sizes on RMSSD, NN50(%), HF(%) and LF/HF (Wilks λ = .530; F = 6.220; p = .041, $_p\eta^2$ = .470; Wilks λ = .474; F = 7.777; p = .027, $_p\eta^2$ = .526; Wilks λ = .469; F = 7.931; p = .026, $_p\eta^2$ = .531; Wilks λ = .525; F = 6.339; p = .040, $_p\eta^2$ = .475, respectively) and statistically significant group $\times$ time interactions with substantial effects, with respect to NN50(%) (Wilks λ = .519; F = 6.483; p = .038, $_p\eta^2$ = .481), HF(%) (Wilks λ = .292; F = 17.006; p = .004, $_p\eta^2$ = .708), LF/HF (Wilks λ = .154; F = 38.579; p < .001, $_p\eta^2$ = .846) and α_{fast} (Wilks λ = .456; F = 8.344; p = .023, $_p\eta^2$ = .544) after controlling for age, gender, BMI and smoking status. A post-hoc group comparison for each time point separately controlling for age, gender, BMI and smoking status, revealed no statistically significant differences between the two groups at Day 1 (*data not shown*). On Day 2 there was a statistically significant difference between the two groups only with respect to LF/HF (F = 5.774, p = .047, FDR analysis indicated a potential type I error here), as patients with depression history had higher values than patients without. Secondary analyses, investigating the impact of peri- and postmenopausal status (1 and 2 women, respectively) on our results, did not alter our findings (*data not shown*).
* p < .05 ; $^\#$ .05 < p < .1

Discussion

The mechanistic understanding of ANS and HPA axis coupling could be of major importance for the understanding of the pathophysiology of stress-related disorders such as MDD and their comorbidity with cardiovascular disease. This study investigated the effects of endocrine HPA axis stimulation through MST on baseline autonomic activity through HRV analysis in MDD patients with and without history of prior depressive episodes (i.e., first vs. recurrent episode). To the best of our knowledge, this is the first study investigating the association of depression recurrence on the direct interplay between the HPA axis, resting ANS activity and reactivity in MDD patients. Therefore, linear and nonlinear (unifractal; detrended fluctuation analysis) methods for analysis of HRV and its dynamics were combined as in previous studies (Agorastos et al., 2013; Agorastos et al., 2019), to examine autonomic effects at rest on two consecutive days (i.e., Day 1: baseline; Day 2: post-MET). HRV constitutes the best-established, non-invasive method of analysis of autonomic activity, while non-linear analysis provides a highly sensitive measure of

autonomic regulation that improves HRV assessment (Meyer and Stiedl, 2003). Unfortunately, to date, this method is still only applied in a relatively limited number of related publications on human data due to its complexity and need of longer ECG recording intervals.

The main findings of this study include: i) no statistically significant differences of baseline autonomic state depending on depression history, but ii) significantly higher post-MET LF/HF values in MDD patients with depression history and iii) statistically significant time x group interactions with respect to NN50(%), HF(%), LF/HF and α_{fast}, suggesting substantial differences on autonomic reactivity to HPA axis stimulation between patients with and without depression history, although the two groups did not differ with respect to plasma endocrine levels of CORT, ACTH and CoP and baseline HRV measures.

Given that increased α_{fast} and LF/HF and decreased HF(%) values reflect reduced tonic parasympathetic (PNS) activity (Meyer and Stiedl, 2003), the metyrapone-related changes of HF(%), α_{fast} and LF/HF in the two groups jointly reflect lower PNS activity in recurrent-episode MDD patients and increased PNS activity in first-episode MDD patients after HPA axis stimulation. Taken together, these findings indicate that HPA axis stimulation in MDD patients leads to inverse vagal response according to MDD history with an HRV reduction in MDD patients with and an HRV increase in MDD patients without prior history of the disease.

Our results support the fact that HRV resting activity and reactivity, although correlated, represent different regulatory processes. The interpretation of our findings should acknowledge the fact that HPA axis stimulation through MET in healthy individuals has been associated with reduced vagal tone in our previous study (Agorastos et al., 2019). This means that MDD patients with prior depression history show similar changes in HRV measures after HPA axis stimulation, as found in healthy samples, while first-episode MDD patients show an inverse reaction. Interestingly, a prior study assessing HRV reactivity to a mental stress task (Liang et al., 2015) showed similar HRV reactions with increased vagal tone in first-episode MDD patients, confirming the results of this study.

This atypical ANS reactivity suggests a more passively disengaged than necessary active physiological stress reactivity over the course of the MDD. Chronic stress system activation, as found in MDD (Otte et al., 2016), might thus lead to a continuous alteration and even inversion of the original autonomic response over the course of a recurrent illness. Such effects have been shown with respect to the HPA axis activity in an animal model of prolonged stress exposure (Marti et al., 1994), and also in MDD patients with recurrent episodes (Ehnvall et al., 2004). Our study is the first that suggests similar effects also with respect to ANS reactivity. Interestingly, there seems to be a close association between developmental risk factors for MDD and a progressive development of an atypical ANS reactivity (Hamilton and Alloy, 2016). The fact that our patients did not show an accompanied inverse reaction of HPA axis measures might rely to the previously suggested progressive divergence of the HPA axis and the ANS activity following chronic stress, possibly through blunted HPA axis feedback circuits with altered glucocorticoid and corticotropin-releasing hormone signaling (Raison and Miller, 2003), as found in MDD (Otte et al., 2016).

Finally, some limitations merit discussion. Because of rigid exclusion criteria, and the combined endocrine challenge, our study investigated a relatively small number of patients. In addition, we did not have access to objective information regarding previous episodes and their duration in patients with depression history. Thus, our findings should be considered preliminary and need replication in larger studies. On the other hand, all subjects were carefully selected to minimize the probability of medical and behavioural confounders and we collected a very large amount of data needed for the nonlinear HRV analysis. Our study is one of the few utilizing nonlinear HRV analysis in MDD. In addition, our patients reported similar depression scores and baseline morning CORT plasma levels as in reference studies (Gili et al., 2012; Otte et al., 2010), suggesting that we included a representative sample of MDD patients. Finally, we acknowledge that nocturnal HPA axis stimulation can only lead to indirect effects of the experimental

treatment conditions 9 hours later, underlining the highly complex and dynamic HPA/ANS interplay.

Individual differences in ANS reactivity are important for adaptive responses and may explain individual differences in stress resilience, suggesting that HRV could represent a significant biomarker of MDD progression. Prospective longitudinal studies are needed to evaluate ANS reactivity along MDD etiology and progression to better understand temporal effects on stress-system-related reactivity alterations and their role in the increased cardiovascular risk found in this population.

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Chapter 7

General Discussion

Synopsis of main findings

Serotonergic, glutamatergic and HPA axis influences on autonomic reactivity and health

Neurobiophysiological theoretical models

Future research and clinical implications

Conclusions

SYNOPSIS OF MAIN FINDINGS

Due to its complexity and limited functional knowledge of the anatomically well described CAN, the molecular and cellular basis for the normal and compromised brain-heart network in stress and stress-related disorders is still a widely unexplored area. Using objective (pharmacological or endocrine) stress provocation challenges and linear and non-linear heart rate variability analyses as a readout of central autonomic activity, this dissertation explored biological factors that objectively modulate central autonomic activity and reactivity to stress in humans.

In **Chapter 2**, our first study assessed autonomic activity and reactivity to stress in association with the genotype of the serotonin transporter genotype 5-HTTLPR in healthy individuals and indicated enhanced sympathetic and/or diminished cardiac vagal activity and blunted autonomic reactivity to stress in subjects with the s/s genotype in comparison to the l/l genotype for the 5-HTTLPR.

In **Chapter 3**, our second study assessed the influence of long-term treatment with SSRIs on autonomic activity and reactivity to stress in healthy individuals and indicated that long-term SSRI treatment with escitalopram shows no significant effects on baseline autonomic activity, but a significant increase of vagal tone and a blunted autonomic reactivity to stress.

In **Chapter 4**, our third study assessed the influence of $mGluR_{2/3}$ agonism on autonomic activity and reactivity to stress in healthy individuals and indicated that $mGluR_{2/3}$ agonism with LY544344 shows no significant effects on baseline autonomic activity, but a significantly enhanced autonomic recovery after stress.

In **Chapter 5**, our fourth study assessed the effects of overnight neuroendocrine HPA axis modulation on resting ANS activity in healthy individuals and indicated that HPA axis stimulation (by using metyrapone) is associated with reduced vagal tone, while HPA axis suppression (by using dexamethasone) has no effect on autonomic modulation of heart function.

In **Chapter 6**, our fifth study assessed the effects of overnight neuroendocrine HPA axis modulation (both stimulation and suppression) on resting ANS activity in non-medicated MDD patients in association with illness history and

indicated that positive history of prior MDD episodes showed no statistically significant effect of baseline autonomic state but distinct effects on autonomic reactivity to HPA axis stimulation (by using metyrapone) with inverse vagal response and lower vagal activity in comparison to first-episode patients.

Taken together, these results underline the complex functional balance of stress system activity and reactivity and highlight an important role for serotonergic and glutamatergic signaling, as well as for HPA axis influence on CAN activity, thereby confirming and extending previous studies. These findings underscore the overlap of main regulatory systems in stress activation and the association between stress-related disorders, CAN dysregulation with compromised neuroautonomic control and somatic, in particular, cardiovascular morbidity and mortality in patients with stress-related disorders.

SEROTONERGIC, GLUTAMATERGIC AND HPA AXIS INFLUENCES ON AUTONOMIC REACTIVITY AND HEALTH

In contrast to the physiological, time-limited compensatory stress response to acute stressors, the stress system activation may be altered either by excessive types of stress (prolonged persistent or repeated stressors, traumatic stress, early life stress, etc.) or by psychological/cognitive or biological individual factors, that surpass the physiological regulatory capacity of the organism, leading to a vulnerable phenotype characterized by disrupted HPA axis and ANS reactivity [1-3]. The main objective of this dissertation was to explore biological factors that objectively modulate central autonomic reactivity to stress in humans using objective (pharmacological or endocrine) stress provocation challenges and heart rate variability analyses as a readout of central autonomic activity.

Serotonergic signaling, stress and ANS reactivity

The central serotonergic system is heavily involved in the regulation of stress and anxiety [4-6]. Exposure to acute stress has repeatedly shown to provoke central

serotonergic responses with an increase of 5-HT synthesis, release and turnover in various brain areas. However, activation of the central serotonergic system can stimulate both anxiogenic and anxiolytic pathways, depending on different serotonin receptors types stimulated and specific brain region [7]. Anatomical and functional evidence supports the existence of projections from the medial PFC moderating (activation and inhibition) the activity of 5-HT neurons of the dorsal raphe nucleus [8]. Thus, different 5-HT receptor types may have reciprocal roles in mediating effects of stress. Accordingly, chronic stress can lead, for example, to a decrease in 5-HT_{1A} and an increase in 5-HT_{2A} receptor density, the two major receptor types involved in stress neurocircuitry [9]**.** Stress-induced alterations in 5-HT activity particularly occur in brain regions that are part of the stress neurocircuitry and many of these (e.g., amygdala, hippocampus, ventral striatum and mPFC) [10-13] are also implicated in the pathophysiology of depression, PTSD and other psychiatric stress-related disorders.

Central serotoninergic transmission is not only involved in the modulation of emotional and cognitive behaviour [14], but also in autonomic (mainly vagal) regulation [15-20], as shown by the observation that the activity and functional connectivity of the CAN is majorly influenced by serotonergic signaling [21-23]. Consequently, altered serotonin regulation in the CNS is associated with autonomic dysregulation, while reduced CNS serotonergic activity is linked to sympathetic dominance and also elevated autonomic responsiveness to stressors [19, 20, 24].

ANS reactivity and the 5-HTTLPR genotype

Identification of genetic factors that influence stress reactivity is of major importance for the linkage of psychosocial and environmental stress factors to disease outcome [25]. Central serotonergic activity is partly regulated by the serotonin transporter (5-HTT) that lowers serotonin action through its uptake from the synaptic cleft. The 44-base pair insertion/deletion length polymorphism in the 5' regulatory region of the human 5-HTT gene is of specific interest [26]. The common variation in the 5-HTT gene linked polymorphic region (5-HTTLPR) consists

of two alleles, labeled “s” (short) and “l” (long), and the short variant is associated with reduced transcriptional efficiency of the 5-HTT gene [27].

By assessing autonomic responses to a defined pharmacological stress challenge in association with variation of the serotonin transporter genotype, **our first study (Chapter 2)** indicated enhanced sympathetic and/or diminished cardiac vagal activity and blunted autonomic reactivity in subjects with the 5-HTTLPR s/s genotype supporting an important role of 5-HTTLPR genotype in the modulation of the magnitude of acute cardiovascular responsiveness, which may affect susceptibility to stress-related disorders [28]. Thus, our study provides novel data supporting claims that the s/s genotype represents a genetic vulnerability factor [29] associated with inadequate hyporeactivity to stress and extends current knowledge on the impact of central serotonergic neurotransmission for autonomic regulation.

These findings are in accordance to the study by Ellis et al. [30], reporting reduced resting respiratory sinus arrhythmia as indirect measure of ANS activity in s/s allele carriers. The initial reactivity hypothesis proposed *enhanced* cardiovascular autonomic reactivity to be associated with increased cardiovascular risk as a mediator of psychosocial and behavioral risk factors [31, 32]. However, in the last years research provided robust evidence that *reduced* cardiovascular reactivity and slower recovery are associated with overall cardiovascular risk [33, 34]. Our results support this hypothesis, because the s/s carriers showed both enhanced sympathetic and/or reduced vagal baseline activity and blunted autonomic reactivity to an objective stress challenge by intravenous application of CCK-4.

Lower stress-related heart rate reactivity to psychological stressors has been linked to sleep deprivation [35], atherosclerosis and history of cardiovascular disease [33, 36], obesity [37], smoking [38], depression [31, 34, 39-41], psychiatric symptom severity [42], childhood adversities or other trauma [42, 43], reduced cortisol reactivity [44], poor cognitive ability [44], higher perceived stress [45] and poor general self-reported health [31]. This fact could possibly indicate a corresponding under-recruitment of brain systems during prolonged mental stress [46]. The s/s genotype has also been associated with these states [47-56]. Thus, our

findings are in accordance with a prior study also reporting higher cardiovascular reactivity in l/l carriers, but question the interpretation of increased cardiovascular risk in the l/l genotype [57] and conclude that it is indeed the s/s genotype, which contributes to increased cardiovascular risk through autonomic hypo-responsiveness [58]. Importantly, the 5-HTTLPR genotype, also seems to have a significant impact on the individual risk for depression and PTSD after severe stress exposure [55, 59, 60].

ANS reactivity and SSRI treatment

Central serotonergic activity of the CNS can be pharmacologically modulated. SSRIs are currently the most frequently prescribed and best characterized therapeutic compounds affecting multiple central projection pathways in the topography of serotonin function [61, 62] and represent the first-line pharmacological treatment option in stress-related disorders such as depression and PTSD [63, 64]. Binding of these drugs to the (5-HTT) leads to its negative allosteric modulation, which effectively inhibits its ability to reuptake serotonin from the synaptic cleft [65]. Although acute SSRI action is associated with rather modest synaptic serotonin increase due to negative feedback loops through somatodendritic 5-HT_{1A} autoreceptors, chronic SSRI administration leads to desensitization of 5-HT_{1A} autoreceptors and downregulation of the negative feedback inhibition and, thus, results in increased serotonin release at postsynaptic heteroreceptor sites [61, 66]. Nevertheless, the existence of functional subpopulations of serotonergic neurons acting at numerous sites of the CNS and the evidence for their tight control by stress hormones [6, 18] suggest a complex interplay of central serotonergic activity with ANS and HPA axis function towards maintenance of homeodynamics during stressful events and adaptation processes impacting on cardiac stress responsiveness.

However, studies about effects of SSRIs on autonomic reactivity using pharmacological stress challenges have not been reported so far in healthy subjects. By assessing autonomic responses to a defined pharmacological panic challenge, **our second study (Chapter 3)** provided first data that chronic SSRI treatment is

associated with reduced autonomic reactivity in healthy subjects. These results, thus, support the important role of central serotonergic activity in the modulation of the magnitude of acute autonomic responsiveness [67].

Following the initial reactivity hypothesis, our data would suggest that the reduced autonomic reactivity to CCK-4 challenge observed in the SSRI group may represent a marker of reduced cardiovascular risk, similarly as postulated in a prior study [57]. This is also supported by the statistically absent differences in baseline HR between the two treatment conditions, as resting HR is considered an independent predictor of cardiovascular risk [68]. SSRI treatment has shown beneficial cardiovascular effects in cardiovascular disease, and these effects were associated with reduced autonomic reactivity. Similarly, advantageous cardiovascular effects of SSRIs leading to normalization of autonomic measures have been also reported in psychiatric disorders, which have been associated with increased [69-71] or reduced [42, 72, 73] autonomic reactivity.

On the contrary, since robust evidence was provided in the last years that it is rather the reduced cardiovascular reactivity and slower recovery that are associated with overall cardiovascular risk [33, 34], our findings of a reduced autonomic reactivity in the SSRI group would support an increased cardiac risk under chronic SSRI treatment in healthy individuals. Unfortunately, our study did not assess data on cardiac recovery after the stress challenge, which is also a major indicator of cardiac reactivity [34]. Thus, it cannot be unambiguously resolved on the basis of the currently used measures whether the reduced stress responsiveness reflects a beneficial ('anxiolytic-like') or a maladaptive ('hypo-responsiveness') state. Nonlinear measures [74] may be beneficial to provide for an unambiguous interpretation of physiological versus pathological change but cannot be applied with short ECG recordings as used here.

Finally, as several long-term SSRI effects (e.g., neuroplastic and neurotrophic changes, effects on gene-expression, anti-inflammatory properties) have been postulated in addition to the simplistic monoaminergic pharmacological

effects of these drugs [66, 75], different pathways of action may be also responsible for the observed autonomic effects.

Glutamatergic signaling, stress and ANS reactivity

An optimal balance between inhibitory and excitatory neurotransmission in the CNS is critical for adaptive and physiological brain function and behavior and relies on the functional interaction between γ-amino butyric acid (GABA – the main inhibitory neurotransmitter) and glutamate (the main excitatory neurotransmitter). Glutamate has a very diverse role in the CNS. In particular, glutamatergic neurotransmission has been implicated in emotion and anxiety/fear regulation, which makes it particularly relevant for stress-related disorders [76]. Glutamatergic neurotransmission is modulated by three families of ionotropic and three groups of metabotropic, G protein-coupled glutamate receptors (mGluR) [77, 78]. This distinct receptor diversity in combination with the complex distribution in the CNS brain areas makes research on glutamatergic modulation of physiological, molecular, and behavioral consequences of stress quite difficult. Nevertheless, accumulating evidence indicates relevance and potential therapeutic usefulness of Group II mGluRs in chronic stress-related disorders [79].

Group II mGluRs ($mGluR_{2/3}$) are predominantly found in CNS regions involved in arousal and emotion and modulate synaptic neurotransmission [80, 81]. Both $mGluR_2$ and $mGluR_3$ are presynaptically localized in mostly preterminal axonal portions, where they act as autoreceptors, decreasing synaptic transmission as a consequence of adenylate cyclase inhibition [82]. $mGluR_2$ are highly expressed in pyramidal neurons of cortical regions and in granule cells of the dentate gyrus, while $mGluR_3$ in the cerebral cortex, the caudate-putamen area, as well as in the granule cells of the dentate gyrus [79]. In addition, $mGluR_{2/3}$ agonism suppresses 5-HT-induced excitatory postsynaptic currents in the PFC [83], while, vice versa, higher 5-HT levels lead to a decrease in glutamatergic signaling and a parallel increase in GABA transmission, particularly in the hippocampus, frontal cortex and cerebellum, suggesting a close coupling between $mGluR_{2/3}$ and serotonergic system [84].

Accordingly, $mGluR_{2/3}$ agonists have been investigated in experimental conditions of fear, anxiety and stress sensitization, and suggested as a new pharmacotherapeutic option in the treatment of anxiety disorders [85]. Interestingly, $mGlu_{2/3}$ receptor antagonists show distinct antidepressant effects similar to that of ketamine in animal research [86], while $mGlu_{2/3}$ receptor agonists and have additionally been investigated in experimental fear, anxiety and stress sensitization animal research [85], but also in humans as a new pharmacotherapeutic option [87].

In addition to their role in emotional and arousal regulation, $mGluR_{2/3}$ also particularly modulate excitability of neurons of the CAN and brainstem circuits and, thus, participate in the fine-tuning of the cardiovascular autonomic control through glutamatergic modulation [88-90]. Nevertheless, to date, no study has reported on autonomic $mGluR_{2/3}$ effects in humans. In this dissertation, first data on $mGluR_{2/3}$ agonism effects on autonomic reactivity in healthy individuals is reported, using HRV data, as one of the best-established non-invasive methods to assess ANS activity [91]. By assessing the effects of an $mGluR_{2/3}$ agonist (LY544344) on autonomic responsiveness, **our third study (Chapter 4)** indicated significantly enhanced autonomic recovery through $mGluR_{2/3}$ agonism to a pharmacological stress challenge with CCK-4 in healthy subjects, confirming the important role of glutamatergic transmission via $mGluR_{2/3}$ in central autonomic regulation. Given that enhanced autonomic recovery is associated with lower cardiovascular risk [33], $mGluR_{2/3}$-agonism effects can be considered beneficial for the organism's adaptive response capacity.

These effects on cardiac ANS reactivity could be mediated directly (i.e., through modulation of the CAN) [88-90] or indirectly (i.e., through modulation of baroreceptor signal transmission) [88, 92, 93]. For example, $mGluR_{2/3}$ modulation of presympathetic neurons in the PVN of the hypothalamus has been shown to be crucial for the regulation of sympathetic output and autonomic vasomotor control [80], with increased glutamatergic excitatory input to the PVN, leading to PVN hyperactivity and increased sympathetic outflow in animal models of hypertension [89, 94]. Interestingly, in hypertensive rats, activation of group II metabotropic

glutamate receptors in the PVN leads to attenuation of increased glutamatergic input and neuronal hyperactivity and inhibits sympathetic vasomotor tone [95]. In addition, $mGluR_{2/3}$ are also involved in the functional connection between NTS neurons and parasympathetic preganglionic neurons of the DMV, as well as to other areas, and modulate vagal sensory input and gastric motor responses [96]. For example, blocking $mGluR_2$ signaling in the dorsal brainstem, an area including NTS (which is involved in baroreflex pathways through glutamatergic transmission), has been shown to lead to higher blood pressure in animal research [97], while $mGluR_{2/3}$ stimulation in the same area can decrease blood pressure and sympathetic nervous activity [98]. Our findings, thus, suggest favorable effects of $mGluR_{2/3}$ agonism on stress-related autonomic responses in accordance with animal data [95, 99] and are relevant to individual stress responsiveness and anxiety and stress-related disorders susceptibility.

HPA axis influence on ANS reactivity

Activity and reactivity of the two major limbs of the stress system (HPA axis, ANS) are crucial for adaptive stress responses and may thus give rise to individual differences in resilience as ability to cope with stressful events and maintain health [100]. The HPA axis and ANS are both functionally and anatomically integrated and interrelated components of the CAN and are increasingly studied together, as their (re)activity normally shows a certain degree of analogy and complementarity at several levels [101-103]. A combined examination of the two systems can provide a more thorough understanding of the association between stress and health. The HPA axis and the ANS show extensive reciprocal innervations but also several neurohumoral mechanisms.

Anatomically, animal studies have shown that CRH neuronal afferents from the hypothalamic PVN project to the LC [104] and noradrenergic neurons from the LC project back to the PVN [105, 106]. Furthermore, amygdala and brain stem neurons of the NTS are critical for both vagal activity and regulation of HPA stress responses, as parasympathetic NTS neurons send direct projections to

hypophysiotropic CRH neurons in the amygdala and herewith also modulate HPA activity [107].

In addition, there are several further neurohumoral interactions between the two systems. For example, CRH receptors have been shown to greatly modulate several SNS and PNS centers in the CNS, including core PNS output areas [108, 109]. CRH has been reported to increase the firing rate of LC neurons and stimulate the release of NA [110]. In turn, NA promotes CRH mRNA expression in the hypothalamic PVN [105]. Accordingly, LC lesions have been shown to attenuate HPA axis responses to stressors in animal studies [111]. CRH has been also suggested to inhibit vagal or activate sympathetic outflow at least in part [112-114], while CRH_1 receptor antagonists increase cardiac vagal and decrease sympathetic activity in rats [115]. On the other hand, CRH released in the BNST during stress, contributes to an activation of the vagal outflow in rats [116].

Human studies have provided further evidence in support of HPA axis and ANS interrelation. In adolescents, higher morning and afternoon cortisol levels have been associated with low HF [117], while elevated cortisol awakening response (CAR) has been associated with reduced LF and HF among young adults [118]. However, only relatively few human studies have simultaneously assessed HPA axis and ANS activity to investigate their direct association [119, 120], while most of these studies are conducted in patients [121] or through subjective stress exposure paradigms [122, 123].

By assessing the direct effects of overnight pharmacoendocrine HPA axis challenges on ANS activity at rest, **our fourth study (Chapter 5)** indicated for the first time that HPA axis stimulation (using metyrapone) is associated with reduced vagal tone, while HPA axis suppression (using dexamethasone) has no effect on autonomic modulation of heart function in young healthy individuals and supports a vital role of the PNS in the interplay between ANS and HPA axis, as well as in the modulation of stress-related cardiovascular responsiveness.

Interestingly, autonomic activity has been proposed to partly reflect the state of this connectivity between mPFC and amygdala [124], with higher HRV

(higher PNS activity) being associated with stronger resting state functional connectivity between the amygdala and the mPFC [125]. Exaggerated amygdala activity leads to mPFC hypofunction and vice versa [126], while this interaction is majorly regulated HPA neuroendocrine levels [127, 128], with HPA axis activation being associated with stronger negative functional connectivity between these areas [129]. This inhibitory control appears to be largely vagally mediated [130-132], as also shown in our study.

HPA axis influence on ANS reactivity in depression

MDD is considered a stress-related disorder. Chronic stress precipitates depression and influences its course [100]. As stress and depression neurocircuitry and neuroendocrinology show a distinct overlap, many of the diverse biological features of MDD actually reflect dysregulations of the stress system [133, 134]. In contrast to the normally time-limited compensatory stress response to acute stressors, stress system activation due to chronic psychological stress as in MDD often leads to a prolonged or repeated activation, which may have long-term, deleterious effects on mental and physical health and may initiate and/or negatively influence disease development, natural course and outcomes [135].

MDD has been, accordingly, associated with a dysregulation of both HPA axis [136-138] and ANS [139-143] activity and reactivity since decades, influencing the whole body and increasing the risk for somatic and particularly cardiometabolic comorbidity, as well as overall mortality [144]. Interestingly, stress system dysregulation follows a complex and continuous path that transits from HPA axis over- to understimulation and is majorly influenced by the duration of exposure [100]. Thereby, the functional interconnection between HPA axis and ANS gets disrupted and the activity of both systems diverges from each other. Accordingly, the progressive divergence of the HPA axis and the ANS activity following chronic stress, has been proposed as a vital pathophysiological trajectory leading to the long-term impact of chronic stress on the stress system and the chronic preservation of symptoms [145], as in MDD. Accordingly, the better understanding of ANS and

HPA axis functional coupling could be of major importance for the understanding of the pathophysiology of stress-related disorders such as MDD and their comorbidity with cardiovascular disease.

However, this dynamic interplay between HPA axis and ANS has been simultaneously assessed only in very few studies in general [146], but also in MDD in particular. Furthermore, no studies have assessed this interaction with respect to depression chronicity/recurrence. By assessing the direct effects of overnight pharmacoendocrine HPA axis challenges on ANS activity at rest in a group of medication-free patients with MDD, **our fifth study (Chapter 6)** indicated for the first time that HPA axis stimulation in MDD patients leads to inverse vagal response according to MDD history and validates the proposition that chronic stress system overactivation, as found in MDD, might lead to a progressive inversion of the original autonomic stress response through HPA axis and ANS divergence over the course of a recurrent illness. This atypical ANS reactivity suggests actually a more passively disengaged than necessary active physiological stress reactivity over the course of the MDD, possibly through a disentanglement of the previously discussed, central neuronal and neurohumoral interactions between HPA axis and ANS, possibly through blunted HPA axis feedback circuits with altered glucocorticoid and corticotropin-releasing hormone signaling [147], as also found in MDD [133].

NEUROBIOPHYSIOLOGICAL THEORETICAL MODELS

The intimate connection between the brain and the heart was enunciated by the French physiologist Claude Bernard over 150 years ago [148]. Commenting on his work, Charles Darwin further proposed that heart and brain were vagally linked, which represents an astonishing statement for the time made [149]. The interaction between these two most important organs of the body is critically associated with normative neurodevelopment and aging [150]. Emotionally of physically driven stress-related changes in brain-heart interaction may affect healthy development and represent a vulnerability to stress and a risk factor for all-cause morbidity and

mortality. In this respect, autonomic reactivity and the interplay between ANS and HPA axis seem to hold a central moderating role.

Autonomic reactivity, emotion and cognition

The Johns Hopkins Tripartite Model of Resistance, Resilience, and Recovery suggests that the individual ability for resistance to stress, rapid and effective rebound from stress and functional improvement after stress is crucial for healthy adaptation, development of stress-related disorders and recovery treatment [151]. Research of the past two decades suggests a correlation between autonomic reactivity, emotion regulation, cognitive function and pathology, with autonomic dysregulation majorly affecting overall mental and physical health and functioning [152]. Importantly, neural circuits of autonomic regulation, attentional regulation and affective regulation that allow the organism to meet the challenges of an ever-changing environment overlap heavily. The two main conceptual-theoretical cornerstones of this perspective are represented by Porges' Polyvagal Theory and Thayer & Lane's Neurovisceral Integration Model, which implicate PNS in particular in the regulation of the central stress system.

According to Porges' polyvagal theory [153, 154], autonomic function is linked to behavior with several neural circuits involved in the regulation of the autonomic state. In particular, Porges suggested that the vagal system contains specialized subsystems with competing roles that regulate adaptive responses, which are crucial to the development of emotional experience and affective processes central to social behavior. Porges suggested that unmyelinated fibers from the dorsal motor complex are involved in regulating the "freeze response", while myelinated nerves from the nucleus ambiguous represent a vagal brake, which allows for self-regulation and ability to inhibit sympathetic outflow. His theory implies that a healthy vagal function of the ANS sets the limits or boundaries for the range of one's emotional expression, quality of communication, and ability to self-regulate emotions and behaviors. A standardized assessment of vagal tone could,

thus, serve as a potential marker for one's ability to self-regulate and mirrors different types or classes of behavior.

The Neurovisceral Integration Model is one of the most influential psychophysiological models addressing the interplay between CNS and somatic functioning (brain-body interaction). The model describes how a set of neural structures involved in cognitive, affective, and autonomic regulation are related to HRV and cognitive performance [102]. Thereby, anatomical and functional interconnections of the CAN are suggested to link the brainstem with forebrain structures and the amygdala with the mPFC through feedback and feed-forward loops and to control visceromotor, neuroendocrine, and behavioral responses critical for goal-directed behavior, adaptability, and overall health. They further propose a central role of the vagus in adaptation to the environment, through providing negative feedback on sympatho-excitatory stress responses [131]. For example, vagally-mediated HRV is linked to higher-level executive functions and the ability to inhibit unwanted memories, and reflects the functional capacity of the brain structures that support working memory and emotional and physiological self-regulation. Taken together, vagal inhibitory processes can be viewed as negative feedback circuits that allow for the interruption of ongoing behavior and the re-deployment of resources to other tasks and are, thus, central in the interplay of cognitive, affective, behavioral and physiological concomitants of normal and pathological emotional states [102].

All our studies (Chapters 2-6) reported in this dissertation support both these models and a central role of the vagal branch of the ANS in the regulation of stress reactivity and also the fact that ANS resting activity and reactivity, although correlated, represent different regulatory processes with different functional and clinical impact. This challenges the completeness of the sympathetic overactivation explanation of stress activation and anxiety. Thereby, vagal activity and its normative increase from childhood to adolescence seem to hold a key role in the proper neurovisceral integration during neurodevelopment on a structural and functional level, subsequent psychological functioning and adaptive regulation [155,

156]. However, continuous neurovisceral integration across the lifespan is constantly affected by ongoing challenges that affect vagal activity with long-term effects on mortality risk [157]. Particularly studies that investigate emotion regulation may yield interesting insights of successful stress inoculation that helps protect people from age-related decline. Thus, interventions that improve brain health also improve heart health and vice versa. [150]

ANS and HPA axis interaction

Several psychobiophysiological theories also highlight that it is actually the inter-relation between the ANS and HPA axis in the coordination of responses to stress that is particularly related to an individual's risk for adverse outcomes. The previous polyvagal theory and neurovisceral integration model actually also support that vagal activity plays an integral role in regulating HPA axis activity. Laboratory experimental research actually supports that both sympathetic and parasympathetic activity can moderate the HPA axis response to stress [130, 158]. Furthermore, Bauer et al. proposed two competing models (i.e., additive or interactive model) [159]. The additive model supports that symmetrical activation of both systems (hyper-arousal: ANS ↑ & HPA axis ↑; hypo-arousal: ANS ↓ & HPA axis ↓) increases overall health risk. In contrast, the interactive model contends that asymmetric activation (i.e., ANS ↑ & HPA axis ↓; or ANS ↓ & HPA axis ↑) decreases risk, as ideal adaptive responses need a balance between ANS and HPA axis activity. Del Giudice et al. extended the previous model by suggesting that distinct response patterns between the ANS and HPA axis are vitally influenced by early life or chronic stress and that the match or mismatch between environmental context and stress response patterns is vital for determining individual risk [160].

Interestingly, there seems to be a close association between developmental risk factors for MDD and a progressive development of an atypical ANS reactivity [139]. Similar diverging effects of the two systems have been shown with respect to the HPA axis activity in an animal model of prolonged stress exposure [161], and also in MDD patients with recurrent episodes [162]. Our **fifth study**

(Chapter 6), indicating that history of prior MDD episodes is associated with inverse vagal response and lower vagal activity in comparison to first-episode patients, is the first that suggests similar effects also with respect to ANS reactivity. Additional prospective longitudinal studies are, however, needed to evaluate ANS reactivity along chronic stress exposure to better understand temporal effects on stress-system-related reactivity alterations and ANS and HPA axis interaction over time.

FUTURE RESEARCH AND CLINICAL IMPLICATIONS

The effects of stress on the human organism are extremely multifaceted and potentially vast, with protection and damage being two sides of the same physiological coin involved in defending the body against every-day challenges [163]. Inadequate, excessive or chronic/repeated stress may, thus, surpass the physiological regulatory capacity of the organism and alter negatively neuroendocrine responses to stress [1-3], leading to a vulnerable phenotype characterized by characteristic psychophysiological alterations, such as impaired GC signaling and disrupted HPA axis and SAM system reactivity during stress. Disrupted stress reactivity exerts profound debilitating effects on homeodynamic balance and adaptivity, development, and mental and somatic health of an individual [163, 164] and may have long-term, deleterious effects on mental and physical health by influencing disease development, natural course and outcomes [135]. Accordingly, stress-related disorders (e.g., MDD, PTSD, etc.) constitute disorders with a huge burden of disease and distinct somatic comorbidity [165-169] and, thus, desperately call for further basic translational and clinical research in order to understand the biological background of risk, development and therapy of these disorders and their comorbidities. In addition, the functional understanding of the brain-heart interaction has to be substantially improved through preclinical and prospective research, since the role of specific brain areas underlying central neuroautonomic dysregulation in stress and stress-related disorders is still essentially unclear.

Stress system dysregulation and chronic somatic comorbidity

Acute and chronic dysregulation of the stress system at different levels has been implicated as a major downstream pathway and link to a broad range of complex behavioural-psychological (e.g., anxiety, depression, eating disorders, post-traumatic stress disorder, sleep disorders, etc.), psychosomatic and somatic diseases (e.g., chronic pain and fatigue syndromes, obesity, metabolic syndrome, chronic inflammation, diabetes type II, hypertension, atherosclerosis, cardiovascular diseases, body composition disorders, cancer, etc.), that all together constitute the so-called "chronic noncommunicable disorders", curtailing life expectancy and plaguing contemporary humanity [100, 147, 170-172]. In fact, a chronically dysregulated stress system has been estimated as a common risk factor of 75-90% of chronic, non-communicable diseases [173]. The high comorbidity of stress-related disorders and cardiovascular disease [141, 165, 166, 168, 174, 175], in particular, suggests an important pathophysiological link between these disorders and autonomic control [176-178].

Beyond these diseases, chronic stress particularly affects the immune system, with altered GC levels influencing all aspects of cellular, humoral, innate and adaptive immunity, thus contributing to increased susceptibility to infections (e.g., tuberculosis, common cold, and COVID-19), (auto-)immune and inflammatory disorders, allergies, and cancer [179]. For example, in periods following a major stressor, a hypercortisolemic state may result in immunosuppression, along with a switch from T helper-1 (cellular) to T helper-2 (humoral) immunity, while a chronic-stress-induced hypocortisolemic state may result in immune system overactivity and increased inflammatory responses due to a decrease in the suppressive effects of cortisol [1, 180]. The mild systemic inflammation observed in chronic stress with devastating rather than salutary effects has been called "para-inflammation".

However, although much research is taking place in the field of acute and chronic stress, there is still less known about all possible factors that regulate stress reactivity and may help stress get "under the skin" to influence disease development [181]. In addition, there is still little recognition of the importance of the stress

system neuroendocrinology within most medical disciplines, and only few stress-system-related implications flow into broad clinical practice. Novel approaches are needed for the proper neuroendocrine assessment and efficacious management of stress system dysregulation in the individualized treatment of both mental and physical stress-related disorders, especially in view of the particular challenges of the evolving new lifestyle of modern societies.

Objective stress challenges for the assessment of stress reactivity

Although the precise brain processes and mechanisms regulating stress reactivity are still unclear, it is presumed that besides biological, particular individual/cognitive factors crucially modulate the complex CAN activity and are related to psychological, behavioral, and health processes [124, 182]. In particular, forebrain neural circuits including the mPFC, but also central CAN brain regions (e.g., amygdala) are involved in individual psychological appraisal and context-related cue-sensitization [183]. Specifically, mPFC activity seems to be a major modulator of the CAN [124, 131], with PFC hypofunction underlying the deficient rational control of emotional responses resulting in exaggerated amygdala activity [126]. This demonstrates the importance of emotionally challenging conditions for the emergence of cardiac risk and fatal outcomes as reported in epidemiological studies [184]. Accordingly, for any objective assessment of biological factors influencing stress reactivity, emotionally challenging psychological/mental stress paradigms should rather be avoided. Instead, experimental endocrine or neurochemical stress provocation that do not primarily affect forebrain circuits should be preferred in future research in combination with neurophysiological autonomic assessment methods, for an objective readout of central autonomic reactivity.

Serotonergic influence on autonomic reactivity

With respect to the influence of serotonergic signalling on autonomic reactivity, future studies are needed to further explore the functional contribution of 5-HTTLPR genotype and serotonin reuptake inhibition effects through the regulation of the different serotonin receptor subtypes to mechanistically understand their role in

both health and disease [185]. Since some studies suggest a non-linear gene dose effect of the 5-HTTLPR [186], inclusion of s/l subjects in further studies with a considerably larger sample size is necessary to precisely characterize genotype differences. In addition, as our studies only assessed a homogeneous male sample to allow finding effects in a small sample, HRV effects still need to be investigated in women, as gender differences cannot be ruled out. Similarly, our results should be replicated in individuals of older age, since age-related changes in 5-HT transmission and SSRI effects have been reported [187].

Heart rate variability in human research

In human research, HRV reflects the capacity of the organism for regulated physiological and emotional responding, while recent studies in mental health research have used HRV as a marker of emotional regulation in order to monitor physiological changes in several psychiatric disorders, including depression and anxiety disorders. Reduced HRV characterizes emotional dysregulation, decreased psychological flexibility and defective social engagement, which in turn are linked to PFC hypoactivity [142]. The collection of HR data in exposure settings and its correlation with various neurochemical and neuroendocrinological markers in plasma and CSF should be also considered for a better understanding of neuroautonomic alterations, as potential consequence of altered fear circuitry affecting emotional states and responses in stress-related disorders and its association with altered HR dynamics [188, 189]. HRV assessment and especially utilization of nonlinear methods may improve our interpretations of autonomic dysregulation on the basis of HR dynamics and serve as a sensitive clinical biomarker with even potential prognostic value in future research of stress-related disorders. In particular, using nonlinear methods can help towards a full translation of results of neuroautonomic control of the heart [190], as nonlinear measures are better suited to determine physiological versus pathological changes and can unravel pathological states of dynamics that can be easily misinterpreted as beneficial based on increased HR variability and blunted responsiveness to stressors in mice [17].

Expanded data analysis based on nonlinear methods may improve our interpretations of dysregulation on the basis of HR dynamics as an even better diagnostic and potentially therapeutic tool in the future research since it allows to bridge basic and clinical research as fully translational measure from rodent to man.

Future studies are needed to replicate our findings and further explore the role of autonomic stress reactivity and diurnal variability as potential biological mechanisms conveying an elevated risk for the development of stress-related disorders and cardiac comorbidity. Respectively, seriously challenging conditions, despite their ethical problems, should be explored more thoroughly, particular with respect to the recovery of the observed HR responses, and to determine any therapeutic efficacy also on autonomic responsiveness. Studies investigating HR measures should focus on unmedicated patients and consider a range of important exclusion criteria that may otherwise impact on the results and its conclusions when investigating disease cohorts.

Diurnal stress system activity and reactivity

Another factor that crucially affects stress system activity and reactivity is circadian timing. The human circadian system is a major temporal modulator of the stress system activity in order to prepare the organism for the higher energetic demand of the waking phase [191]. Direct and indirect neuronal projections from the (SCN reach i) CRH and AVP containing neurons of the medial parvocellular PVN modulating the CRH and AVP secretion and ii) preautonomic hypothalamic neurons, affecting autonomic centres in brain stem and spinal cord and, indirectly also the adrenal medulla and the catecholamine release [192]. Stress system activity in humans, thus, shows a distinct diurnal pattern at the “resting” state, with circulating GC levels exhibiting a sharp rise in the latter part of the night, peaking in the early morning, and a nadir preceding the habitual inactive phase [193]. Similarly, major ANS markers (e.g., heart rate, blood pressure, baroreflex, heart rate variability, plasma epinephrine and norepinephrine) also show robust circadian variations with a distinct peak of SNS activity and nadir of PNS activity in the morning hours [192].

By doing so, HPA axis and ANS prepare the organism for the higher energetic demand of the waking phase [191].

Given the distinct diurnal changes in stress system activity, stress exposure at different times can differentially affect the stress system [194]. Acute psychological stress, involving higher brain areas and the limbic system, as well as acute physical external stress (i.e., restraint/immobilization, foot shock, shaking stress in animal models) elicit the largest stress response during the rest phase, when the HPA axis is hypoactive and the GC-sensitivity is at its peak [192, 195]. Inversely, acute physiological internal stress (i.e., hypoglycaemia, peripheral haemorrhage), relayed to the PVN and brainstem, elicit the largest stress response at the beginning of the activity phase, when these brain areas are activated [192, 195]. This appears reasonable, as internal physiological stress represents a greater threat during higher physical activity, while acute external physical and psychological stress (e.g., predator attack) during the inactive phase. Circadian timing should, thus, be carefully included in any future research protocol, in order not to affect stress system activity or reactivity measurements.

The role of early life stress and childhood trauma on stress reactivity

Stress effects on the organism depend on myriad different factors including nature of stressors, subjective appraisal, duration of exposure, circadian timing, gender, (epi-)genetic background, emotional state, coping and support mechanisms and even personality, which makes the research in this domain extremely difficult and sometimes even controversial. Hereby, one of the most important factors affecting stress system activity and reactivity in the long run is developmental timing of stress exposure [196, 197]. Stress exposure during critical periods of brain development characterized by elevated neuroplasticity and increased sensitivity to epigenetic effects of stress, could exert a long-lasting programming effect on particular neuronal networks that lead to enduring neuroendocrine alterations [198]. Early life stress (e.g., childhood trauma) could, thus, exert a programming effect on sensitive neuronal brain networks related to the stress response and lead to enduring hyper-

or hypo-activation of the stress system and altered glucocorticoid signalling even decades later. In particular, early life stress during the first 2-3 years of life may lead to hyperactivity and hyperresponsiveness of the HPA axis in adolescence and a hypoactive and hyporesponsive HPA axis at later stages of life [199-202]. These age-dependent differences in HPA axis plasticity could be also reflected on the specific risk for a mental and somatic disorder in adulthood. Human genetic background and epigenetic modifications through stress-related gene expression could then interact with these alterations and explain inter-individual variation in vulnerability or resilience to stress and total risk for the development of chronic non-communicable diseases.

Accordingly, besides the well-established negative association between early life stress and general adult mental and physical health-related quality of life [203-207], several larger-scale studies and meta-analyses also suggest a close association of early life stress with cardiovascular, gastrointestinal, neuromusculoskeletal, pulmonary, inflammatory and metabolic diseases, chronic pain syndromes, frequency of medical consultations, as well as number of medical diagnoses [208-215]. The severity of physical and psychological consequences may be also associated with the number of experienced early life stress events [206, 216-218]. More recent studies confirmed that increasing number of events may result in higher adult risk for psychopathological complexity and severity, mental comorbidities, prescribed psychotropic medication, poor mental and physical quality of life, as well as several physical conditions (e.g., chronic pain syndromes, cephalgia, heart disease, asthma, diabetes mellitus and arthritis) [211, 219-226]. All these findings suggest that early life stress may trigger a stress-system-related health risk cascade and be conceptualized as a common developmental risk factor and cumulative health risk mediator, associated with a chronic dysregulation of the stress system reactivity and an increased physical and mental morbidity and all-cause mortality in later life [213, 216, 227-236]. Future studies should, thus, prospectively investigate putative mediators and their temporal sequence, while considering the potentially delayed time-frame for their phenotypical expression.

Finally, better screening strategies for early life stress events are needed for a better individual prevention and treatment.

Future clinical considerations

The neurovisceral integration model has proposed that altered stress system reactivity plays an important role in all major allostatic systems in the pathway from toxic stress to chronic disease [131]. The understanding of the risk factors leading to chronic stress system malfunction may yield important insights into the etiopathology, course, prevention and treatment of the most important major public health concern of chronic, non-communicable diseases [237]. Especially when functional interactions between ANS reactivity and somatic subsystems are considered, diverse patterns of behavioral maladjustment can be subsumed into a single disease model. In this model, autonomic imbalance may be a final common pathway to increased morbidity and mortality from a host of conditions and diseases, while assessment of autonomic imbalance may provide a unifying framework in order to investigate the impact of risk factors, including biological, behavioral, psychosocial and environmental factors on health and disease [148, 238]. Especially chronic activation of inhibitory cortico-subcortical circuits leading to low parasympathetic activation and prefrontal hypoactivity may structurally, as well as functionally, link psychological processes with health-related physiology [239]. In addition, altered autonomic reactivity shows also significant age-related changes (e.g., diminished autonomic reactivity and poorly coordinated autonomic discharge), leading to an impaired ability to adapt to environmental or intrinsic visceral stimuli in the elderly [240]. Thereby, measures of ANS reactivity can be viewed as an autonomic, transdiagnostic biomarker of self-regulation, cognitive control and overall health state [241]. HRV in particular, can be easily used to assess autonomic imbalance in the staging of chronic diseases and classification of morbidity and mortality risk [238].

The incorporation of the autonomic reactivity into a broader perspective on emotions, mental health, and good cognitive and physical functioning should, however, not only be used as a theoretical ground for further research, but also as

a starting point for clinical applications [152]. Nevertheless, although particularly autonomic imbalance can be easily measured and also influenced by methods that are already available in primary care, these possibilities are generally overlooked by clinicians [242]. Simple strategies for autonomic function improvement and increasing cortical blood flow could be used to improve autonomic activity and reactivity [240]. **For example, as physical activity influences both resting autonomic activity but also autonomic reactivity,** regular moderate aerobic exercise (e.g., walking) could have a positive effect on ANS reactivity [243]. Furthermore, primary, secondary and tertiary prevention of stress-related effects on individuals could incorporate additional behavioral and life-style modification strategies, such as stress management techniques, sleep hygiene, healthy nutrition, smoking cessation, positive psychology and emotional self-regulation strategies and social engagement/support strengthening strategies alone or in terms of a cognitive-behavioral psychotherapeutic process. Here, though, the responsibility lies not only at the individual, but also at policy makers for the implementation at a societal level.

Additional clinical intervention strategies could include more specific treatment alternatives, such as pharmacotherapy and somatic afferent stimulation (e.g., stroking skin, acupuncture, vagus nerve stimulation, HRV coherence training/biofeedback), in order to restore autonomic balance. Instead of exclusively targeting sympathetic activation as in the past years, physicians should rather attempt to increase vagal tone. In particular, there has been increasing interest in treating a wide range of disorders with implanted pacemaker-like devices for stimulating the vagal afferent pathways for a broad range of diseases (e.g., obesity, depression, anxiety, epilepsy, migraine, chronic pain, etc. [244-246]). In addition, drugs affecting CAN activity (e.g., SSRIs) and circadian rhythm, substances reducing oxidative stress or inflammation, or influencing stress-system dysregulation effects in the periphery (e.g., GR modulators), or even metabolism altering agents hold a potential of effectively disrupting the chronic vicious cycle of stress progression and its effects on the body.

CONCLUSIONS

Variation in the activity of the stress-responsive ANS and HPA axis is particularly important for adaptive stress responses and may thus give rise to individual differences in resilience as ability to cope with stressful events. Individual differences in stress reactivity may vitally affect adaptive responses and possibly explain individual differences in stress resilience and progression of stress-related disorders. Identification of such biological factors that influence stress reactivity is, thus, of major importance for the linkage of psychosocial and environmental stress factors to disease outcome.

Our studies used HRV analyses, as one of the most valid and applicable biomarkers mirroring central autonomic activity to explore the effects of several biological factors on autonomic reactivity to stress by pharmacoendocrine challenges. Our results indicated an important role of central serotonergic and glutamatergic activity, as well as of the vagal nervous system in the modulation of the CAN activity, and underlined the vital importance of the interplay between ANS and HPA axis and the modulation of stress-related cardiovascular responsiveness, thus affecting susceptibility to stress-related disorders and representing an important pathway towards the higher cardiac mortality seen in stress-related disorders.

Future studies need to replicate and extend these findings in both sexes and in healthy as well clinical populations in order to further explore factors influencing autonomic stress reactivity as potential biological mechanism conveying an elevated risk for the development of stress-related disorders and their effect over time. Our results especially underline the utility of HRV as a transdiagnostic potential biomarker for stress system sensitivity and vulnerability to stress-related disorders and underline a much broader use in reach and clinical practice. In addition, ANS or HPA axis-related treatment alternatives may play a future role in the ideal adjustment of stress reactivity and, thus, represent an additional therapeutic option in the treatment of stress-related disorders.

Devising novel prevention strategies for chronic non-communicable disorders will depend on the careful elucidation and inclusion of the common, stress-related pathways for developing chronic health risks. Thereby, especially stress system reactivity deserves additional consideration by researchers, clinicians and policymakers as a target for early interventions to prevent chronic stress-related disorders.

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Addendum

Summary

Samenvatting

Impact paragraph

Acknowledgments

Curriculum vitae

Publications list

SUMMARY

Biomedical research has shown that the impact of stress on human physiology and pathophysiology is pervasive and enormous. The human stress response is mediated and modulated by the body‘s stress system, which has both central and peripheral components. The two main peripheral effector limbs of the stress system are the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS). It is especially the precise regulation of organ and tissue functions through a fine-tuning of the two main ANS tracts (sympathetic nervous system, SNS; parasympathetic nervous system, PNS) that is crucial for optimal stress reactivity, adaptive responses and, hence, overall health. ANS function is mainly regulated by the central autonomic network (CAN), an internal central autonomic regulation system essential for survival. CAN dysregulation can be critically involved in stress-related disorders, as it may affect downstream autonomic core centers. Thereby, CAN dysregulation can alter peripheral ANS activity and cardiac function and is associated with decreased dynamic adaptability, increased morbidity and mortality. Variation in the activity and reactivity of the CAN to stress may, thus, give rise to individual differences in resilience as ability to cope with stressful events.

Experimental evidence suggests that the activity and functional connectivity of the brain regions involved in the CAN is partly influenced by several signaling systems and their related brain areas. Especially serotonergic and glutamatergic signaling, but also the central HPA axis regions are heavily involved in CAN activity modulation and, thus, also in the regulation of stress and anxiety. Stress-induced alterations in those systems are implicated in acute or prolonged autonomic dysregulation and the pathophysiology stress-related disorders. In fact, as neurocircuitry of stress-system and depression show a distinct overlap, many of the diverse and deleterious biological features of major depression could actually reflect prolonged or repeated dysregulation of autonomic brain regions. However, due to its complexity and limited *functional* knowledge of the anatomically well described CAN, the molecular and cellular basis for the normal and compromised brain-heart network in stress and stress-related disorders is still a widely unexplored

area. Identification of such biological factors that influence stress reactivity is, thus, of major importance for the linkage of psychosocial and environmental stress factors to disease outcome.

The studies of this dissertation assessed the role of the central serotonergic and glutamatergic system, as well as the influence of the HPA axis on the CAN, in order to further explore biological factors that objectively modulate central autonomic reactivity activity and reactivity to stress in humans. As stress reactivity is often influenced by subjective/cognitive factors, the following studies employed only objective stress challenges using endocrine and pharmacological stress provocation, while linear and non-linear heart rate variability (HRV) analyses were applied as one of the best established and widely used non-invasive methods for the quantitative and translational assessment of ANS activity.

In the first three studies (Chapter 2-4), the cholecystokinin tetrapeptide (CCK-4) paradigm was utilized as a pharmacological stress/panic challenge to investigate autonomic reactivity in healthy humans using linear HRV measures. In **Chapter 2**, our first study assessed autonomic activity and reactivity to CCK-4 stress challenge in association with the genotype of the serotonin transporter (5-HTT-linked polymorphic region) genotype 5-HTTLPR in a group of 30 healthy young men, 15 of each with the long/long (l/l) or short/short (s/s) genotype for the 5-HTTLPR. Our results indicated enhanced sympathetic and/or diminished cardiac vagal activity and blunted autonomic reactivity to stress in subjects with the s/s genotype in comparison to the l/l genotype for the 5-HTTLPR. In **Chapter 3**, using the same provocation challenge, our second study assessed the influence of long-term selective serotonin reuptake inhibitor (SSRI) application on autonomic activity and reactivity to stress in 30 healthy young men in a double-blind, placebo (PLA)-controlled, randomized, within-subject cross-over design. Our results indicated that long-term SSRI treatment with escitalopram shows no significant effects on baseline autonomic activity, but a significant increase of vagal tone and a blunted autonomic reactivity to stress. In **Chapter 4**, our third study assessed the influence of group II metabotropic glutamate receptor ($mGluR_{2/3}$) agonism on autonomic activity and

reactivity to CCK-4 stress challenge in a double-blind, randomized placebo-controlled, cross-over study in healthy humans. Our results indicated that $mGluR_{2/3}$ agonism with LY544344 shows no significant effects on baseline autonomic activity, but a significantly enhanced autonomic recovery after stress.

In the two final studies, we utilized overnight pharmacoendocrine HPA axis challenges with dexamethasone (suppression) and metyrapone (stimulation) on two consecutive days to investigate the influence of the HPA axis on autonomic activity the following day using linear and non-linear HRV measures. In **Chapter 5**, our fourth study assessed the direct effects of HPA axis suppression and stimulation on ANS activity at rest in 39 young healthy individuals and indicated that HPA axis stimulation (using metyrapone) is associated with reduced vagal tone, while HPA axis suppression (using dexamethasone) has no effect on autonomic modulation of heart function. In **Chapter 6**, using also an endocrine challenge paradigm, our fifth and final study investigated the influence of prior depression history on the modulation of resting autonomic activity by HPA axis stimulation (using metyrapone) in a a group of 14 physically healthy, antidepressant-free patients with clinical, non-psychotic major depression. Our results indicated that positive history of prior MDD episodes showed no statistically significant effect of baseline autonomic state but distinct effects on autonomic reactivity to HPA axis stimulation with inverse vagal response and lower vagal activity in comparison to first-episode patients.

Taken together, our results underline the complex functional balance of stress system activity and reactivity, and highlight an important role of central serotonergic and glutamatergic activity, as well as of the vagal nervous system in the modulation of the CAN activity, and additionally show a vital importance of the interplay between ANS and HPA axis and the modulation of stress-related cardiovascular responsiveness, thereby confirming and extending previous studies. Thus, our findings suggest a major overlap of main regulatory systems and the association between stress-related disorders, CAN dysregulation with compromised neuroautonomic control and somatic, in particular, cardiovascular morbidity and

mortality in such patients. Future studies need to replicate and extend these findings in both sexes and in healthy as well clinical populations in order to further explore factors influencing autonomic stress reactivity as potential biological mechanism conveying an elevated risk for the development of stress-related disorders and their effect over time. Finally, our results especially underline the utility of HRV as a transdiagnostic potential biomarker for stress system sensitivity and vulnerability to stress-related disorders and support a much broader use in research and clinical practice.

Devising novel prevention and treatment strategies for chronic non-communicable disorders will depend on the careful elucidation and inclusion of the common, stress-related pathways for developing chronic health risks. Individual differences in stress reactivity may vitally affect adaptive responses and possibly explain individual differences in stress resilience and progression of stress-related disorders. Thereby, especially stress system reactivity deserves additional consideration by researchers, clinicians and policymakers as a target for early interventions to individually treat and prevent stress-related disorders.

SAMENVATTING

Biomedisch onderzoek heeft aangetoond dat de impact van stress op de menselijke fysiologie en pathofysiologie alomtegenwoordig en enorm is. De menselijke stress respons wordt gemedieerd en gemoduleerd door het stresssysteem van het lichaam, dat zowel uit centrale als perifere componenten bestaat. De twee belangrijkste perifere onderdelen van het stress systeem zijn de hypothalamus-hypofyse-bijnieras (HPA) en het autonome zenuwstelsel (ANS). Het is vooral de nauwkeurige regeling van de twee belangrijkste ANS-componenten (sympathisch zenuwstelsel, SNS; parasympathisch zenuwstelsel, PNS) die cruciaal is voor een optimale stressreactiviteit, adaptieve reacties en uiteindelijk voor de algehele gezondheid. De functie van het ANS wordt voornamelijk gereguleerd door het centraal autonoom netwerk (CAN), een intern centraal autonoom reguleringssysteem dat essentieel is voor overleving. Ontregeling van het CAN kan een cruciale rol spelen bij stress-gerelateerde aandoeningen, omdat het downstream autonome kerncentra kan beïnvloeden. Bovendien kan ontregeling van het CAN de perifere ANS-activiteit en de hartfunctie veranderen: het is het geassocieerd met een verminderd dynamisch aanpassingsvermogen, verhoogde morbiditeit en mortaliteit. Variatie in de activiteit en reactiviteit van het CAN op stress kan dus leiden tot individuele verschillen in veerkracht om met een stressvolle gebeurtenissen om te gaan.

Experimenteel bewijs suggereert dat de activiteit en de functionele connectiviteit van de hersengebieden die betrokken zijn bij het CAN deels worden beïnvloed door verschillende neurotransmittersystemen en hun gerelateerde hersengebieden. Vooral serotonerge en glutamaterge signalen, maar ook de centrale gebieden van de HPA-as zijn sterk betrokken bij de modulatie van de CAN-activiteit en dus ook bij de regulering van stress en angst. Veranderingen in deze systemen die door stress veroorzaakt zijn, zijn betrokken bij acute of langdurige autonome ontregeling en de pathofysiologie van stress-gerelateerde aandoeningen. Aangezien de neurocircuits van het stress systeem en depressie sterk overlappen, zouden veel van de diverse biologische kenmerken van ernstige depressie het gevolg

kunnen zijn van langdurige of herhaalde ontregeling van autonome hersengebieden. Echter, vanwege de complexiteit en de beperkte *functionele* kennis van het anatomisch goed beschreven CAN, is de moleculaire en cellulaire basis voor het normale en betrokken hersen-hart netwerk bij stress en stress-gerelateerde stoornissen nog een grotendeels onbekend gebied. Identificatie van dergelijke biologische factoren die stressreactiviteit beïnvloeden is dus van groot belang voor de koppeling van psychosociale- en omgevingsstressfactoren aan ziekte-uitkomsten. De studies van dit proefschrift evalueerden de rol van het centrale serotonerge en glutamaterge systeem, evenals de invloed van de HPA-as op het CAN, om de biologische factoren die de centrale autonome reactiviteit en de reactiviteit op stress bij mensen objectief moduleren verder te onderzoeken. Aangezien stress reactiviteit vaak beïnvloed wordt door subjectieve/cognitieve factoren, is het volgende onderzoek verricht.

In de eerste drie studies (hoofdstukken 2-4) werd het cholecystokinine tetrapeptide (CCK-4) paradigma gebruikt als een farmacologische stress/paniek challenge om autonome reactiviteit te onderzoeken bij gezonde mensen met behulp van lineaire HRV metingen. **Hoofdstuk 2** bevat onze eerste studie, over de autonome activiteit en reactiviteit op een CCK-4 stress challenge. in samenhang met het genotype van de serotonine transporter (5-HTT-gekoppelde polymorfe regio) genotype 5-HTTLPR. Deelnemers waren een groep van 30 gezonde jonge mannen, 15 van elk met het lange/lange (l/l) of korte/korte (s/s) genotype voor het 5-HTTLPR. Onze resultaten wezen op een verhoogde sympathische en/of verminderde cardiale vagale activiteit en een verminderde autonome reactiviteit op stress bij proefpersonen met het s/s genotype in vergelijking met het l/l genotype voor het 5-HTTLPR. In **hoofdstuk 3** wordt onze tweede studie beschreven, met dezelfde stress challenge. In deze studie werd de invloed onderzocht van langdurige toediening van selectieve serotonine heropname remmers (SSRI's) op de autonome activiteit en de reactiviteit op stress. De studie werd uitgevoerd bij 30 gezonde jonge mannen in een dubbelblind, placebo (PLA)-gecontroleerd, gerandomiseerd, "within-subject cross-over" design. Onze resultaten toonden aan dat langdurige SSRI-behandeling met

escitalopram geen significante effecten heeft op de autonome activiteit op baseline, maar er was wel een significante toename van de vagale tonus en een verminderde autonome reactiviteit op stress te zien. In **hoofdstuk 4** werd in onze derde studie de invloed onderzocht van groep II metabotrope glutamaat receptoren (mGluR2/3) op de autonome activiteit en de reactiviteit op een CCK-4 stress challenge, Ook dit was een studie in gezonde mensen, dit keer in een dubbelblind, gerandomiseerd placebo-gecontroleerd, cross-over design. Onze resultaten gaven aan dat mGluR2/3-agonisme met LY544344 geen significante effecten heeft op de autonome activiteit bij baseline, maar wel een significant verbeterd autonoom herstel veroorzaakt na stress.

In de twee laatste studies gebruikten we 's nachts op twee opeenvolgende dagen farmaco-endocriene HPA-as challenges (dexamethason (suppressie) en metyrapone (stimulatie)) om de invloed van de HPA-as op autonome activiteit de volgende dag te onderzoeken met behulp van lineaire en niet-lineaire HRV-maten. **Hoofdstuk 5** betreft onze vierde studie, naar de directe effecten van HPA-as onderdrukking en stimulatie op de ANS-activiteit in rust bij 39 jonge gezonde proefpersonen. De resultaten tonen aan dat HPA-as stimulatie (met metyrapone) geassocieerd is met verminderde vagale tonus, terwijl HPA-as onderdrukking (met dexamethason) geen effect heeft op de autonome modulatie van de hartfunctie.

In **hoofdstuk 6** onderzochten we in onze vijfde en laatste studie, met dezelfde HPA-as challenge, de invloed van een voorgeschiedenis van depressie op de modulatie van autonome activiteit in rust. Dit werd onderzocht in een groep van 14 lichamelijk gezonde, antidepressivumvrije patiënten met een ernstige (maar niet psychotische) depressie. Onze resultaten gaven aan dat een positieve voorgeschiedenis van eerdere episoden van ernstige depressie geen statistisch significant effect had op de basale toestand van het autonome zenuwstelsel. Er was echter wel een duidelijke effect op de autonome reactiviteit na HPA-as stimulatie (met metyrapone) met een omgekeerde vagale respons en lagere vagale activiteit in vergelijking met patiënten met een eerste episode.

Samengevat benadrukken onze resultaten het complexe functionele evenwicht van activiteit en reactiviteit van het stress systeem, en suggereren ze een belangrijke rol van centrale serotonerge en glutamaterge activiteit, alsook van het vagale zenuwstelsel in de modulatie van de CAN-activiteit. Bovendien tonen de resultaten het grote belang aan van de wisselwerking tussen het ANS en de HPA-as en van de modulatie van stress-gerelateerde cardiovasculaire reactiviteit. Hiermee worden eerdere studies bevestigd en uitgebreid. Onze bevindingen wijzen dus op een grote overlap tussen de belangrijkste regulerende systemen en op de associatie tussen stress-gerelateerde stoornissen en somatische, met name cardiovasculaire, morbiditeit en mortaliteit bij dergelijke patiënten. Toekomstige studies moeten deze bevindingen repliceren en uitbreiden bij beide geslachten en bij zowel gezonde als klinische populaties. Het is van belang om de factoren te onderzoeken die de autonome stress reactiviteit beïnvloeden, evenals het potentieel biologisch mechanisme voor de ontwikkeling en het tijdsverloop van stress-gerelateerde aandoeningen. Ten slotte benadrukken onze resultaten vooral het nut van HRV als transdiagnostische potentiële biomarker voor de gevoeligheid van het stress systeem en de kwetsbaarheid voor stress-gerelateerde stoornissen en onderstrepen ze een veel breder nut in onderzoek en de klinische praktijk.

Het ontwerpen van nieuwe preventie- en behandelingsstrategieën voor chronische niet-overdraagbare aandoeningen zal afhankelijk zijn van de zorgvuldige opheldering van de gemeenschappelijke, stress-gerelateerde mechanismen voor het ontwikkelen van chronische gezondheidsrisico's. Individuele verschillen in stress reactiviteit kunnen van groot belang zijn voor adaptieve reacties en mogelijk individuele verschillen in stressbestendigheid en progressie van stress-gerelateerde aandoeningen verklaren. Daarom verdient vooral de reactiviteit van het stress systeem extra aandacht van onderzoekers, clinici en beleidsmakers als een doelwit voor vroegtijdige interventies om stress-gerelateerde aandoeningen individueel te behandelen en te voorkomen.

IMPACT PARAGRAPH

Research impact embodies real change in the real world and includes all the diverse ways that knowledge generated through research is applied to society and benefits individuals and specific target groups through increasing effectiveness of public services and policies, improving quality of life, overall health, or economic benefits. However, every-day routine may put up barriers between researchers, the research work itself and those who may benefit from it or can apply it to make change. Keeping research impact in mind, thus, helps keep us focused on the overall purpose, rather than the process, of research. A focus on impact can therefore help ensure the best possible return from the investments that societies are making in research.

Aim and key findings

The overall aim of this dissertation is to explore biological factors that objectively modulate central autonomic activity and reactivity to stress in humans using stress provocation challenges. In particular, the studies of this dissertation assessed the role of the central serotonergic and glutamatergic system, as well as the influence of the HPA axis on central autonomic reactivity. As stress reactivity is often influenced by subjective/cognitive factors, the following studies employed only objective stress challenges using endocrine and pharmacological stress provocation. Heart rate variability analyses were applied as a readout of central autonomic activity. In order to increase the translational comparability of the findings, both linear and non-linear heart rate variability measures were included, where possible.

In **Chapter 2**, our first study indicated enhanced sympathetic and/or diminished cardiac vagal activity and blunted autonomic reactivity to stress in subjects with the s/s genotype in comparison to the l/l genotype for the 5-HTTLPR. In **Chapter 3**, our second study indicated that long-term SSRI treatment with escitalopram shows no significant effects on baseline autonomic activity, but a significant increase of vagal tone and a blunted autonomic reactivity to stress. In **Chapter 4**, our third study indicated that $mGluR_{2/3}$ agonism with LY544344 shows no

significant effects on baseline autonomic activity, but a significantly enhanced autonomic recovery after stress. In **Chapter 5**, our fourth study indicated that HPA axis stimulation (metyrapone) is associated with reduced vagal tone, while HPA axis suppression (dexamethasone) has no effect on autonomic modulation of heart function. In **Chapter 6**, our fifth study indicated that positive history of prior episodes in patients with major depression showed no statistically significant effect of baseline autonomic state but distinct effects on autonomic reactivity to HPA axis stimulation (metyrapone) with inverse vagal response and lower vagal activity in comparison to first-episode patients.

Taken together, our results underline the complex functional balance of stress system activity and reactivity and highlight an important role of central serotonergic and glutamatergic activity, as well as of the vagal nervous system in the modulation of the CAN activity, and additionally show a vital importance of the interplay between ANS and HPA axis and the modulation of stress-related cardiovascular responsiveness, thereby confirming and extending previous studies. Finally, our results especially underline the utility of HRV as a transdiagnostic potential biomarker for stress system sensitivity and vulnerability to stress-related disorders and underline a much broader use in reach and clinical practice.

Individual impact

Stress research suggests that the individual ability for resistance to stress, rapid and effective rebound from stress and functional improvement after stress is crucial for healthy adaptation. Disrupted individual stress reactivity exerts profound debilitating effects on homeodynamic balance and adaptivity, development, and mental and somatic health of an individual and may have long-term, deleterious effects on mental and physical health by influencing disease development, course and outcome. Stress responsiveness and particularly autonomic reactivity has been linked to increased overall health risk as a measure of an individual's psychobiologic response to challenges in the environment and a mediator of psychosocial and behavioral risk factors. Respectively, in the last years, research provided robust

evidence that indeed reduced autonomic reactivity and slower recovery are associated with higher cardiovascular and overall (physical and mental) morbidity and mortality risk. However, although much research is taking place in the field of acute and chronic stress, there is still less known about individual biological factors that regulate stress reactivity and may help stress get "under the skin" to influence disease development. Thereby, the identification of distinct biological factors influencing individual stress reactivity is of vital importance for a personalized medical approach, as well as for the linkage of psychosocial and environmental stress factors on pathophysiology of disease development. The acknowledgement of such biological risk factors influencing stress reactivity could be used in individual risk assessment, as well as personalized prevention and treatment approaches. Our results support, for example, that the s/s 5-HTTLPR genotype might represent a genetic risk factor for developing stress-related, cardiometabolic and other chronic, non-communicable diseases, as well as the fact that pharmacological or endocrine serotonergic, glutamatergic and HPA axis modulation might have clinical utility for the individual stress reactivity and, respectively, for personalized treatment.

Socioeconomic impact

Stress system dysregulation is considered endemic in contemporary societies, with about 2/3 of the population at the age of 55 years suffering from a "syndrome of chronic stress and inflammation". Acute and chronic stress system dysregulation with altered stress reactivity has been linked to a broad range of complex behavioural-psychological (e.g., anxiety, depression, eating disorders, post-traumatic stress disorder, sleep disorders, etc.), and psychosomatic and somatic diseases (e.g., chronic pain and fatigue syndromes, obesity, metabolic syndrome, chronic inflammation, diabetes type II, hypertension, atherosclerosis, cardiovascular diseases, body composition disorders, cancer, etc.), that all together constitute the so-called "chronic noncommunicable disorders", curtailing life expectancy. Interestingly, a chronically dysregulated stress system has been found as a common risk factor of 75-90% of all chronic, non-communicable diseases, excreting a huge

health-related socioeconomic burden on modern humanity. The high comorbidity of stress-related disorders and cardiovascular disease, in particular, suggests an important pathophysiological link between these disorders and autonomic control. Beyond these diseases, chronic stress particularly affects the immune system, with altered GC levels influencing all aspects of cellular, humoral, innate and adaptive immunity, thus contributing to increased susceptibility to infections (e.g., tuberculosis, common cold, and COVID-19), (auto-)immune and inflammatory disorders, allergies, and cancer. This huge burden of disease caused by stress-related pathophysiology desperately calls for further basic translational and clinical research in order to understand the biological background of risk, development and therapy of these disorders and their comorbidities, which actually affect all our lives from birth to older age. The understanding of the biological risk factors leading to chronic stress system malfunction may yield important insights into the etiopathology, course, prevention and treatment of the most important major public health concern of chronic, non-communicable diseases. Better understanding of biological factors affecting the development of stress-related disorders can further help in primary, secondary and tertiary prevention in the general population, in target groups at risk and in patients. Especially individuals with early-life stress and trauma experience (i.e., childhood abuse/neglect), individuals exposed to shift work, patients with stress-related disorders (e.g., depression, anxiety disorders, PTSD), and patients with chronic non-communicable diseases (i.e., cardiovascular, immune, autoimmune, metabolic, malignant) could greatly profit from targeted prevention and treatment alternatives tailored according to specific biological risk factors influencing their stress system reactivity. Alleviating this huge burden of disease through stress-related chronic non-communicable diseases would represent one giant step towards lower morbidity and mortality, lower health expenditure costs in every country and better societal productivity and prosperity.

Scientific impact

Due to its complexity and limited functional knowledge of the anatomically well described central stress system, the molecular and cellular basis for the normal and compromised brain-heart network in stress and stress-related disorders is still a widely unexplored area. Therefore, the National Institute of Mental Health (NIMH) has recently identified a set of priorities for stress biology research aimed at creating the basic and clinical knowledge bases for reducing and alleviating health burden across the lifespan. Accordingly, our studies provide relevant evidence in the better understanding of biological factors contributing to individual stress system reactivity through different objective stress paradigms, embrace different subsystems and their interaction to exploit the complexity of the stress response and apply translational methods (i.e., non-linear HRV analyses) that seek to test mechanistic hypotheses across species. Our studies combine expertise and methods from different experimental fields, including psychoneuroendocrinology, psychoneurophysiology, psychoneuropharmacology, experimental clinical research and clinical psychiatry to help zoom in on individual biological aspects of stress response in both health and disease and establish functional mechanistic links across different levels of stress response. For this purpose, our studies have followed very strict and timely precise methodological protocols and only objective stress challenges for an objective readout of central autonomic reactivity, in order to avoid cognitive, personality, circadian, and other influences that may affect individual stress responses. Our results underline the complex functional balance of stress system activity and reactivity and highlight an important role for serotonergic and glutamatergic signaling, as well as for HPA axis influence on CAN activity, thereby confirming and extending previous studies. These findings underscore the overlap of main regulatory systems of autonomic, affective and attentional regulation and the association between stress-related disorders, CAN dysregulation with compromised neuroautonomic control and somatic, in particular, cardiovascular morbidity and mortality in such patients. Autonomic imbalance may be a final common pathway to increased morbidity and mortality from a host of conditions

and diseases, while assessment of autonomic imbalance may provide a unifying framework in order to investigate the impact of risk factors, including biological, behavioral, psychosocial and environmental factors on health and disease. Thereby, measures of autonomic reactivity can be viewed as a transdiagnostic biomarker of self-regulation, cognitive control and overall health state. HRV assessment and especially utilization of nonlinear methods may improve our interpretations of autonomic dysregulation and serve as a sensitive clinical biomarker with potential prognostic value in the staging of chronic diseases and classification of morbidity and mortality risk.

Our studies also support a central role of the vagal branch of the ANS in the regulation of stress reactivity and also the fact that ANS resting activity and reactivity, although correlated, represent different regulatory processes with different functional and clinical impact. This model, thus, challenges the completeness of the sympathetic overactivation explanation of stress activation and anxiety. However, the functional understanding of stress reactivity has to be substantially improved through further preclinical and prospective research. Thereby, vagal activity and its normative increase from childhood to adolescence seem to hold a key role in the proper neurovisceral integration during neurodevelopment on a structural and functional level, subsequent psychological functioning and adaptive regulation. Thereby, factors as developmental timing, (epi)genetics, duration and nature of stressors among others play an important moderating and modulating role. An improved understanding of mechanisms underlying stress responses and the functional consequences of stress can and will speed translation from basic research to predictive markers of risk and to improved, personalized interventions for mental and chronic illness. For example, as neurocircuitry of stress-system and depression show a distinct overlap, many of the biological factors influencing stress reactivity could actually be responsible for prolonged or repeated dysregulation of brain regions in the pathophysiology of depression.

Clinical Impact

Despite that basic and clinical research have already offered great insights of stress system pathophysiology, there is still little recognition of the importance of the stress system within most medical disciplines, and only few stress-system-related implications flow into broad clinical practice. Novel approaches are needed for the proper neuroendocrine and neurophysiological assessment of stress system reactivity and efficacious management of stress system dysregulation in the individualized treatment of both mental and physical stress-related disorders, especially in view of the particular challenges of the evolving new lifestyle of modern societies. Thereby, our results especially underline the utility of HRV as a transdiagnostic potential biomarker for stress system sensitivity and vulnerability to stress-related disorders and underline a much broader use in research and clinical practice. In addition, simple strategies for autonomic function improvement and increasing cortical blood flow (i.e., regular moderate aerobic exercise) could be used to improve autonomic activity and reactivity in prevention and treatment. Further clinical intervention strategies could include more specific treatment alternatives, such as pharmacotherapy and somatic afferent stimulation (e.g., stroking skin, acupuncture, vagus nerve stimulation, HRV coherence training/Biofeedback), in order to restore autonomic balance. Instead of exclusively targeting sympathetic activation as in the past years, physicians should rather attempt to increase vagal tone. In particular, there has been increasing interest in treating a wide range of disorders with implanted pacemaker-like devices for stimulating the vagal afferent pathways for a broad range of diseases (e.g., obesity, depression, anxiety, epilepsy, migraine, chronic pain, etc.). In addition, drugs affecting CAN activity (e.g., SSRIs) and circadian rhythm, substances reducing oxidative stress or inflammation, or influencing stress-system dysregulation effects in the periphery (e.g., GR modulators), or even metabolism altering agents hold a potential of effectively disrupting the chronic vicious cycle of stress progression and its effects on the body.

Future directions

The results of this dissertation argue for a broader implementation of easily accessible stress-system biomarkers into clinical practice for the better assessment of chronic health risks, especially in the general and targeted prevention, monitoring and personalized treatment of patients with chronic non-communicable diseases and mental disorders. Nevertheless, more targeted research in broader patient groups is needed before such biomarkers can be applied in clinical routine settings. Healthcare insurers could play a facilitating role by including such examinations for patients in their refunding list and research foundations by enhancing biological stress research also outside the scope of mental health and psychiatry, but including it to all related disciplines.

Future studies are needed to replicate our findings and further explore the role of autonomic stress reactivity and diurnal variability as potential biological mechanisms conveying an elevated risk for the development of stress-related disorders and physical comorbidity. Respectively, seriously challenging conditions, despite their ethical problems, should be explored more thoroughly, particular with respect to the recovery of the observed autonomic responses, and to determine any therapeutic efficacy also on autonomic responsiveness. Studies investigating HR measures should focus on both medicated and unmedicated patients and consider a range of important exclusion criteria that may otherwise impact on the results and its conclusions when investigating disease cohorts. In particular, since some studies suggest a non-linear gene dose effect of the 5-HTTLPR, inclusion of s/l subjects in further studies with a considerably larger sample size is necessary to precisely characterize genotype differences. In addition, HRV effects still need to be investigated in women, and in individuals of older age, since age-related changes in 5-HT transmission and SSRI effects have been reported. Future studies should also prospectively investigate putative mediators and their temporal sequence, while considering the potentially delayed time-frame for their phenotypical expression. Finally, the broader inclusion of HRV as a transdiagnostic measure of emotional and

autonomic biomarker into clinical research in further patient groups outside the psychiatric clinical context is of importance.

Furthermore, primary, secondary and tertiary prevention of stress-related effects on individuals could incorporate additional behavioral and life-style modification strategies to the assessment of biomarkers, such as stress management techniques, sleep hygiene, healthy nutrition, smoking cessation, positive psychology and emotional self-regulation strategies and social engagement/support strengthening strategies alone or in terms of a cognitive-behavioral psychotherapeutic process. Hereby, the implementation at a societal level supported by health policy makers is of crucial importance, in order to have an important impact at a general socioeconomic level. Policy makers could be involved and informed via expert groups that contribute to the development of policies. This suggests also that organizations with strong health-related or societal infrastructure should acknowledge and include stress-related applications and interventions into their functional algorithms, as technology-based interventions at nodal social hubs, are now scientifically and medically possible. Furthermore, training of the stress detection and management basics very early in life (e.g., school courses) and continuous training in important life stages (e.g., college, work, marriage, parenthood, etc.) could be fruitful as a general prevention strategy and increase the socio-psycho-somatic resilience to stress in societies, but also an individual level. The findings from this study could be used by policy makers to inform, to comprehend and to convince people that biological factors can alter their stress resilience and that improving lifestyle is not only good for their general health, but also specifically for their brain and mental health.

Dissemination of knowledge

Results of this dissertation were nationally shared at national and international congresses and symposia with colleagues in both research and clinical field (*12th World Congress of Biological Psychiatry 2015*, Athens, Greece; *27th ECNP Congress* 2014, Berlin, Germany; *DGPPN National German Psychiatry and Psychotherapy*

Congresses 2014-2016). Internationally, the results of this dissertation were published in internationally high-ranked peer-reviewed scientific journals of relevant fields [*J Psychiatr Res* 2020 (IF 2019: 3.74); *Psychoneuroendocrinology* 2019 (IF 2017: 4.73); *Int Clin Psychopharmacol* 2016 (IF 2015: 2.41); *Int J Neuropsychopharmacol* 2014 (IF 2013: 5.26); *J Psychiatr Res* 2014 (IF 2013: 4.09)], in which the quality of the studies is evaluated by experts in the field, and have been already cited over 62 times (23.09.2022). The working method and first results were disseminated within internal science meetings at the Department of Psychiatry and Psychotherapy, University Medical Center Hamburg-Eppendorf, Germany and deliberated on with international colleagues during work visits to the Center of Neurogenomics and Cognitive Research, VU University of Amsterdam. Results of these studies were used as basis for the candidate's granted application (PI) for one year's research scholarship through the Excellent Young Investigator Research Founds of the Medical Faculty of the Hamburg University, Germany (2014) and the candidate's granted application (co-PI) for a clinical research grant through the Werner-Otto Foundation, Germany (2015).

Conclusion

Biomedical research has shown that the impact of stress on human physiology and pathophysiology is pervasive and enormous. Thereby, individual differences in stress reactivity may vitally affect adaptive responses and possibly explain individual differences in stress resilience and, thus, deserve additional consideration by researchers, clinicians and policymakers as a target for early interventions to individually treat and prevent stress-related disorders. Identification of biological factors that influence stress reactivity is, thus, of major importance for the linkage of psychosocial and environmental stress factors to disease outcome and may yield important insights into the etiopathology, course, prevention and treatment of the most important major public health concern of chronic, non-communicable diseases and mental health disorders.

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CURRICULUM VITAE

Agorastos Agorastos was born on August 3rd, 1981 in Athens, Greece and graduated Greek Highschool with distinction in 1999. He received his MD at the Medical University of Vienna, Austria. He then moved to Hamburg, Germany and completed his complete clinical and psychotherapeutic training (Board Approval for CBT) and German doctorate (German Dr. med.) at the Department of Psychiatry and Psychotherapy of the University Medical Center Hamburg-Eppendorf. Afterwards, he moved to U.S.A. for his postgraduate research fellowship in the PTSD research group of the VA Center of Excellence for Stress and Mental Health (CESAMH) at the University of California, San Diego (USCD), La Jolla, and then moved back to Hamburg, where he served as attending physician at the University Department of Psychiatry and Psychotherapy and clinical coordinator of the outpatient depression service and depression research programme. He then served as Head of the Outpatient Psychiatric Services of the University Department of Psychiatry and Psychotherapy, as well as Team Leader of the Psychoneuroimmunology and Psychoneurophysiology Research Group of the Laboratory of Biological Psychiatry, while he also completed his MSc in Affective Neuroscience at the Universities of Maastricht and Florence. In 2017, he moved back to Greece, where he currently serves as Consultant Physician in the II. University Dept. of Psychiatry at the Psychiatric Hospital of Thessaloniki, Greece, while he also holds an International Partnership with the CESAMH at USCD.

Fields of clinical expertise include stress- and trauma-related disorders, anxiety disorders, OCD and treatment-resistant/post-partum depression, while his current research focus centers in the neurobiology of stress and stress-related disorders with particular emphasis on psychoneuroendocrinology, -immunology and -physiology of stress, PTSD and depression and their neuropsychological correlates, as well as on the circadian system and chronodisruption as vital components of neuropsychiatric disease development. Dr. Agorastos has received a large number of international scholarships (e.g., by EPA, ISPNE, SOBP, APS, ECNP, WFSBP, King's College, University of Hamburg), research grants (Werner-Otto Foundation, Bial

Foundation, University of Hamburg), awards (EPA, ISPNE, University of Hamburg), honors and certifications (e.g., by EABCT, ECNP, University of Maastricht, EAP), is active member of >10 international, European and national professional societies, has served as ad hoc reviewer in >30 international scientific journals and associate editor and/or editorial board member in several international scientific Journals. Since 2010, he has published 74 PubMed listed articles in high-rank international journals (50 of them as first/last author) with a total impact factor of 320.45 and holds an h-index of 28 and an i10-index of 49 with a total of 2781 citations. According to Expertscape, Dr. Agorastos has been listed as Expert among the top 1% cited scientists of the world in all four categories of his research focus: Posttraumatic Stress Disorder, Autonomic Nervous System, Hypothalamus-Pituitary-Adrenal Axis and Circadian Rhythms.

LIST OF PUBLICATIONS

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