“Salivary alpha-amylase: More than an enzyme
Investigating confounders of stress-induced and basal amylase activity”

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This thesis is dedicated to my parents
for their love, endless support
and encouragement

Die Erkenntnis, daß mein sogenanntes Ich nur ein Muster ist, ein Tanz: das ist der wahre Kern der Entdeckung, in welchem Zeitraum der Atome des Gehirns durch andere Atome ersetzt werden. die Atome gelangen in mein Hirn, tanzen ihren Reigen und verschwinden wieder - da sind laufend neue Atome, doch sie tanzen, des Reigens von gestern eingedenk, immer wieder den gleichen Tanz.

(Richard Feynmann, Öffentliche Ansprache auf der Herbsttagung 1955 der Nationalen Akademie der Wissenschaften)

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Table of Contents

Figure and Table Index

List of Abbreviations

A:	Adrenaline
AAR:	Amylase awakening response
ACh:	Acetylcholine
ACTH:	Adrenocorticotropic hormone
AHS:	Adrenomedullary hormonal system
AL:	allostatic load
(rm) ANOVA:	(repeated measurement) analysis of variance
ANS:	Autonomic nervous system
ATP:	Adenosine triphosphate
AUC:	Area under the curve
AUC_g:	Area under the curve with respect to the ground
AUC_i:	Area under the curve with respect to increase
AVP:	Arginine vasopressin
BMI:	Body mass index
BP:	Blood pressure
bpm:	Beats per minute
cAMP:	Cyclic adenosine monophosphate
CAR:	Cortisol awakening response
CLIA:	Chemi-luminescence-immunoassay
CN:	Cranial nerve
CNS:	Central nervous system
CRH:	Corticotrophin-releasing hormone
CSSS:	Chronic Stress Screening Scale
DFG:	Deutsche Forschungsgemeinschaft (German Research Foundation)
ECG	Electrocardiogram
EDA:	Electrodermal activity
GC:	Glucocorticoids
HDL:	High density lipoprotein
HPA axis:	Hypothalamic-pituitary-adrenal axis
HR:	Heart rate
HRV:	Heart rate variability
h/wk:	hours per week
LC:	Locus coeruleus
LLM:	Lipid lowering medication
min:	Minute

NA:	Noradrenaline
OC:	Oral contraceptives
PNS:	Parasympathetic nervous system
PSS:	Perceived Stress Scale
PVN:	Paraventricular nucleus
RER:	Rough endoplasmic reticulum
RMSSD:	Root mean square of successive differences
rpm:	Rounds per minute
sAA:	Salivary alpha-amylase
SAM system:	Sympathetic-adrenomedullary system
SCN:	Suprachiasmatic nucleus
sec:	Seconds
SEM:	Standard error of the mean
SFR:	Salivary flow rate
SNS:	Sympathetic nervous system
SSRI:	Selective serotonin reuptake inhibitor
STAI:	State-trait-anxiety inventory
TICS:	Trier Inventory for the Assessment of Chronic Stress
TSST:	Trier Social Stress Test
TSST-C:	Trier Social Stress Test for Children
VAS:	Visual analogue scale

1. Introduction

The neuroendocrine and the autonomic nervous system (ANS) are two of the major systems playing a role in the adaptation of organisms to developmental changes that threaten homeostasis, i.e. stability of the internal milieu (Chrousos & Gold, 1992). The hypothalamus-pituitary-adrenal (HPA) axis is an important neuroendocrine system and involves the secretion of glucocorticoids (GC), including cortisol, into the circulatory system. The ANS is classically divided into its two subsystems the parasympathetic nervous system (PNS) using mainly acetylcholine (ACh) as its neurotransmitter and the sympathetic nervous system (SNS) stimulating the release of the catecholamines adrenaline (A) and noradrenalin (NA)[1] into the blood stream.

Numerous studies have been published that introduced salivary cortisol to assess HPA axis activity and therefore strengthens its role as an easy obtainable biomarker in stress research that can be monitored easily and frequently (Kirschbaum & Hellhammer, 2000). In contrast, measurement of salivary catecholamines has been difficult because of the low concentrations, rapid degradation and the difficulty to stabilize these chemicals. However, recent findings suggested a possible surrogate marker of catecholamines: salivary alpha-amylase (sAA). It was reported that this enzyme increases under a variety of physiological and psychological stress conditions. Furthermore, it seems to be predictive of plasma catecholamine levels even if those associations were relatively low (Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007). Thus, stimuli that increase plasma catecholamines may also activate autonomic input to the salivary glands followed by an increase in sAA activity. Furthermore, numerous studies failed to find correlations between cortisol and sAA, suggesting that both salivary measures are independent biomarkers for each of the two principal stress responsive systems. Up to date, clearly additional methodological research is needed for a better understanding of the advantages and disadvantages of sAA activity in comparison to already established markers of ANS activity. The aim of the present thesis is to further our knowledge of confounders of sAA activity under basal and acute stress conditions and to strengthen the validity of this enzyme as an easy obtainable alternative for adrenergic activity testing.

First, classical as well as more modern stress concepts will be summarized followed (chapter 2.1.) by an introduction into stress system physiology (2.2.) and a discussion of a possible relationship between the HPA and the ANS. In chapter 3 the reader will learn how salivary glands are innervated by the nervous system leading to secretion of salivary proteins and an alteration in salivary flow rate (SFR). Afterwards, sAA will be introduced (chapter 4.) and a more nuanced review of methodological considerations of alpha-amylase

[1] Adrenaline and noradrenaline are the terms of these chemicals approved in Great Britain while epinephrine and norepinephrine are United States approved terms. This thesis will use the terms adrenaline and noradrenaline.

determination (chapter 5.) will show gaps of knowledge concerning sAA usefulness as a marker of ANS activity. Afterwards, the aims of this thesis will be illustrated (chapter 6.) followed by the presentation of four empirical studies. Study 1 describes sAA activity under psychological stress conditions in different age groups (chapter 7.). The following three studies measured sAA activity under basal conditions in different age groups (chapter 8.), in older adults suffering from high blood pressure and treated with antihypertensive drugs (chapter 9.), and in adults of different ages under different light conditions in the morning (chapter 10.). The last part of this thesis (chapter 11.) summarizes the findings, provides further methodological considerations concluded from the results, and integrates results in a broadly based context.

2. Stress

In this chapter, the theoretical background of measuring the psychobiology of stress within a multisystem approach is provided. Recent technical advances enabled the simultaneous noninvasive assessment of neuroendocrine and ANS reactivity in saliva adding new opportunities to the study of health and human development. At first, chapter 2.1. will introduce traditional stress concepts beginning in the late 19th century up to modern concepts. Afterwards, both major physiological stress systems, HPA axis and ANS, will be shortly introduced and the relationship between both will be elaborated in chapter 2.2.

2.1. Stress concepts

2.1.1. Traditional concepts of stress

The French physiologist Claude Bernard was the first who described a biologic principle that for life there must be fixity of the “Milieu intérieur” despite physiological or psychological demands from the external environment. As early as 1878 his principle of a balanced internal state maintained by biochemical and physiologic processes implied a static constancy (Bernard, 1878). Later, Cannon called this principle of the equilibrium of physiological systems *homeostasis* (Cannon, 1932) and thereby described the “coordinated physiological process which maintains most of the steady states of the organism”. Any change within the organisms` internal environment leads to a compensatory process called “righting” response that minimizes changes. Providing further insight into the concept of homeostasis, Dubos (1965) introduced homeostasis and adaptation as two complementary concepts being necessary for balance. In his opinion *absolute constancy is only a concept of the ideal* with homeostatic processes occuring quickly in response to stress and adaptive processes resulting in structural or functional changes over time. In 1915, Cannon also coined the term “fight or flight” to describe the response of animals to external threats (Cannon, 1915).

In the mid 1930´s, Cannon (1935) and Selye (1936) introduced the concept of stress. This was the beginning of a long way of attempts to narrow this concept and find a definition most precise (Levine, 2005). Cannon himself used this term to describe threats of homeostasis. Selye was the first who conceptualized the physiology of stress as (1) consisting of a set of responses that he called “general adaptation syndrome” and (2) leading to a pathological state when there is ongoing, unrelieved stress. He was inspired by the finding that every irritating substance injected in mice resulted in the same symptoms: atrophy of the thymus, swelling of the adrenal cortex, and gastric and duodenal ulcers. Same was true for other “noxious agents” like cold, heat, or x-rays. In his early career, Selye used the term “stress” to indicate the stimuli. In his later work, he preferred the term “stressor” to define the different stimuli while “stress” describes the physiological response. In his 1974

work he described stress as the “nonspecific response of the body to any demand made upon on it”. In his view, the system whereby the body responds to and copes with stress is the HPA axis. He defined glucocorticoids as the primary mediators and the adrenal cortex as the “organ of integration” (Selye, 1974). Comparing the concepts of Cannon and Selye reveals the inclusion of psychological and emotional stimuli in the concept of Cannon while Selye only refers to non-psychological stimuli. In 1968, Mason criticized Selye´s concept of nonspecificity and assumed that physiologic threatening stimuli evoke endocrine responses solely because of their simultaneous impact on psychological processes (Mason, 1968). Thus, it is not the stressor itself but rather psychological influences known to affect HPA activity. According to Mason, factors most reliable stimulating a stress response are novelty, unpredictability, uncontrollability, uncertainty, ego-involvement, and trying. Those factors of stress perception are processed by the central nervous system (CNS) and taken into account to evaluate the situation and if a stress response is required (Mason, 1968). In the early 1990´s, Levine and Ursin tried to integrate different stress concepts and proposed a structuring into (a) stress stimuli, (b) the individual coping with stress, and (c) stress responses. According to this model, a stimulus (= input), called “load”, becomes a stressor (= output) after interpretation within a multilevel process of appraisal (Levine & Ursin, 1991). This multidimensional concept classifies possible stress responses into subjective-verbal, behavioral, and physiological responses. Furthermore, the authors pointed out that short lasting stress responses are less likely to have adverse effects while long lasting excessive responses come along with substantial health risks. Nowadays, more modern concepts try to clarify this paradox situation where stress is protective under acute threat conditions but also results in chronic hyperactivity and pathology in the long run. One of those modern theories is the concept of Allostatis and Allostatic Load (AL) first introduced by McEwen and Stellar (1993). The next chapter will give a short review of this concept and provide a critical discussion of strengths and weaknesses of this theory.

2.1.2. Allostasis and Allostatic Load

It is often discussed that only prolonged activation can lead to pathogenic states possibly leading to disease (Linden, Earle, Gerin, & Christenfeld, 1997). This concept was already included in the stress theory of Selye (1974). However, only a few scientists added this concept into their theories. In the early 1980´s, Ursin and co-workers introduced the concept of “sustained activity” (Ursin & Murison, 1983). But not until the late 1990´s - with the implication of the concept of Allostasis and AL (McEwen, 1998) - the stress theory was expanded by prolonged physiological activation and stress recovery. In its original meaning allostasis describes the adaptation of the cardiovascular system to changes in activity levels, i.e. situations that did not challenge survival (McEwen & Stellar, 1993). This “stability through

change" requires coordinated alteration in different body systems in response but also in anticipation of demands. The physiological mechanisms that maintain stability are called allostatic mediators, e.g. hormones, cytokines, as well as behavior. According to McEwen and co-workers, the term Allostatic Load refers to "the wear and tear that the body experiences due to repeated cycles of allostasis as well as the inefficient turning-on or shutting off these responses" (McEwen, 1998; McEwen & Wingfield, 2003a). Four distinct types of AL were outlined. Being exposed to multiple stressors during short time periods is the first type of AL (*repeated "hits"*). The second form of AL involves the experience of the same repeated stressor eliciting a physiological response that fails to habituate (*lack of adaptation*). In the third form of AL, the physiological response may be normal in frequency or magnitude while the physiological recovery is delayed (*prolonged response*). The fourth form of AL involves a stress response that is very weak or absent leading to imbalances between stress systems (*inadequate response*). When AL occurs too high or too long, or when demands exceed the energy that can be obtained from the environment, *allostatic overload* results (McEwen & Wingfield, 2003a). Therefore, *allostatic overload* is the state in which serious pathophysiological changes may occur. Validating the AL concept, higher AL summary scores were predictive of a higher risk of cardiovascular diseases, physiological and cognitive decline, and mortality (McEwen & Seeman, 1999).

A major strength of the AL model is the incorporation of circadian, circannual, and life-related changes, i.e. the idea that physiological parameters change with lifetime. The concept of AL as a measure to model the wear and tear of the body is another strength since this may indicate how well prepared the individual is when exposed to future stressors (McEwen & Seeman, 1999). A third strength is the prediction of when does accumulated AL turns into *allostatic overload* and the generation of testable hypotheses dealing with this question. On the other side there are several weaknesses. One weakness is related to the usefulness of energy expenditure and consumption as a measure of allostasis. Energy use not only depends on different contexts but it is also quite obvious that not all energy mobilization is equivalent. As McEwen and Wingfield (2003b) have also recognized, the AL model may draw too much attention on only measuring glucocorticoids (GC) as markers of energy use. This assumption seems to be too strict since corticosteroids led to increases of blood glucose, basis for the assumption that GC´s mobilizes energy (Sapolsky, Romero, & Munck, 2000), in fasted but not in fed animals (Remage-Healey & Romero, 2001). Furthermore, not all stress responses are accompanied by significant increases in energy expenditure, e.g. freezing of animals in the presence of a predator. In addition, there are a number of concepts that are not easily incorporated into the AL concept, e.g. early developmental effects believed to be mediated through neural and/or epigenetic mechanisms (Szyf, Weaver, Champagne, Diorio, & Meaney, 2005). More recent work now tries to

incorporate a network of mediators involved in the stress response as well as genetic contributors to AL (for a review see Juster, McEwen, & Lupien, in press).

2.2. Stress system physiology

The main endocrine systems of the body are the HPA axis which triggers the release of GC´s from the adrenal cortex and the sympathetic-adrenal-medullary (SAM) axis, as part of the ANS, triggering the release of catecholamines from the adrenal medulla. Therefore, the following chapter will describe the physiology and indicators of HPA axis (2.1.1.) as well as ANS activity (2.1.2.).

2.2.1. The hypothalamic-pituitary-adrenal (HPA) axis

2.2.1.1. Physiology

Disruption of the homeostasis stimulates and induces the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus. In conjunction with arginine vasopressin (AVP), CRH stimulates the production of adrenocorticotropic hormone (ACTH) in the pituitary. This hormone enters the bloodstream and stimulates the release of GC´s (mainly cortisol in humans) from the adrenal gland. In turn, GC´s suppress hypothalamic CRH and pituitary ACTH production in a negative feedback loop. Because of being bound to binding proteins in blood, only 5-10% of total plasma cortisol is biologically active, the so called free cortisol. In humans, cortisol binds to intracellular steroid receptors. Since those receptors are present in nearly every organ and tissue of the body, cortisol mediates various processes. Therefore, activity of the HPA has been studied extensively showing that HPA axis dysfunction is involved in the development and/or progression of different disease states including the metabolic syndrome, diabetes, hypertension, depression, and cognitive impairments (Belanoff, Gross, Yager, & Schatzberg, 2001; Bjorntorp, 2000; Chrousos & Gold, 1992; Holsboer, 2000).

2.2.1.2. HPA axis activity indicators

In humans, cortisol is the final product of the HPA and is most commonly measured in blood serum or plasma, urine, or saliva. The usefulness of these assessment methods is proven for a variety of research questions including acute stress paradigms or paradigms capturing circadian profiles. Compared to blood samples, saliva sampling is non-invasive, low-cost, and easy to obtain even without supervision. Only free cortisol appears in saliva. However, salivary cortisol and values of the free cortisol fraction in blood correlate very well (Kirschbaum & Hellhammer, 1994). If longer time periods are of interest, urine collections are another sampling technique. However, because of its limited feasibility (collection might be bothersome, e.g., when being not at home) this integrative cortisol measurement is non-applicable for periods longer than 24 hours. Just recently, a new method of analyzing cortisol

in human hair was introduced that captures longer time periods depending on the length of the hair and allowing the retrospective determination of cortisol secretion (e.g., Kirschbaum, Tietze, Skoluda, & Dettenborn, 2009).

Experimental studies have investigates the effects of different types of stress on HPA (re-) activity. Pharmacological challenges, physical exercise, or psychological stress are used to stimulate the HPA axis. Furthermore, it is also necessary to differentiate between real-life stressors and laboratory stressors under either acute or chronic stress conditions. One advantage of pharmacological stimulation is that this may act at different levels of the HPA axis and helps to determine e.g. the capacity or sensitivity of different components of the HPA axis. With regard to laboratory stress protocols, there is a large diversity of designs having different potencies to evoke HPA axis responses (for a review see Biondi & Picardi, 1999). According to Dickerson and Kemeny (2004) tasks that contain elements of uncontrollability and social evaluation elicit largest cortisol responses and longest recovery periods. One of those well-evaluated stress protocols is the Trier Social Stress Test (TSST, Kirschbaum, Pirke, & Hellhammer, 1993). This high standardized test consists of a 3 minute preparation period, a 5 minute speech in front of an unknown auditory, and a 5 minute mental arithmetic task. Studies applying this psychosocial stressor repeatedly revealed HPA axis responder rates of > 70% (Kudielka, Hellhammer, & Kirschbaum, 2007), what underlies the importance of psychological elements in stress system reactivity.

2.2.2. The autonomic nervous system (ANS)

The ANS innervates nearly every organ and thereby regulates various functions such as cardiovascular, respiratory, endocrine and exocrine, electrodermal, or gastrointestinal in a continuous manner through the action of autonomic reflexes (Oribe, 1999).

2.2.2.1. Physiology

The peripheral part of the ANS is composed of the SNS, the PNS, and the enteric nervous system. Therefore, neurons and nonneuronal cells of the sympathetic, the parasympathetic, and enteric ganglia and the chromaffin cells of the adrenal form the involuntary control of many organs and muscles. Neurons of the ANS are under control of the central nervous system through preganglionic neurons. Those are located in the spinal cord and receive inputs from different brain regions, mainly neurons of the locus coeruleus/noradrenaline (LC/NA) nuclei of the brain stem.

The SNS originates in the thoracic and lumbar spinal cord. The general function of the SNS is to induce the “fight-or-flight”-response and to mobilize the body but also to maintain homeostasis through sustaining a constant level of activity. The shorter preganglionic nerves travel to a ganglion where they synapse with a postganglionic neuron. Here, the preganglionic neurons release the neurotransmitter ACh that binds to nicotinic ACh receptors

on the postganglionic neurons triggering the release of NA what activates adrenergic receptors on target tissues. There are two important exceptions: neurons innervating sweat glands release ACh and stimulate muscarinic receptors, and the adrenal medulla who acts as a modified sympathetic ganglion. Here, pre- and postganglionic neurons synapse and the postganglionic neurons directly release NA (20%) and A (80%) as well as a small amount of dopamine into the blood stream (Goldstein, 1995; Shepard & West, 1951). The adrenal medulla is responsible for essentially all A secreted into the body while adrenergic neurons produce the majority of NA. Due to presynaptic uptake only 10 to 15% is secreted into the blood stream, the so called free NA.

The neurons of the PNS originate in the sacral region of the spinal cord and in the medulla. Here, the cranial nerves (CN) III, VII, IX, and X, also called vagus nerve, form the preganglionic nerve fibers projecting to ganglia close to the target tissue. Both, the synapses between pre- and postganglionic parasympathetic fibers as well as postganglionic projections to the target organs use ACh as its neurotransmitter. Main functions of the PNS are salivation, lacrimination, urination, it promotes digestion and defecation. Summarizing those actions, the function of the PNS may be described as “rest-and-digest”. In sum, the traditional assumption that SNS and PNS typically function antagonistic should be better understood as complementary and synergistic what will be discussed with respect to salivary gland innervation in more detail below (chapter 3.).

2.2.2.2. ANS activity indicators

Autonomic activity and reactivity are of main interest in psychophysiological research since nearly every organ is innervated by autonomic nerve fibers. Most important non-invasive indicators of peripheral autonomic activity are electrodermal activity (skin conductance), cardiovascular activity (heart frequency, heart rate variability, and blood pressure), and respiration. Furthermore, A and NA as well as its metabolites may be directly measured in serum, urine, and saliva.

Electrodermal activity (EDA) describes changes in the ability of the skin to conduct electricity. Eccrine sweat glands that are sympathetically innervated via ACh and the activity of myoepithelial cells form the basis of measuring EDA. Changes in hydration status of sweat glands can be translated into skin conductance or skin resistance since filled with salt-water, sweat glands show less resistance to electricity or conversely greater conductance. Non-invasive measurement of EDA involves two electrodes usually placed on the first and second of either one or both hands. An electrical signal is passed between both electrodes whereby the skin acts like a series of resistors. Changes in sweat gland activity are indicated by variations in the skin conductance responses. Electrodermal measures are seen as correlates of psychophysiological states of arousal or activity and related to various affective and cognitive responses (Oribe, 1999).

Due to the role of the ANS in modulating heart activity and its influence on vascular tonus, heart rate, and blood pressure are further indicators of ANS activity. Non-invasive determination of systolic and diastolic blood pressure is in most cases performed using the Riva Rocci Korotkoff method providing punctual measurements to study responses to arousing stimuli, either physiological or psychological, immediately. Another application is the assessment of 24-hour blood pressure monitoring. This method allows the reliable assessment of blood pressure during day and night to detect circadian rhythmicity. Throughout the course of a day, various stimuli (activity, work, emotions) can alter blood pressure what might be of interest not only for evaluation and treatment of hypertension (Schächinger & Langewitz, 1997). The electrocardiogram (ECG) is another important device to assess heart activity. During each heart beat the heart muscle depolarizes leading to potential differences that can be detected on the skin surface. Most important parameters that can be obtained from the ECG are heart rate (HR) and heart rate variability (HRV). Heart rate variability is thought to reflect the ability of the heart to quickly adapt to changing environments and thereby, to assess the regulatory input of the ANS on cardiac activity. While there is no possibility to isolate sympathetic and parasympathetic influences on heart rate, analysis of the HRV helps to separate both autonomic impact factors. Heart rate variability measures the variability in the beat-to-beat-intervals that is analyzed to give different variables that mainly can be grouped under time-domain and frequency-domain measures (for an overview see Stein & Kleiger, 1999; Task Force of the European Society of Cardiology and The North American Society of Pacing and Electrophysiology, 1996). There are multiple factors that influence the validity of HRV: respiration, blood pressure, peripheral blood flow, thermoregulatory processes, hormones, sleep-wake cycle, physical activity, and stress (e.g. van Ravenswaaij-Arts, Kollée, Hopman, Stoelinga, & van Geijn, 1993) what might be considered when measuring HRV.

According to Goldstein (2003), there are three peripheral catecholamine systems: the SNS, the adrenomedullary hormonal system (AHS), and the DOPA-dopamine autocrine/paracrine systems. The author suggested that, depending on the stressor, there is a difference between SNS and AHS stress responses what questions the notion of a unitary SAM system. This new concept postulates that NA, also described as marker of overall SNS activity, is mainly responsible for appropriate distribution of blood volume and homeostasis of blood pressure e.g. during orthostasis or moderate exercise. On the other hand, A, described as marker of the AHS system, is rather responsive to global or metabolic threats, such as exercise beyond an anaerobic threshold or emotional distress. All three endogenous catecholamines can be assessed in biochemical analyses from different body fluids. Due to neuronal re-uptake, clearance rates of specific tissues, and the speed of blood flow, only a small amount (5-10%) of NA is added into the blood stream (Esler et al., 1990). In blood

obtained from an arm vein, plasma catecholamines reflect catecholamines secreted from all body organs (Dimsdale & Ziegler, 1991). Despite there is evidence for peripheral (plasma clearance) NA reflecting central (secretion) noradrenergic activity (Lambert et al., 1997), techniques that directly record sympathetic nerve traffic may be more valid and reliable than plasma NA. Microneurography or NA radiotracer methods allow discriminating central from peripheral triggered plasma NA levels and estimating regional sympathetic neural function. For an extended review of different techniques to assess sympathetic activity see Grassi and Esler (1999).

2.2.3. Relationships between stress systems

Centrally, the HPA axis together with the SAM system connects the brain with the periphery. The CRH and AVP neuronal network of the PVN of the hypothalamus forms the central basis of the HPA. Noradrenergic neurons of the LC/NA nuclei of the brain stem are the basis of central SNS activity. Both systems have a baseline circadian rhythmicity and they are well known as the major stress responsive systems. There is evidence for a close interaction between the HPA axis and sympathetic functioning with e.g. heightened vulnerability to develop addictive disorders due to a higher sensitivity to the effects of cortisol mediated via an increased activity of mesencephalic dopaminergic neurons (Piazza & Le Moal, 1996). Furthermore, in vitro studies were able to show the effectiveness of neurotransmitters to affect adrenal steroidogenesis (Delarue et al., 2001). In the review mentioned above, Goldstein (2003) concluded that there is a closer association between the HPA axis and the ANS than between AHS and SNS responses. Bornstein and Ehrhart-Bornstein (2000) were able to show a ten times higher production of GC´s in adrenocortical cells when co-cultured with chromaffin cells. Furthermore, circulating catecholamines are known to influence the secretion and the effects of CRH and AVP in the hypothalamus and the secretion of ACTH in the pituitary (Plotsky, Cunningham, & Widmaier, 1989). To sum up, despite the close interaction between both stress systems, there is also evidence for a differentiated response and sensitization to stress that may serves different demands.

3. Saliva and salivary glands

3.1. Physiology

3.1.1. Anatomy, origin, and composition

Due to their important role for eating, aiding digestion and taste, and acting as a pH buffer, salivary glands are present in amphibians upward. There are three main paired salivary glands in mammals starting to develop in set positions as outpouchings of oral ectoderm into the surrounding mesoderm. In humans, the submandibular and the sublingual glands lie under the tongue, and the parotid glands are situated under and in front of the ears having long ducts that drain saliva opposite to the first to second molar. Additionally, there are a large number of minor salivary glands (> 700) throughout the oral cavity including the labial, buccal, lingual and palatinal glands. The major salivary glands not only develop at different sites but also have a very different histology and produce different types of saliva. Generally, salivary glands are composed of acini producing saliva, a secretary duct, and a collecting duct system transporting saliva into the oral cavity (Humphrey & Williamson, 2001 and Figure 3.1). The parotid glands have a tree-like architecture and its acini produce proteinaceous, waters and serous saliva. During gustatory and olfactory stimulation two thirds of salivary flow originates from these glands. Submandibular saliva is high mucin with mainly viscous/serous secretion and under basal conditions whole saliva mainly consists of this saliva. Sublingual gland saliva is higher in mucin content than submandibular gland saliva. Minor salivary glands are purely composed of mucinous acini. Myoepithelial cells are parenthesized between the acini and the basement membrane. Those cells have a contractile function and drive the saliva into the acinar lumen and the duct system. Whole saliva consists of about 99.5% water and 0.5% large organic molecules including proteins, glycoproteins and lipids, small organic molecules including glucose and urea, and electrolytes. While non-stimulated whole saliva mainly consists of saliva originating from the submandibular and sublingual gland (up to 70%, up to 20% from the parotid glands, up to 10% from the minor glands) stimulated whole saliva consists of up to 50% from the parotid glands, up to 50% from the submandibular and sublingual glands and only marginal from the minor glands (Humphrey & Williamson, 2001; Screebny, 2000).

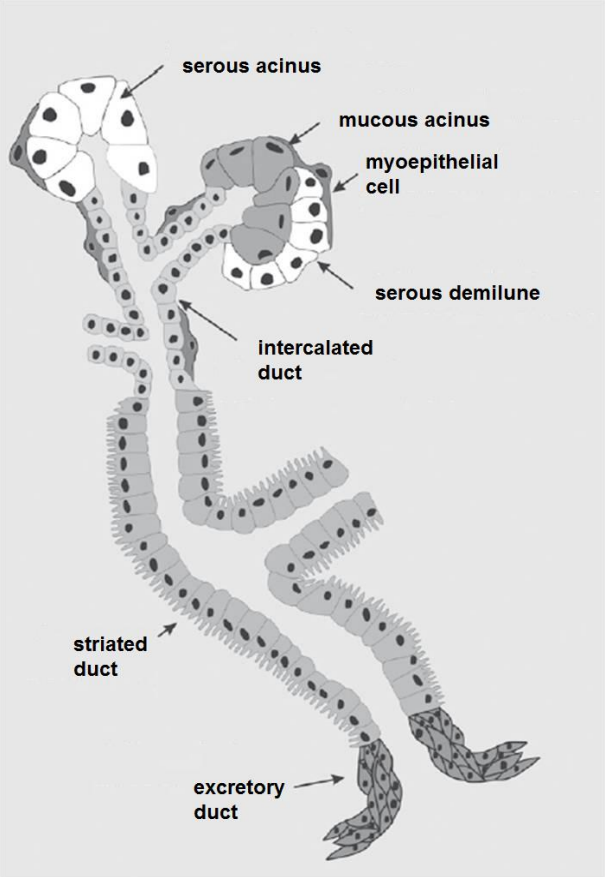

Figure 3.1 Structure of the salivary gland (Edgar, O´Mullane, & Dawes, 2005 p. 7).

3.1.2. Innervation

Salivary glands are innervated by the sympathetic and parasympathetic branches of the ANS. Salivary glands receive their parasympathetic input via cranial nerves. The glossopharyngeal nerve innervates the parotid gland, while the submandibular and the sublingual glands are innervated by the facial nerve (CN VII) via the submandibular ganglion. These nerves release ACh and substance P as neurotransmitters that act as first messengers communicating with intracellular second messengers which have direct control of cellular secretory pathways (see also 3.3.). Sympathetic innervation takes place either directly, described below, or indirectly via the innervations of blood vessel that supply the salivary glands. Direct sympathetic innervation is carried via preganglionic nerves in the thoracic segments T1-T3. These nerves synapse in the superior cervical ganglion with postganglionic neurons releasing NA. This activates beta-adrenergic receptors on the acinar and ductal cells of the glands leading to an increase of the second messenger cyclic adenosine monophosphate (cAMP) and thus increasing saliva secretion. Important to note is that in this regard sympathetic as well as parasympathetic signaling stimulates saliva secretion. Normal salivary secretion depends on a more complementary than antagonistic interplay between the sympathetic and the parasympathetic nervous system. While the PNS

is primary involved in fluid secretion, it has a limited role in protein secretion and exocytosis. Stimulation via the SNS results in low volume of saliva with high protein content. Thus, with regard to the stimulation of salivary flow, the SNS is synergistic to the PNS that is dominant (Witt, 2006).

3.1.3. Salivary gland physiology with aging

Structural changes of salivary glands are well known with aging. Especially a less ordered lobule structure and acini that vary more in size and atrophy have to be mentioned. Furthermore, the fibroadipose tissue increases (Azevedo, Damante, Lara, & Lauris, 2005). Other changes that take place include shrinkage of cells, dilation of ducts, oncocytic transformation, increased adiposity, fibrosis, microcalcification, and chronic inflammation (Scott & Path, 1986). Despite these changes, parotid glands have a large secretory reserve (Fischer & Ship, 1999) that nevertheless decreases with age (Segawa et al., 2000). A decreased flow in submandibular glands and minor salivary glands has been reported with age, but not within the parotid glands (Wu, Baum, & Ship, 1995). Changes observed within the parotid glands are a decrease in electrolyte content (Chauncey, Feller, & Kapur, 1987) and histanins (Johnson, Yeh, & Dodds, 2000), increases in Immunglobulin A (Childers et al., 2003; Wu et al., 1995), and decreases in mucin content (Denny et al., 1991) accompanied by decreases in sAA (Ben-Aryeh et al., 1986). In contrast, Aguirre, Levine, Cohen, and Tabak (1987) reported that sAA levels remained unchanged in stimulated parotid saliva. In sum, despite profound morphological changes with aging salivary output remains relatively unaffected.

3.2. Saliva and salivary flow

Humans secrete between 0.5 and 1.5 L of generally tasteless saliva daily. Saliva is composed of a variety of organic and inorganic components. Inorganic components are electrolytes such as sodium, calcium, magnesium, bicarbonates, phosphates, and nitrogenous products such as ammonia. The vast majority of the organic components of saliva are proteins such as immunglobulins, enzymes, and mucins. These are actively transported and either produced in the acinar cells, in the ducts, or transported into saliva from blood. Parotid gland saliva is much higher in protein content than the other major salivary glands. Thus, the final saliva is an aggregate of saliva produced by several glands showing different secretory characteristics. This leads to a variability of composition at any particular time enhanced by the fact that salivary gland function changes with type of stimulus that drives the saliva production (Cummings, 2005). In healthy humans, saliva has a slightly alkaline pH and therefore buffers acids (Witt, 2006). One of the major problems when studying this body fluid is that salivary flow rates (SFR) vary by as much as 45%

intraindividual (Ghezzi, Lange, & Ship, 2000) stabilizing after the age of 15 years (Humphrey & Williamson, 2001) and showing only slight changes with aging (Sevón et al., 2008). Furthermore, SFR was not influenced by the phase of menstrual cycle (Laine, Pienihäkkinen, Ojanotko-Harri, & Tenovuo, 1991) while postmenopausal women showed slightly lower flow rates compared to menstruating women (Dural, Hatipoğlu, & Çağirankaya, 2006). In a study examining the impact of aging and estrogen status on flow rates of all three major salivary glands, results revealed no differences for parotid and whole saliva, either stimulated or non-stimulated, whereas submandibular/sublingual flow rates differed significantly (Streckfus et al., 1998). There were no difference between those taking estrogens and those without.

3.3. Protein secretion

Most protein secretion occurs in the acinar cells and is largely under sympathetic control and evoked by beta-adrenergic stimulation in particular (Busch & Borda, 2002; Garrett, 1987; Proctor & Carpenter, 2007). The cervical ganglion of the sympathetic chain sends sympathetic postganglionic fibers to salivary glands. Sympathetic neurons release NA that binds to alpha- and beta-adrenergic receptors on the acinar cell. Intracellular Calcium is elevated due to alpha-receptor activation while beta-receptor activation results in elevation of intracellular cAMP. Once stimulated, amino acids are actively transported into acinar cells from the interstitium. Proteins and other macromolecules such as sAA and glycoproteins are produced intracellularly in the rough endoplasmic reticulum (RER). After synthesis, salivary proteins are segregated in the cisternal space of the RER and intracellular transport via adenosine triphosphate (ATP) mechanism carries secretory products to the golgi complex. Temporary storage takes place in secretory granules. Following activation by cAMP and calcium activated kinases (in response to beta-adrenergic stimulation) lead to exocytosis and thus to the release of contents extracellularly (Castle & Castle, 1998; Smith, 2004).

4. Alpha-amylase in saliva

Salivary alpha-amylase is the most important and abundant protein in saliva. It constitutes about 10% of overall salivary protein content (Witt, 2006). In humans, total sAA production is estimated to be 1.5 g whereby 60% are released by the pancreas and 40% by salivary glands, mainly parotid glands (up to 70%).

4.1. Chemical characteristics

The common name of the enzyme is alpha-amylase (also ptyalin). The chemical name is 1,4 alpha-D-glucan glucanohydrolase consisting of 511 amino acids. According to its atomic composition its formula is $C_{2589}H_{3857}N_{715}O_{752}S_{23}$. Its instability index of 23.58 classifies the protein as stable (Gasteiger et al., 2005). In humans, there are 5 isoforms encoded by the AMY1A, AMY1B and AMY1C gene in saliva and the AMY2A and AMY2B gene in the pancreas. Alpha-amylase is a calcium-containing metalloenzyme that catalyses the endohydrolysis of 1-4-alpha-D-glucosidic linkages in polysaccharides containing three or more 1,4-alpha-linked D-glucose units. Additionally, sAA has been shown to have an important role in bacterial clearance (Scannapieco, Torres, & Levine, 1993).

4.2. Secretion of alpha-amylase

Neuronal stimuli such as neurotransmitters and specific bioactive peptides play a major role in stimulating sAA release. Using blocking or stimulating pharmacological agents or electrical stimuli helps to understand secretory mechanisms. One of the first studies indicating sAA as a marker of sympathetic activity is a study conducted by Speirs, Herring, Cooper, Hardy, and Hind (1974) who either administered beta-adrenergic active substances (isoprenaline a stimulating agent or propanolol a blocking agent) or immersed subjects in cold water (4-5 °C). While propanolol led to a reduction in sAA, cold water and isoprenaline increased sAA in parotid saliva. Using beta-blocking agents such as timolol maleate, atenolol or metoprolol, different studies examined the quantity and quality of saliva secretion controlled by beta-adrenoceptors in healthy subjects (Laurikainen, Laurikainen, Tenovuo, Kaila, & Vilja, 1988; Nederfors, Ericsson, Twetman, & Dahlöf, 1994) and hypertensive subjects (Nederfors & Dahlöf, 1996). More recent studies revealed that propanolol attenuates sAA increases in response to a stress test compared to a placebo group (van Stegeren, Rohleder, Everaerd, & Wolf, 2006). To test the hypothesis that sAA increases might reflect interacting sympathetic and parasympathetic stimulation via central nervous noradrenergic input, Ehlert, Erni, Hebisch, and Nater (2006) examined the effect of yohimbine hydrochloride, an alpha-2-adrenergic receptor antagonist, on sAA release. As expected, larger increases in sAA activity were found in the yohimbine condition. Furthermore, there is

a well known effect of mechanical and gustatory stimulation on salivary amylase secretion what will be discussed elsewhere within this thesis (see chapter 5.3.5.5.).

4.3. Diagnostic value of alpha-amylase

As also reviewed in the next chapter and based on the findings concerning the impact of stress and its physiological mechanisms in the secretion of sAA, one might conclude that sAA activity could serve as an index of pathological ANS dysregulations. Different psychiatric conditions are accompanied by changes in sAA activity (see 5.3.5.). However, up to date, only few studies examined sAA as an autonomic marker in psychiatric patients. More is known about sAA alterations in somatic diseases such as immunological diseases (see 5.3.5.). Based on those findings in clinical populations, the usefulness of sAA to measure the effects of therapeutic interventions should also be considered. This possible application of sAA measures is just in the fledging stages. Kalman and colleagues (2008) used sAA to assess stress levels and the therapeutic effect of Relora® (a plant-based medication to reduce anxiety and its symptoms). Similarly, to measure sAA as an endpoint of a treatment was applied in a study using reflexology in dementia patients (Hodgson & Andersen, 2008). Results revealed significant reductions in observed pain and sAA in reflexology receiving patients. Patients undergoing a surgery and being exposed to natural sounds showed decreased levels of sAA compared to patients who had no sounds (Arai et al., 2008). Other contexts where sAA might be a useful marker seem to be pain (Bugdayci, Yildiz, Altunrende, Yildiz, & Alkoy, 2010) or sleep (Räikkönen et al., 2010) research. Especially ambulatory settings, where an easy and non-invasive technique is necessary, sAA measurement may be particularly useful. The reader is referred to chapter 11 for a more detailed illustration.

5. Methodological considerations of alpha-amylase determination

When measuring sAA activity several methodological aspects have to be taken into account. Researchers have to decide which sampling method to take, how to storage samples and which determination method to use. Furthermore, there are multiple factors that might influence sAA activity. This chapter will describe those issues in more detail.

5.1. Collection methods and preparation

5.1.1. Saliva collection

Methods of saliva collection can be divided into two subgroups: those methods collecting whole saliva and those for collecting specific saliva, either under stimulated or unstimulated conditions. According to Navazesh (1993) there are four methods of collecting unstimulated saliva: (1) the draining method where the subject is asked to tilt the head forward and to allow saliva to drain from the lower lip into a storage container, (2) the spitting method where saliva should be accumulated in the mouth and after about a minute spitted in a storage container, (3) the suction method where saliva is drawn out of the mouth into a storage container, and (4) the swab absorbent method where a cotton role is placed in the mouth what should absorb saliva. In contrast, stimulated whole saliva can be stimulated by mechanical (chewing) or gustatory stimuli (citric acid) as already discussed in chapter 4. However, given the fact that gustatory stimuli might confound the results, mechanical stimulation should be preferred. One of the most common used techniques to collect stimulated whole saliva is the use of salivettes (Sarstedt, Nümbrecht, Germany). This device consists of a plastic vessel, a centrifugation tube, and a cotton swab. In 2009, Sarstedt introduced the Salivette®Cortisol with a synthetic swab for precise cortisol measurement. While the use of salivettes is currently viewed as gold standard when assessing cortisol levels (Kirschbaum & Hellhammer, 1999), sAA activity measures might be confounded by stimulation of saliva flow since salivary proteins also responds to mechanistic stimuli per se (see chapter 3). This issue will be discussed in the next chapter.

5.1.2. Impact of flow rate

It is known that the amount of sAA in humans has its short-term functionality in the digestion of starches as soon as they enter the mouth. Salivary flow rate and composition are influenced by primary oral stimulation like chewing and food properties (Froehlich, Pangborn, & Whitaker, 1987; Mackie & Pangborn, 1990) and by alteration of parasympathetic nervous system activity (Anderson et al., 1984; Garrett, 1987). As previously shown, changes of sAA activity are a primary response to oral stimulation alone since oral stimulation would have effectively changed only its secretion rate but not its concentration (Froehlich et al., 1987; Mackie & Pangborn, 1990). A higher sAA secretion rate parallels increased SFR values, so

that more sAA is available for digestion while concentration in saliva remains unaffected. Given the general assumption that the sympathetic and the parasympathetic branches of the ANS are functionally opposing and mutually inhibitory, stress induced increases of sympathetic tone would therefore inhibit parasympathetic tone. As the PNS is the primary mediator of SFR, this inhibition would decrease SFR. Together with unchanged protein secretion from acinar cells this theoretically leads to higher protein concentrations without increased protein secretion per se. This hypothesis was tested by Rohleder, Wolf, Maldonado, and Kirschbaum (2006) who could show that stress-induced increases of sAA levels were correlated with increases of amylase output but not with flow rate. Furthermore, flow rate increased only when sampled by passive drooling but not when using salivettes. The authors suggested that sAA reactivity is independent of flow rate and that salivettes are a valid sampling technique whereby the assessment of flow rate is unnecessary.

5.1.3. Impact of pH-value

It has been reported that stimulation or chewing changes the pH value of saliva (Polland, Higgins, & Orchardson, 2003) and that a lower pH increases flow rate (Guinard, Zoumas-Morse, & Walchak, 1997). It is well known that enzymatic activity is affected by changes in pH. So, it could be suggested that chewing-stimulated increase of salivary flow leads to alterations of pH value and thereby to changes of enzymatic activity. To test this hypothesis, we changed sample pH value with hydrochloride or sodium hydroxide. We therefore obtained 52 saliva samples with the help of salivettes and divided them into three portions. Untreated saliva samples showed a mean pH value of 8.12 ± 0.53. After adding hydrochloride mean pH value decreased to 6.18 ± 0.60 and after adding sodium hydroxide values increased to 10.15 ± 0.51. Salivary alpha-amylase was measured by a quantitative enzyme kinetic method (see 5.2.). Analyses revealed no difference between the treated and untreated samples (all $t_{51} < 0.8$, $p < 0.44$) with untreated samples showing a mean sAA activity value of 59.32 ± 31.45 U/ml, hydrochloride treated samples 56.39 ± 41.83 U/ml and sodium hydroxide treated samples 60.44 ± 36.39 U/ml. These data indicate that there is no alteration of sAA activity level after changing the pH value (Strahler, unpublished data).

5.2. Biochemical determination

Different measures may indicate sAA. The most common index of sAA activity is the determination of the enzyme level per volume of saliva (enzyme units per milliliter, U/ml) which will be also used in this thesis. However, this is only an indirect measure of sAA concentration. Therefore, total enzyme output in a fixed time interval is also often given as enzyme units per minute (U/min). Since the specific activity in relation to other salivary

proteins may also be of interest, enzyme units per milligram protein (U/mg) are another index.

As already discussed, sAA is classified as a relative stable protein (Gasteiger et al., 2005). Nevertheless, it is recommended to store samples at -20°C or lower temperatures if longer storage (up to 12 months) is planned (for an excellent review of methodological considerations see Rohleder & Nater, 2009). However, little protein may precipitate after freezing (Francis, Hector, & Proctor, 2000). There are multiple techniques to analyze sAA whereby enzyme kinetic methods are most commonly used. In this thesis, we primarily focus on one method performed using 96-well microtiter plates and a standard laboratory absorbance reader (Sunrise-Basic Tecan, Tecan Austria GmbH, Grödig, Austria). After centrifugation, saliva was diluted 1:625 with double-distilled water. Standard was prepared from ‘‘Calibrator f.a.s.’’ solution (Roche Diagnostics, Mannheim, Germany) ranging from 5.01 to 326 U/L amylase, and double-distilled water as zero standard. Twenty µl of diluted saliva and standard were transferred into transparent 96-well microplates and 80 µl of substrate reagent (alpha-Amylase EPS Sys; Roche Diagnostics) was added. In contrast to other studies using a waterbath at 37°C, the microplate was heated in an incubator at 43°C for 90 sec. Our laboratory has established this procedure because internal tests revealed constant temperatures of 37°C in each well at this ambient air temperature. After this first incubation a first measurement at 405 nm using a standard interference photometer (Sunrise-Basic Tecan) was done. Afterwards, the plate was incubated for another 5 min at 43°C, and the second measurement was done. The activity of sAA was than determined using a linear regression calculated for the standard curve on each microplate (GraphPad Prism 4.0c for MacOSX, GraphPad Software, San Diego, CA). Here, increases of absorbance of samples were transformed into activity. Intra- and inter-assay precision expressed as percent coefficient of variation was below 10% in all studies described in this thesis.

5.3. Interindividual differences in sAA activity

When looking at modulating factors of sAA activity, we have to differentiate between determinants of basal values, and influences on acute alterations of this enzyme. Recent studies support the view that sAA activity could be described as fast-reacting physiological response (Nater et al., 2005, 2006; Rohleder, Nater, Wolf, Ehlert, & Kirschbaum, 2004a; Rohleder, Chen, Wolf, & Miller, 2008). But not only acute factors could have an immediate impact on amylase secretion, also determinants having more lasting effects like age, sex, smoking, and physical fitness are factors influencing basal levels in general as well as acute sAA secretion. In the following chapter, basal activity as well as acute reactivity of sAA will be briefly summarized and major determinants will be reviewed. Afterwards, the aim of this

thesis will be briefly summarized showing how this work will provide further evidence for the usefulness of sAA as a marker of ANS activity.

5.3.1. Basal activity

Organisms not only regulate their physiological functioning in response to acute environmental demands, but also rely on biological rhythms to continuously adapt to the environment. Same seems to be true for salivary gland function. In the early 1970s, Dawes and colleagues were one of the first showing a significant diurnal rhythmicity of salivary flow and protein content in whole saliva and stimulated parotid saliva from eight healthy women and men (Dawes, 1972). One of the first publications summarizing circadian rhythms in human parotid saliva reviewed seven studies who measured sAA. One study reported higher morning sAA values and in four others higher afternoon values were reported (Ferguson, Fort, Elliott, & Potts, 1973). Following studies replicated those findings in stimulated and unstimulated whole saliva from healthy students (Artino et al., 1998; Jenzano, Brown, & Mauriello, 1987; Li & Gleeson, 2004; Rantonen & Meurman, 2000) and from diabetic patients (Artino et al., 1998). Using a handheld electronic measurement device, Yamaguchi, Deguchi, and Miyazaki (2006) did not observed significant changes over time in n=15 healthy male students. In relation to diurnal rhythm, more recent studies using salivettes found a pronounced daily profile of sAA activity characterized by a strong decrease in the morning and steadily increasing values peaking in the late afternoon. This pattern was shown for young adults (Nater et al., 2007; Rohleder et al., 2004a; Rohleder et al., 2008) as well as children and adolescents (Wolf, Nicholls, & Chen, 2008). More specifically, Nater and colleagues (2007) reported that sAA activity levels increased an average of 17% with each hour across the day. One study could not find this drop within the first 30 minutes after awakening (Michaud et al., 2006). These authors also found that morning sAA rhythms were not influenced by noise, i.e. sleep disturbances, and sleeping at home vs. in the laboratory.

5.3.2. Acute responses

Salivary alpha-amylase has shown to be a correlate of adrenergic reactivity to psychosocial stress (e.g. Rohleder et al., 2004a) and physiological stress conditions (Chatterton, Vogelsong, Lu, Ellman, & Hudgens, 1996). Furthermore, reduction of sAA concentrations in human saliva by beta-adrenergic blockers clearly indicated that this salivary constituent is a measure of sympathetic activity (Speirs et al., 1974; Van Stegeren et al., 2006).

5.3.3. Age effects

5.3.3.1. Basal amylase activity

Due to its role in digestion of starch and a possible coincidence with the eruption of teeth the development of sAA activity in newborns is of great interest. In the late 1970s and early 1980s sAA was measured in whole saliva from newborns and shown to be nine times lower than in adults (Bellavia, Moreno, Sanz, Picas, & Blanco, 1979) and positively associated with increasing age from 26 to 42 weeks gestational age (Hodge, Lebenthal, Lee, & Topper, 1983). Looking at a larger time slot, Sevenhuysen, Holodinsky, & Dawes (1984) found basal amylase activity to be very low to undetectable in the newborn and after then continually increasing to reach adult levels within the first three months. Increasing values of sAA activity in whole saliva were also found in infants from one to twelve months (Ruhl, Rayment, Schmalz, Hiller, & Troxler, 2005) and from 13 to 30 months of age (Dezan, Nicolau, Souza, & Walter, 2002). In unstimulated whole saliva, alpha-amylase activity was shown to be different between infants and toddlers only (Ben-Aryeh, Fisher, Szargel, & Laufer, 1990) while activity levels remained stable into older adulthood. In this study, same was also true for stimulated whole saliva. However, the activity of sAA in resting and stimulated parotid saliva was lower in older age groups (Ben-Aryeh et al., 1986). Other studies could not find any age effects on sAA activity in stimulated parotid saliva (Aguirre et al., 1987) and unstimulated whole saliva (Salvolini et al., 1999). Even in frail elderly persons, sAA was not correlated with age as well as diseases, drug use, or dentate status (Pajukoski et al., 1997). Overall, all studies described above are subjected to the methodological limitation that saliva was obtained at one single time-point. Since amylase is known to be responsive to acute stress and was found to show a pronounced diurnal rhythm, those results are of limited value. Only few studies looked at full diurnal profiles of sAA activity and their age ranges are too small to draw any conclusions. Studies looking at daily changes of sAA activity are still rare. While pronounced changes of sAA activity are found in young adults (e.g. Nater et al., 2007; Rohleder et al., 2004a; Rohleder et al., 2008) and children (Maldonado et al., 2008; Wolf et al., 2008), none have looked at older populations.

5.3.3.2. Stress-induced amylase activity

Nearly all our previous knowledge concerning acute stress-induced changes of sAA activity stems from studies with adult participants. Studies investigating sAA responses to acute stress in younger age groups are rare and again, none have looked at older populations. The development of sympathetic innervation of the salivary glands occurs postnatally (Knox & Hoffman, 2008). The youngest age group of whom saliva samples were collected to measure sAA activity after acute stress has been neonates. Schäffer and colleagues (2008) could not find significant increases of sAA in response to a heel prick test

in normal weight as well as small for gestational age neonates. Same non-responses to age-appropriate challenging tasks were also found in toddlers (Fortunato, Dribin, Granger, & Buss, 2008). Looking at toddler´s sAA responses to an anger-inducing task, Spinrad et al. (2009) could not find a difference between pre-test values and values ten minutes post stressor. In contrast, Davis & Granger (2009) reported sAA increases to a painful stressor in infants aged six and twelve months, but not at two or 24 months of age. The authors attributed this non-response in 24 months old infants to already elevated levels prior testing due a possible anticipation response. Much more evidence exists for acute sAA responses in older children and adolescents. While results of these studies implicate that adolescents respond in similar magnitudes as adults do, none of these studies directly compared youth and adults and no one has looked at older populations. Subjecting youth between nine and 14 years of age to a modified version of the TSST, alpha-amylase increased significantly in unstimulated whole saliva (Gordis, Granger, Susman, & Trickett, 2006, 2008). Furthermore, no association with age was found (Gordis et al., 2006). Just recently, Stroud and colleagues (2009) compared children (aged seven to twelve years) and adolescents (13 to 17 years) on their stress responses to a modified version of the TSST for Children (TSST-C; Buske-Kirschbaum et al., 1997) and to a peer-rejection task. No significant sAA increases were found in response to the TSST-C while the rejection task led to higher sAA levels especially pronounced in adolescents.

In sum and keeping the methodological limitations of some results in mind, basal amylase activity does not change over lifespan and acute stress responses are absent in newborns, can be measured in infants as young as six months, develop during childhood and reach adult magnitude in adolescence. No data are available on diurnal and stress-induced changes in older adulthood.

5.3.4. Sex differences

Besides the fact that many endocrine systems show sex differences, as for example the HPA axis (Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999), the ANS also show a well described sexual dimorphism due to e.g. differences in body fat mass and/or sexual hormonal status. On the one hand basal catecholamines vary over the menstrual cycle (Hirshoren et al., 2002) and on the other hand women were shown to display lower NA changes after various activating stimuli (Frankenhaeuser, Dunne, & Lundberg, 1976; Zouhal, Jacob, Delamarche, & Gratas-Delamarche, 2008) possibly depending on their menstrual cycle phase (Carter & Lawrence, 2007; Mills et al., 1996).

5.3.4.1. Basal amylase activity

Some studies examined possible effects of gonadal steroid changes, i.e. menstrual cycle, on the secretion of saliva and its components. One early study investigating 14 women not using oral contraceptives (OC) during their menstrual cycle revealed no difference in sAA levels in stimulated whole saliva between menstruation and ovulation (Tenovuo, Laine, Söderling, & Irjala, 1981). Same was true in a study conducted by Laine et al. (1991) who could not find an effect of menstrual cycle phase when comparing eleven women using OC´s, eleven women not using OC´s and ten men. Studies using larger sample sizes confirmed these results (Bhoola, Matthews, & Roberts, 1978). Findings regarding sAA activity level changes during pregnancy remain inconclusive. Some authors could not find any differences throughout pregnancy (Laine et al., 1988) and in contrast to controls (Ciejak et al., 2007; D´Alessandro, Curbelo, Tumilasci, Tessler, & Houssay, 1989). In contrast, Salvolini, Di Giorgio, Curatola, Mazzanti, & Fratto (1998) reported higher sAA activity levels in women at ten and 21 weeks of gestation in comparison to non-pregnant controls and to women at 40 weeks of gestation. In the few studies assessing diurnal rhythmicity of sAA no significant differences between men and women were found (Rantonen & Meurman, 2000; Rohleder et al., 2004a), neither in average concentrations nor in slopes (Nater et al., 2007). Looking at studies assessing sAA activity on a single time-point, no differences between the sexes appeared (Ben-Aryeh et al., 1986; Dezan et al., 2002; Harm & Schlegel, 2002). In contrast, van Stegeren, Wolf, and Kindt (2008) reported higher overall values in men. However, it is too early to draw any conclusions since the number of participants included in the studies described above was pretty low.

5.3.4.2. Stress-induced amylase activity

Up to date, studies investigating sex effects on acute sAA responses to stress revealed no differences between men and women. This was true for sAA responses to physical strain including competitive elements (Kivlighan & Granger, 2006), viewing an eye surgery video (Takai et al., 2007), in response to cold pressure stress (van Stegeren et al., 2008) as well as an oral academic examination (Schoofs, Hartmann, & Wolf, 2008), giving a lecture (Filaire et al., 2009) or the TSST (Preuß & Wolf, 2009). While there was no difference between women using oral contraceptives and those without (Schoofs et al., 2008), pregnancy had a profound effect on sAA responses to the TSST. Nierop and collagues (2006) could show that women in the second trimester showed lower stress responses than non-pregnant women, and women in the third trimester showed even more attenuated sAA changes after stress.

In sum, basal amylase activity seems to be comparable in men and women and up to date no sex differences in acute stress responses have been reported. Concerning the impact of steroid hormone status, existing data suggests that basal sAA activity does not vary within the menstrual cycle while the impact of pregnancy seems inconclusive. Acute

sAA responses seem to be attenuated in pregnant women. To the best of our knowledge, there have been no systematically studies examining acute stress-induced sAA changes in different phases of the menstrual cycle.

5.3.5. Modulating factors influencing amylase (re-)activity

When salivary measurements are employed into studies several exogenous factors need to be considered that may be associated with individual differences in salivary hormones and enzymes. The following chapter describes major confounding variables of salivary alpha-amylase activity - (1) life style factors such as tobacco smoking, alcohol, caffeine, adiposity, food intake, and exercise, (2) factors associated with disease such as acute and chronic somatic and psychiatric diseases, medical drugs, and (3) external factors such as the impact of sunlight on morning values.

5.3.5.1. Impact of smoking

It is a fact that nicotine activates the ANS/SNS (for a review see Adamopoulos, van de Borne, & Argacha, 2008). Thus, cigarette smoking should be associated with increased sAA activity. However, at least two studies reported no association between the habitual consumption of tobacco and sAA levels (Nagaya & Okuno, 1993; Zuabi et al., 1999), while the majority of studies show that smoking is associated with decreased activity after acute tobacco intake, either in vivo (Zappacosta et al., 2002) or in vitro (Greabu et al., 2007; Nagler et al., 2000). Besides an assumed neurovascular reflex, one of the favorite mechanisms explaining this effect, noxious effects of acid aldehydes present in tobacco smoke on oral tissues as well as salivary proteins are also considered. Saturated and unsaturated aldehydes react with thiol compounds of salivary enzymes, leading to an alteration in structure and function of these proteins (Leuchtenberger, Leuchtenberger, and Zbinden, 1974; Weiner, Levy, Khankin, & Reznick, 2008). Weiner, Khankin, Levy, and Reznick (2009) proposed that the formation of adducts at SH-groups of the sAA active site is a mechanism responsible for the decrease of sAA activity.

These findings raise the question whether there is a difference between habitual smokers and non-smoker according to their sAA activity. In the mid 80´s, Callegari and Lami (1984) found a significant reduction of sAA activity in 32 habitual smokers (aged 30 - 65 years, three - 20 pack years, currently 20 cigarettes/day) compared to 40 non-smokers. The documentation of the impact of habitual smoking on acute sAA stress responses in the literature is rare. Up to date, only two studies attend to this question. While the stress protocols failed to increase sAA, baseline levels were lower in habitual smokers in both studies (Goi et al., 2007; Granger et al., 2007a). Results of Granger et al. (2007a) also revealed that the impact of second hand tobacco smoke needs to be considered. The authors reported that children of mothers who smoked showed lower sAA activity. Because

these children had higher cotinin levels as well, this was probably due to smoke exposure. Up to date, there is only one study assessing diurnal sAA activity and smoking. Nater et al. (2007) showed that the decrease of sAA in the morning was much more pronounced in smokers while following levels throughout the day did not differ. Furthermore, in the 7% of measurements where one to three cigarettes were smoked in the hour before sampling, no association with subsequent sAA activity levels were found.

In sum, since acute tobacco consumption has a toxic and thus inhibitory effect on sAA and since most studies reported lower overall values in habitual smokers, smoking status is a major confounder in amylase research and studies need to be controlled for.

5.3.5.2. Impact of alcohol

Different chemicals - including alcohol - have the potential to reflexly induce saliva secretion (Hector & Linden, 1999). To assess the impact of acute alcohol consumption on saliva flow rate and amylase activity, Enberg, Alho, Loimaranta, & Lenander-Lumikari (2001) exposed 24 healthy nonalcoholic participants to a body weight- and sex-matched amount of alcohol. Stimulated, but not unstimulated whole saliva decreased significantly (40%). Amylase activity decreased in both simulated and unstimulated whole saliva. In a more recent study, ten healthy volunteers consumed either 300ml top-fermented beer (5.2%) or non-alcoholic beer in a cross-over design (Brand, Bruins, Veerman, & Nieuw Amerongen, 2006). While a short-term decrease in stimulated saliva flow rate was found (15%), no effect was shown on sAA concentration and output.

The effect of chronic alcohol consumption on salivary gland function is not fully understood (Dutta, Orestes, Vengulekur, & Kwo, 1992; Proctor & Shori, 1996; Scott & Berry, 1989). In rat studies, results remain contradictory with one study showing a decreased salivary flow rate and sAA activity (Maier, Born, Veith, Adler, & Seitz, 1986) and another study not revealing changes in sAA under similar conditions (Scott & Berry, 1989). In humans, same results appeared. While Nagaya and Okuno (1993) could not find differences between light drinker and controls, Dutta et al. (1992) found salivary flow and sAA to be decreased in alcoholic subjects.

In sum, evidence suggests that acute and chronic alcohol consumption is associated with dampened sAA activity. Until mechanisms of these functional changes are not fully understood, studies should control for chronic alcohol intake and ask their participants to be abstinent before interventions.

5.3.5.3. Impact of caffeine

Caffeine ingestion is associated with increased SNS activity (Laurent et al., 2000). Thus, it could be speculated that acute as well as chronic coffee consumption may be related to changes of sAA activity. In a study conducted in eleven endurance-trained men, caffeine

ingestion led to an increased sAA activity and secretion rate not accompanied by changes in saliva flow (Bishop, Walker, Scanlon, Richards, & Rogers, 2006). Interestingly, these changes did not return to baseline at the last measurement 3.5h later. Same caffeine induced sAA increase was shown in a study by Morrison, Haas, Shaffner, Garrett, and Fackler (2003) who found caffeine intake to be a predictor of higher sAA activity. Just recently, it was shown that there was no relation between habitual caffeine consumption and diurnal sAA activity (Wingenfeld et al., in press). Since it is not clear, whether acute responses of sAA differ between habitual caffeine consumers and non-consumers, studies should ask their participants not to drink any coffee before experiments.

5.3.5.4. Impact of high body fat and obesity

Given the fact that increases in body fat are accompanied by alteration of ANS/SNS functioning and that individuals who are more obese may be more responsive to stress (Benson et al., 2009; Epel et al., 2000), it might be asked if high body fat and obesity is a possible confounder of basal and stress-induced sAA activity. Just recently, Brydon (in press) could show that women with larger waists had greater stress-induced increases in cytokines leptin, interleukin-1 receptor antagonist, and impaired post-stress recovery of diastolic blood pressure. Furthermore, these data suggest that leptin may potentiate SNS activity during stress. There is only one study investigating sAA activity in obese individuals compared to controls. Aydin (2007) could show that alpha-amylase levels were significantly higher in 20 obese diabetic patients compared to healthy normal weight controls. However, there was no difference to normal weight diabetic patients. However, to the best of our knowledge, there are virtually no studies investigating sAA stress reactivity and diurnal rhythmicity in individuals who are more obese compared to normal weight controls. In sum, it might be cautiously concluded that especially visceral adiposity tends to stimulate the SNS. Thus, studies investigating sAA activity should control for this factor.

5.3.5.5. Impact of food intake

Several stimuli are associated with changes in salivary flow rate and composition. Mechanical as well as gustatory stimuli are often used to stimulate saliva flow, e.g. chewing on paraffin wax (Mackie & Pangborn, 1990) or citric acid (Froehlich et al., 1987). In 10 healthy volunteers, citric acid, but not sodium chloride, sucrose and starch stimulated sAA activity in parotid saliva (Froehlich et al., 1987). Mackie & Pangborn (1990) could show that oral stimulation effectively changed only sAA secretion rate but not sAA concentration. Investigating sAA responses to real food intake revealed much more pronounced changes in sAA compared to a sham intake (Harthoorn, Brattinga, van Kekem, Neyraud, & Dransfield, 2009) or non-eating (Toda & Morimoto, 2007). Looking at a possible relation between sAA activity and satiety, Harthoorn (2008) found sAA systematically increasing upon food intake

and satiation. The author therefore suggests that sAA could serve as an objective measure of satiety. Investigating the effects of specific diets or dietary changes of sAA revealed no differences cross-sectionally (Lenander-Lumikari, Ihalin, & Lähteenoja, 2000; Mazengo et al., 1994) as well as longitudinal (Johansson & Birkhed, 1994). Employing a more evolutionary approach, Perry and colleagues (2007) found that sAA gene copy numbers were positively correlated with sAA levels, and that individuals from traditionally high-starch diet populations had more gene copies than those with low-starch dietary habits. Since most previous studies were conducted in populations characterized by high-starch dietary habits, i.e. Europe/Northern America or Japan, future studies should address the issue of ethnical differences in basal and stress-induced sAA activity in more detail.

Taken together, there is some evidence for an association between long-term (evolutionary) diet and sAA production. Conclusive evidence concerning the impact of gustatory and mechanical stimuli as well as real food intake on acute sAA secretion point attention to the control of time of food intake in studies measuring diurnal as well as stress induced sAA levels.

5.3.5.6. Impact of physical exercise

It is known that SNS activity increases progressively with intensity of exercise (Stainsby & Brooks, 1990) what in turn alters salivary composition (Bishop, Blannin, Armstrong, Rickman & Gleeson, 2000; Chicharro, Lucía, Pérez, Vaquero, & Ureña, 1998; Walsh, Montague, Callow, & Rowlands, 2004). Salivary alpha-amylase increases in response to multiple different kinds of physical stimulation including running (Dawes, 1981; Nexø, Hansen, & Konradsen, 1988; Steerenberg et al., 1997), cycling (Chatterton et al., 1996; Walsh et al., 1999; de Oliveira et al., 2010) or treadmill exercise (Gilman, Thornton, Miller, & Biersner, 1976). As already shown for acute sAA responses to psychosocial stress, sAA activity increases faster than e.g. cortisol levels and declines rapidly after finishing the stressor, either in morning or afternoon exercises (Li & Gleeson, 2004). Interestingly, caffeine intake prior physical activity potentiates sAA increases (Bishop et al., 2006). One biological mechanism that might be discussed is the effect of exercise-induced dehydration on saliva flow and protein content. Walsh et al. (2004) could show that acute dehydration led to a decrease in saliva flow accompanied by increasing values of total protein concentration while secretion rates did not change. In contrast to the multitude of studies on acute sAA responses to exercise, only little is known about the impact of fitness and training status on diurnal sAA activity. To the best of our knowledge there are no studies investigating whether endurance trained differ from sedentary individuals. However, there is evidence that sAA could serve as a non-invasive marker of aerobic fitness as it highly correlates with lactate levels (Calvo et al., 1997; Bronas et al. 2002). In summary, extensive physical activity prior

testing should be avoided, while low intense activity seems to be less relevant (Nater et al., 2007).

5.3.5.7. Impact of somatic and psychiatric diseases

Saliva is essential for oral health and cavity, and changes of sAA have been related to oral pathologies such as periodontitis (Da Rós Gonçalves et al., 2010; Henskens et al., 1996), oral cancer (Shpitzer, Bahar, Feinmesser, & Nagler, 2007) or dental caries (Scannapieco et al., 1993). Furthermore, different systemic diseases such as breast cancer (Streckfus et al., 2008), Sjögren´s syndrome (Fleissig et al., 2009; Ryu, Atkinson, Hoehn, Illei, & Hart, 2006), diabetes mellitus (Aydin, 2007), cystic fibrosis (Chernick, Eichel, & Barbero, 1964), systemic sclerosis or rheumatoid arthritis (Daniels & Whitcher, 1994; Helenius et al., 2005; Matthews, Bhoola, Rasker, & Jayson, 1985) are associated with changes in sAA levels due to their oral complications. Accordingly, changes in sAA have been found in children suffering from asthma and atopic dermatitis (Crespi et al., 1982; Wolf et al., 2008), Parkinson´s disease patients (Tumilasci et al., 2006) and chronic obstructive pulmonary disease (COPD) patients (Yigla, Berkovich, & Nagler, 2007). Just recently, Bugdayci and colleagues (2010) found sAA to be of dynamic nature in different periods of migraine headache (Bugdayci et al., 2010) with significant lower levels in attack period compared to post-attack period, and decreasing to normal values during interval period. Furthermore, a significant correlation was found between sAA levels and pain ratings (Shirasaki et al., 2007). Less well known is about sAA in cardiovascular disease. To the best of our knowledge there are virtually no studies investigating sAA levels in subjects suffering from high blood pressure.

Studies investigating sAA levels in psychiatric diseases are rare. There is clear evidence for an activation of the SNS and chronic augmentation of sympathetic tone in depression (Esler, 2009; Scalco et al., 2009). Thus, it was suggested that sAA may be elevated in patients suffering from depression and distress (Vale, 2007). Rohleder et al. (2008) reported an association between daily sAA values and self-reported depression in young women (Rohleder et al., 2008). Just recently, Vigil, Geary, Granger, & Flinn (in press) reported higher sAA activity in women exposed to Hurricane Katrina compared to non-exposed controls. However, a small negative relation between higher sAA and lower depressive symptoms for the control group, but not for the Katrina group was found. So far, no final conclusion could be drawn for sAA activity in depression. Furthermore, anxiety related conditions are associated with autonomic alterations (Coupland, Wilson, Potokar, Bell, & Nutt, 2003; Geracioti et al., 2001). There is preliminary evidence, that sAA might be useful as a marker for anxiety reports. In healthy participants, sAA peak levels were found to be highly correlated with state anxiety measures (Noto, Sato, Kudo, Kurata, & Hirota, 2005; Takai et al., 2004). To date, only few studies investigated sAA in psychiatric patients. One of the first

reports of an altered sAA activity is a study by Labudda, Wolf, Markowitsch, and Brand (2007) who assessed sAA in pathological gamblers. Only those patients who showed less disadvantageous decision-making patterns had an increase of sAA during a laboratory task of decision making. Furthermore, daily sAA levels seem to be altered in posttraumatic stress disorder (PTSD) patients. Unpublished data indicates changes of circadian sAA patterns in PTSD patient with lower levels after awakening and strongly increasing values throughout the day (Rohleder, Joksimovic, Wolf, & Kirschbaum, 2004b). In contrast, Reinhardt (2008) found slightly lower basal values in inpatients at a single time-point. Another disorder which is accompanied by hyperarousal, such as increased heart rate, is generalized social anxiety disorder. Van Veen and colleagues (2008) could show that the ANS is also involved in this disorder with 43 patients having higher diurnal and post-dexamethasone sAA levels compared to 43 controls. Eating disorders are another group of psychiatric disorders associated with changes in sAA activity possibly due to vomiting (Kinzl, Biebl, & Herold, 1993) and its oral complications. In a study conducted in 45 patients with eating disorders (twelve restrictive anorexia nervosa, 13 bulimic anorexia nervosa, 20 bulimia nervosa) and 30 healthy controls all patients showed higher concentrations and secretion rates of sAA (Scheutzel & Gerlach, 1991). In the 14 patients whose salivary glands were swollen, sAA secretion in resting saliva was decreased. Just recently, changes of basal sAA activity were also reported in patients with schizophrenia (Inagaki et al., 2010). Here, sAA activity was higher in the patient group compared to healthy controls and was also positively correlated to psychiatric symptoms.

5.3.5.8. Impact of medical drugs

Since sAA activity could be increased via activation of beta-adrenergic receptors and reduced by blockade of these receptors (Ehlert et al., 2006; van Stegeren et al., 2006), studies should control for or even exclude the use of adrenergic agonists and antagonists, i.e. asthma or antihypertensive medication. Von Knorring and Mörnstad (1986) reported increases in basal sAA in patients taking nonselective antidepressive drugs. This effect was shown after one single dose and remained after 14 days of treatment. In a more recent study, de Almeida and colleagues (2008) could not find an effect of antidepressants (selective serotonin reuptake inhibitors, SSRI´s) and benzodiazepine on sAA values. Other classes of drugs that may have an impact on sAA activity due to diminishing SFR and protein composition are anticholinergics, diuretics and antihypertensive agents, and other psychopharmaca (Parvinen, Parvinen, & Larmas, 1984). Nederfors & Dahlöf (1996) could show that the intake of metoprolol, an antihypertensive drug, led to decreases of sAA, disappearing on drug withdrawal and decreasing again on drug re-exposure. However, nothing is known about the impact of chronic administration of adrenergic active substances on diurnal rhythmicity and stress responses of sAA.

5.3.5.9. Impact of sunlight on diurnal amylase

While it was often shown that sunlight has an impact on salivary cortisol awakening responses via retinal gland cells - nucleus suprachiasmaticus (SCN) - nucleus paraventricularis pathways (Bear, Connors, & Paradiso, 2006), nothing is known about the impact of sunlight on diurnal sAA activity. Animal studies could show that rats chronically exposed to constant light showed an increase of sympathetic activity in salivary glands (Bellavía & Gallará, 2000). Furthermore, animal studies revealed the rhythmic expression of clock genes and Amylase 1 Gene in salivary glands of rats, influenced by feeding (Furukawa et al., 2005). These results might highlight the functional importance of SCN outputs in the control of peripheral circadian oscillators indicating that also human salivary proteins, including sAA, might be influenced by external factors such as sunlight.

6. Aims and outline of the present work

The aim of this thesis was to highlight the usefulness of salivary alpha-amylase as indicator of autonomic functioning and to investigate possible confounders against the background of the psychosocial stress response and circadian rhythmicity. In summary, since pronounced changes of sAA in response to psychosocial stress are found in young adults (e.g. Nater et al., 2005, 2006; Rohleder, et al. 2004a) and children (e.g. Gordis et al., 2006, 2008), none of these studies directly compared youth and adults and no one has looked at older populations. Therefore, individuals´ reactivity to acute psychosocial stress at different ages has been investigated (Study 1). Furthermore, children as well as young adults show pronounced diurnal changes of sAA activity throughout the day (e.g. Nater et al., 2007; Wolf et al., 2008). Again, no studies have looked at older populations. In contrast to the multitude of studies on acute sAA responses to stress, only little is known about the impact of repeated, chronic stress on diurnal sAA activity. This thesis therefore examines daily rhythms of sAA activity in competitive ballroom dancers of different ages compared to age-matched healthy controls to capture the effects of age as well as chronic stress on basal sAA (Study 2). Two other confounding factors of particular interest in research using sAA as a prognostic indicator of pathological processes are the intake of adrenergic active substances and the stimulus of light. Therefore, sAA levels in older adults suffering from high blood pressure as well as the impact of chronic administration of adrenergic active substances on diurnal sAA rhythmicity have been investigated (Study 3). To examine the impact of sunlight on changes of sAA after awakening, we obtained sAA morning profiles in adults waking up in the dark compared to waking up with daylight in a natural setting (Study 4). In all studies presented here, sex was considered and analyzed as another confounding variable.

7. Study 1: Salivary alpha-amylase stress reactivity across different age groups[2]

7.1. Introduction

Physiological stress responses are important determinants of health and disease (McEwen & Stellar, 1993). Since vulnerability to disease and disease prevalence patterns change with age, it is important to investigate stress reactivity of people in different age groups. So far, the main focus has been on hypothalamus-pituitary-adrenal (HPA) axis reactivity and salivary cortisol, while there is a relative lack of knowledge regarding sympathetic nervous system (SNS) markers. One potential reason is that SNS reactivity has been more difficult to assess than HPA axis markers. Recently, salivary alpha-amylase (sAA) has been suggested as a non-invasive saliva based marker for SNS activity (see Nater & Rohleder, 2009 and Rohleder & Nater, 2009, for a summary). In order to use sAA for investigating acute stress responses throughout the life span, we set out to establish sAA response patterns to a standardized stressor in three relevant age groups across the life span. We chose childhood between 6 and 10 years, because it is a sensitive period of growth and development, and thus plays an important role for later life health. For example, an association between childhood health and morbidity in later life was found for cancer, lung disease, cardiovascular conditions, and arthritis/rheumatism (Blackwell, Hayward, & Crimmins, 2001). Young adults were included to represent the most studied human age group as a reference. Older adults between 59 and 61 years were investigated, because at this age the course is set for the further development of a person's health in later life, and because autonomic stress responses in older age might be important determinants of cardiovascular and inflammatory aging.

Acute stress responses of salivary sAA have been proposed as markers for sympathetic stress responses. Since Chatterton et al. (1996) reported a significant correlation between sAA, adrenaline (A) and noradrenaline (NA) after physical exercise, several studies included sAA as an indicator of physiological and psychological stress. Psychosocial stress has been shown to induce a rapid increase in sAA activity (Nater et al., 2005). However, looking at the association between stress-induced cortisol, sAA, A, and NA release, correlations could not be found consistently (Nater et al., 2006; Rohleder et al., 2004a). Pharmacological studies (Ehlert et al., 2006; Van Stegeren et al., 2006) provide direct evidence for the relevance of central autonomic mechanisms on sAA release. Ehlert et al. (2006) suggested that sAA might be an indirect indicator of the central (noradrenergic) sympathetic, system which is not

[2] **Acknowledgment**

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necessarily associated with peripheral catecholamine release. Despite the increasing number of researchers integrating sAA into their study designs, nearly all previous data concerning psychosocial stress-induced sAA responses stems from studies with children, youth, and young adults (Gordis et al., 2006, 2008; Nater et al., 2005; Stroud et al., 2009; Van Stegeren et al., 2008), while no studies have looked at older populations. Therefore, we need to know how healthy older adults' sAA levels respond to stress and how comparable these responses are to that of children and young adults.

In addition to age differences, research on another stress system, i.e., the hypothalamus-pituitary-adrenal (HPA) axis, produced evidence for diverging age-related physiological changes in men and women (Kudielka, Buske-Kirschbaum, Hellhammer, & Kirschbaum, 2004b). Reanalyzing stress-induced HPA axis responses in age groups ranging from children to older adults, Kudielka and colleagues (2004b) reported significant responses to a psychosocial stress task in all age groups. While no sex differences were found in children and younger adults, older men displayed larger free salivary cortisol increases than women as well as the other age groups (Kudielka et al., 2004a). Again, no studies have yet investigated age by sex interactions in sAA stress responses.

However, age and sex differences have been found for various other sympathetic markers, mainly heart rate (HR) and heart rate variability (HRV). HRV is often used as an index of autonomic function (Bigger, Fleiss, Steinman, Rolnitzky, Schneider, & Stein, 1995). Taken together, previous work has established that the primary influence of aging on the human SNS is an elevation in tonic activity rather than responsiveness to stress (Seals & Dinenno, 2004). HRV parameters during stress decrease with aging depending on the measure (Lavi, Nevo, Thaler, Rosenfeld, Dayan, et al., 2006; White, Courtemanche, Stewart, Talajic, Mikes, et al., 1997), and parameters are also sex dependent with values for young females being lower than those for age-matched male participants. However, some authors did not find any age by sex interactions in HRV reactivity (Kunz-Ebrecht, Mohamed-Ali, Feldman, Kirschbaum, & Steptoe, 2003; Mezzacappa, Kelsey, Katkin, & Sloan, 2001; Wright, O'Donnell, Brydon, Wardle, & Steptoe, 2007). Furthermore, there appears to be an age by sex interaction in HR reactivity. While young women displayed stronger HR stress responses than young men (Kudielka, Buske-Kirschbaum, Hellhammer, & Kirschbaum, 2004a), older men and women were comparable in their acute HR stress response (Kudielka et al., 2004a). Finally, age and sex differences have been reported for the catecholamines adrenaline (A) and noradrenaline (NA). There is evidence for sex differences in adrenal medullary catecholamine stress reactivity, with lower responses in women (Honojosa-Laborde, Chapa, Lange, & Haywood, 1999; Matthews, Gump, & Owens, 2001). With aging, higher absolute levels of NA were found (White et al., 1997). Overall there seems to be an increased systemic adrenergic ‘‘drive’’ in parallel with aging whereby age-related differences

in NA levels are markedly attenuated in female participants (White et al, 1997). Overall, despite age-related changes of catecholamine and HRV baseline activity (White et al., 1997), responsiveness to acute stress does not uniformly change with advancing age. However, autonomic reactivity shows sex-related alterations with attenuated responses in either older men or older women according to the involved measurements.

Another factor that should be taken into account when investigating age differences in sAA production is the possibility of age-related changes in salivary gland physiology. Studies have shown that with increasing age morphological changes of oral mucosa appear (Ghezzi & Ship, 2003; Scott, Flower, & Burns, 1987), but only marginal alterations of salivary gland function and saliva composition are seen (Baum, 1989; Ghezzi & Ship, 2003; Wu, Atkinson, Fox, Baum, & Ship, 1993). In a recent study, Nagler and Hershkovich (2005) reported a 62% lower resting saliva flow rate and higher sAA concentrations in elderly participants compared to young adults, but no difference of overall sAA output. Taken together, while studies show that there are age-related changes in saliva production and composition, there is no direct evidence for age-related changes of basal sAA production. Furthermore, stress-induced increases of sAA activity seem to be independent of salivary flow rate (Rohleder et al., 2006).

We set out in the present study to investigate sAA responses to acute psychosocial stress in three relevant age groups representing relevant stages of human development - healthy children, young adults, and older adults of both sexes. To ensure the effectiveness of our stress protocol in triggering a physiological stress response and in view of the importance of physiological stress responses as determinants of health and disease, we aimed to test for associations of sAA with more established stress system markers, i.e., salivary cortisol as outcome measurement of HPA reactivity, HR and HRV as markers for autonomic reactivity. Based on the literature summarized above, we hypothesized that sAA, HR, and HRV responses would be attenuated in older age, while cortisol responses would be increased. Salivary alpha-amylase responses in children would be lower compared to young adults, while we expect no differences in cortisol responses. Furthermore, we hypothesized to find no association between sAA and cortisol, while sympathetic responses (sAA, HR, and HRV) would be associated. It has been shown that chronic stress alters HPA stress responses, but until now, nothing is known about the impact of chronic stress on sAA reactivity. Thus, we set out to examine how subjective chronic stress levels influence sAA as well as HR and HRV stress responses.

7.2. Methods

7.2.1. Participants

Children aged 6 to 10, young adults aged 20 to 31, and older adults aged 59 to 61 were recruited via a notice posted on campus of the Technische Universität Dresden[3], via an advertisement in a local newspaper and via personal contact. The group of children was recruited with the help of a local hospital, through which parents who delivered a baby there between 1998 and 2002 were contacted. Finally, 62 children (32 boys, 30 girls), 78 young adults (45 men, 33 women), and 74 older adults (37 men, 37 women) fulfilled the inclusion criteria. We included participants with a body mass index (BMI) below 30 kg/m2 in children and young adults and below 35 kg /m² in older adults. Smokers and individuals who reported excessive alcohol consumption (43 times a week) were excluded, also individuals under anti-hypertensive medication, asthma medication, anti-rheumatic medication, using psychotropic substances, sleeping pills, or painkillers. Participants were free of psychiatric and severe somatic diseases as evaluated by interview by one of the authors (J.S.). Use of other medication in the presence of changes of cardiovascular functioning (anticoagulants), fat metabolism (antilipemics) and thyroid functioning (thyroid therapeutics), and use of vitamins and natural therapeutics were allowed, and participants were instructed to take their prescribed drugs at least 5 hours before intervention. Since there is no conclusive data available on the impact of the use of oral contraceptives on sAA and menstrual cycle phase (Rohleder & Nater, 2009), women were invited in their luteal phase of menstrual cycle, while those taking oral contraceptives were excluded. Participants were not allowed to drink (anything but water) and eat one hour before intervention, since these factors have been shown to modify the activity of sAA (Rohleder & Nater, 2009).

7.2.2. Study Protocol

All participants were invited to our laboratory on a weekday between 14:00 and 18:00 h in the afternoon. Children were accompanied by at least one of their parents, who also signed the informed consent and stayed in our laboratory until the end of testing. After a 30-min resting period to minimize the impact of physical activity, prior stress, and emotions, during which the adult participants filled in some questionnaires (see Psychometrical analyses), adults were exposed to the Trier Social Stress Test (TSST; Kirschbaum et al., 1993) while children were exposed to the child version of the TSST (TSST-C), developed and evaluated by Buske-Kirschbaum and colleagues (Buske-Kirschbaum et al., 1997). The TSST consists of a 3-min preparation period, a 5-min speech task, during which adult participants have to discourse about their personal characteristics and children have to finish

[3] independent of the journal´s publication standards, we will always use “Technische Universität Dresden” within this thesis

a story, and a 5-min mental arithmetic task, both in front of an audience. After this stress task, participants stayed in our laboratory for another 20 min to collect samples during recovery. The study protocol was approved by the ethics committee of the Technische Universität Dresden.

7.2.3. Measures

7.2.3.1. Saliva sampling

To determine sAA and cortisol stress responses, four saliva samples were collected immediately before, immediately after, 10 and 20 min after the stressor with the help of cotton swabs (Salivettes, Sarstedt, Nümbrecht, Germany). This sampling protocol was chosen based on previous data showing that we would be able to capture peak and recovery of sAA, as well as peak cortisol levels (Rohleder et al., 2006). Since the focus of the present study was to evaluate sAA stress responses, we accepted not being able to assess cortisol recovery for economic reasons. A common problem when collecting saliva is that older individuals do not secrete enough saliva without stimulation. We therefore decided to instruct all participants to chew on the cotton rolls for 1 min to stimulate saliva flow. A previous study from our group has shown that acute stress responses are independent of sampling technique (Rohleder et al., 2006). Cotton swabs were then transferred to the plastic containers and stored at -20°C until analysis.

7.2.3.2. Heart rate and heart rate variability

HR and HRV parameters were measured as additional markers of autonomic activity in adults. We were not able to obtain HR data from children for technical reasons. HR and HRV variables were derived from cardiovascular measurements during the whole time of the study using Polar S810i cardiac monitors (Polar Electro Ltd., Kempele, Finland). For analyses, artifact free time points of 2-min duration were chosen corresponding to the time points of sAA measurements. HR and HRV were calculated for one additional 2-min interval starting 8 min into the TSST as a read-out for HRV during stress. For quantification of HR and HRV, we focused on time domain variables because these are equivalent to frequency-domain variables as well as easier to perform. In accordance with current recommendations (Task Force, 1996), only the root mean square of successive differences (RMSSD) from time domain analysis was obtained, reflecting the short-time HRV and being predominantly a response to changes in parasympathetic tone. RMSSD represents fast alterations of heart frequency in the respiratory frequency range and might therefore be a measure of fluctuating variations of vagal tone (Task Force, 1996). Despite being highly correlated to power spectral measures of respiratory sinus arrhythmia (RSA), it was suggested that RMSSD is not significantly affected by changes in the breathing rate (Penttilä, Helminen, Jartti, Kuusela,

Huikuri, et al., 2001). All analyses were performed with HRV Analysis Software (Biomedical Signal Analysis Group, University of Kuopio, Kuopio, Finland).

7.2.3.3. Biochemical analyses

Salivary alpha-amylase was measured by a quantitative enzyme kinetic method. After thawing, saliva samples were centrifuged at 3000 rpm for 3min. After this, saliva was diluted 1:625 with double-distilled water. Twenty µl of diluted saliva and standard were transferred into transparent 96-well microplates. Standard was prepared from ‘‘Calibrator f.a.s.’’ solution (Roche Diagnostics, Mannheim, Germany) ranging from 5.01 to 326 U/L amylase, and double-distilled water as zero standard. After that, 80 µl of substrate reagent (alpha-Amylase EPS Sys; Roche Diagnostics) was added. The microplate was then heated in an incubator at 43°C for 90 s. Our laboratory has established this procedure because internal tests revealed constant temperatures of 37°C in each well at this ambient air temperature. After a first interference measurement at 405 nm using a standard interference photometer (Sunrise-Basic Tecan, Tecan Austria GmbH, Grödig, Austria), the plate was incubated for another 5 min at 43°C, and the second measurement was done. Increases of absorbance of samples were transformed to alpha-amylase activity using a linear regression calculated for the standard curve on each microplate (GraphPad Prism 4.0c for MacOSX, GraphPad Software, San Diego, CA). Intra- and inter-assay precision expressed as percent coefficient of variation was below 10%. Concentrations of salivary free cortisol were measured using a commercially available chemiluminescence-immuno-assay (CLIA; IBL, Hamburg, Germany).

7.2.3.4. Psychometrical analyses

In order to control for possible influences of perceived stress on acute SNS reactivity, adult participants filled in the German version of the Perceived Stress Scale (PSS; Cohen, Kamarck, & Mermelstein, 1983). Additionally chronic stress was measured using the 12-item Chronic Stress Screening Scale (CSSS; Schulz, Schlotz, & Becker, 2004) in order to examine possible influences on HPA stress responses as well as autonomic reactivity, i.e., sAA, HR, HRV stress responses. Assessing the subjective stress experience while testing, participants were asked to answer questions regarding anxiety and mood pre and post-stressor. Anxiety was measured with help of the state version of the State-Trait Anxiety Inventory Scale (STAI-S; Laux, Glanzmann, Schaffner, & Spielberger, 1981). After cessation of the TSST, adult participants rated the stressfulness of the task on eight visual analog scales (VAS). All of these questionnaires have shown high internal consistency and validity. No such psychometric data was available for children, but excessive stress experiences of the children in the last 2 weeks prior testing were excluded by parent interview.

7.2.4. Statistical analyses

Data were tested for normal distribution and homogeneity of variance using a Kolmogorov–Smirnov and Levene's test before statistical procedures were applied. These analyses revealed significant deviations from normality of some absolute sAA and cortisol levels. Amylase and cortisol values were therefore log-transformed prior to analyses, which restored normality of distribution. ANOVAs for repeated measures were used to analyze sAA and cortisol responses to the stressor with the between subjects factors group (children vs. younger adults vs. older adults) and sex (male vs. female), and the within-subjects factor sampling time (4 times). Similar ANOVAs were calculated for the HR and HRV responses, but only for young and older adults and a within-subjects factor with 5 levels. All results were corrected by the Greenhouse-Geisser procedure where appropriate (violation of sphericity assumption). Furthermore, because of significant differences between the age groups with respect to BMI, sAA and cortisol baseline values, perceived stress (PSS) and chronic stress level (CSSS), those variables were included as covariates where appropriate. Stress-induced increases of sAA activity were indexed as delta score between sAA levels immediately pre-task and the post-intervention maximum, increases of cortisol activity were indexed as delta score between cortisol scores immediately pre-task and mean of 10 and 20 min post task, and stress-induced HR and HRV alterations were indexed as delta scores between immediately pre-task and 8 min after stressor onset. Furthermore, area under the curve with respect to ground (AUC_g) as well as increase (AUC_i) were calculated for each biomarker according to Pruessner, Kirschbaum, Meinlschmid, & Hellhammer (2003). Univariate ANOVAs were computed for comparisons of the age groups. Post hoc analyses using the LSD method were conducted to determine subgroup differences. To test for associations with chronic stress levels, Pearson correlations were computed. Finally, hierarchical linear regression equations were used to predict the three different indices for the sAA response (delta increase, AUC_g, AUC_i) by indices for cortisol, HR, and RMSSD responses, controlling for age, BMI, and sex, as well as chronic stress levels. For significant results, we report partial eta squared (η^2) as a measure for effect size. For all analyses, the significance level was $\alpha = 5\%$. All results shown are the mean ± standard error of mean (SEM).

7.3. Results

7.3.1. Sample characteristics

Main characteristics of the study groups are shown in Table 1. The results of the stress questionnaires (PSS, CSSS) indicated that younger adults experienced a significant higher amount of chronic stress than older adults (PSS: $t_{153} = 3.651$, $p < .001$; CSSS: $t_{139} = 53.665$, $p > .001$), but neither group was experiencing significant subchronic or chronic stress during

3 months before the stress test according to normative data (Cohen & Williamson, 1988; Schulz et al., 2004).

Table 7.1 Sample characteristic of children, young adults and older adults

	children (n = 62)		young adults (n = 78)		older adults (n = 74)	
Variable	**Mean (SD)**	**Range**	**Mean (SD)**	**Range**	**Mean (SD)**	**Range**
age (years)*	7.9 (1.0)	6 - 10	24.1 (2.5)	20 - 31	59.8 (0.6)	59 - 61
weight (kg)	28.7 (6.3)	18 - 47	70.1 (11.2)	50 - 105	77.1 (12.7)	53 - 106
height (cm)	133.4 (8.4)	116 - 154	176.2 (9.3)	160 - 194	170.4 (8.4)	148 - 189
BMI (kg/m²)*	**16.0 (2.0)**	11 - 23	**22.5 (2.4)**	18 - 31	**26.5 (3.5)**	20 - 35
PSS*	-	-	**21.9 (6.4)**	7 - 40	**18.4 (5.3)**	6 – 38
CSSS*	-	-	**16.4 (7.8)**	0 - 35	**12.5 (5.4)**	1 - 26
amylase baseline (U/ml)*	**101.5 (52.3)**	1 - 213	**47.7 (34.6)**	6 - 175	**46.6 (30.2)**	7 - 140
cortisol baseline (nmol/l)*	**3.1 (2.1)**	0.5 - 10.9	**9.0 (5.7)**	0.8 - 24.6	5.7 (3.1)	1.4 - 16.4

SD = standard deviation; BMI = Body Mass Index; PSS = Perceived Stress Scale, age specific norms: young adults 21.1 ± 7.2, older adults 18.3 ± 8.1; CSSS = Chronic Stress Screening Scale, age specific T-score norms (mean 50+10z): young adults 7-24, older adults 4-19. * group difference: t-test: $p < 0.001$

7.3.2. Subjective stress response

Analyses revealed no differences between the two adult groups as well as men and women with regard to their evaluation of stressfulness of the TSST. The stress paradigm led to a significant change in subjective anxiety ($F_{1,147} = 12.887$, $p > .001$, $\eta^2 = 0.081$), but without significant group or sex differences (all $p > .05$).However, we found a significant subjective anxiety by sex by group interaction ($F_{1,147} = 4.884$, $p = .029$, $\eta^2 = 0.032$), with older women showing the highest increase of subjective anxiety (data not shown).

7.3.3. Physiological stress response

7.3.3.1. Salivary alpha-amylase

There was a highly significant baseline difference between the age groups with respect to sAA activity ($F_{2,210} = 23.9$, $p < .001$, $\eta^2 = .185$). Post hoc analyses revealed that children had a significantly higher baseline than both adult groups (all $p < .05$). ANOVA for repeated measurements revealed significant sAA changes in response to the TSST in all age groups (time effect: $F_{2.8,580.3} = 3.9$, $p = .009$, $\eta^2 = .019$). Furthermore, we found a significant main effect of age group ($F_{2,204} = 8.9$, $p < .001$, $\eta^2 = .081$), as well as a significant time by age group interaction ($F_{5.7,580.3} = 7.7$, $p > .001$, $\eta^2 = .071$) with children and older adults showing attenuated acute stress responses (Figure 7.1A). Univariate ANOVA of delta scores revealed a significant main effect of group ($F_{2,206} = 35.9$, $p < .001$, $\eta^2 = .259$). Post hoc tests showed that all groups differed significantly (all $p < .05$), with young adults showing the highest stress response and children showing the lowest (Figure 7.1B). Same was true for AUC_i ($p < .001$). Further analyses showed a significant main effect of group on AUC_g ($p < .001$) with post hoc

analyses revealing significantly higher values in children compared to both adult groups (all $p < .001$, data not shown). No sex effects or age group by sex interactions were found. Pearson correlations between indices of stress levels (CSSS, PSS) and summary indices (delta response, AUC_i, AUC_g) revealed no association between the parameters.

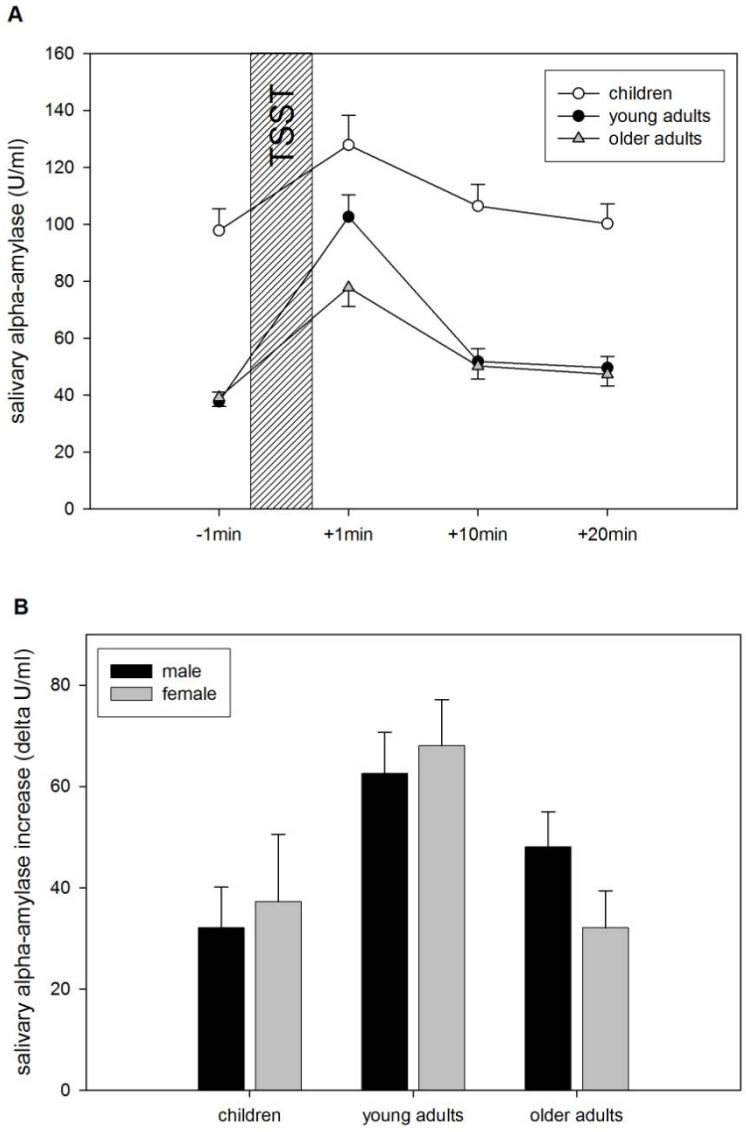

Figure 7.1 (A) mean salivary alpha-amylase levels (± standard error) after the Trier Social Stress Test (TSST) in children, young adults and older adults, (B) mean salivary alpha-amylase increase (± standard error) after the Trier Social Stress Test (TSST) in children, young adults and older adults.

7.3.3.2. Salivary cortisol

There was a highly significant baseline difference between the age groups with respect to salivary cortisol concentrations ($F_{2,209} = 27.2$, $p < .001$, $\eta^2 = .206$). Post hoc analysis revealed that children had significantly lower baseline levels than older adults, and both had significantly lower baseline levels than young adults. ANOVA for repeated measurements revealed highly significant salivary cortisol changes in response to the TSST in all age

groups (time effect: $F_{1.5,302.6} = 31.3$, $p < .001$, $\eta^2 = .133$). Furthermore, we found a significant main effect of age group ($F_{2,203} = 6.1$, $p = .003$, $\eta^2 = .056$), as well as a significant time by age group interaction ($F_{3.0,302.6} = 12.0$, $p < .001$, $\eta^2 = .106$), with young adults and children reaching their maximum level 10 min post stressor while older adults displayed a prolonged response (Figure 7.2A). No sex effect, but a significant time by group by sex interaction ($F_{1.5,302.6} = 2.9$, $p = .033$, $\eta^2 = .028$) was found. Univariate ANOVA of delta scores revealed a significant main effect of group ($F_{2,204} = 6.2$, $p = .002$, $\eta^2 = .058$). However, post hoc tests showed no difference between the groups, although older participants showed the highest mean stress response. While there was no main effect of sex, a significant group by sex interaction ($F_{2,204} = 3.6$, $p = .028$, $\eta^2 = .034$) was found. As shown in Figure 7.2B, acute cortisol stress responses increased with age in male participants but remained constant through all age groups in female participants. This main effect of group and the group X sex interaction were also found for AUC_i as well as AUC_g (all $p < .05$, data not shown). Furthermore, post hoc analyses showed a lower AUCi as well as AUC_g in children compared to both adult groups (all $p > .05$). Pearson correlations between indices of stress levels (CSSS, PSS) and summary indices (delta response, AUC_i, AUC_g) revealed no association between the parameters.

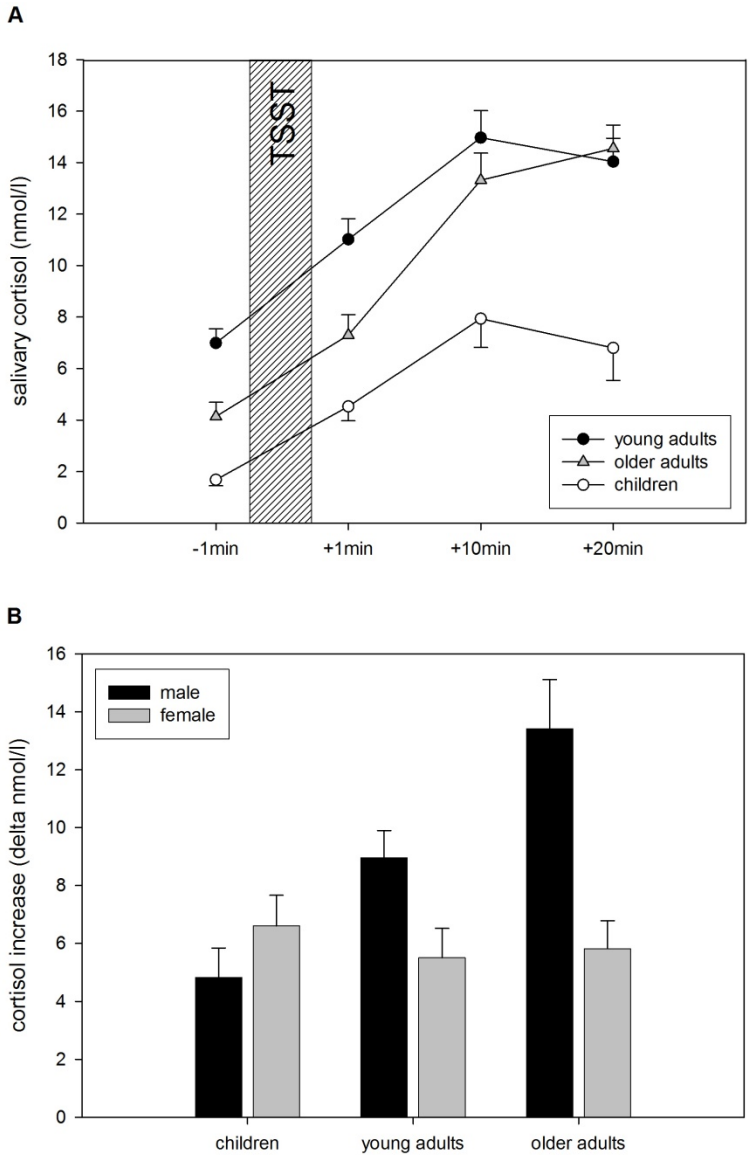

Figure 7.2 (A) mean salivary cortisol levels (± standard error) after the Trier Social Stress Test (TSST) in children, young adults and older adults, (B) mean salivary cortisol increase (± standard error) after the Trier Social Stress Test (TSST) in children, young adults and older adults.

7.3.3.3. Heart rate

HR measurements were obtained only from the two adult groups. There was a significant baseline difference between the age groups with respect to HR ($F_{1,129}$ = 4.2, p = .043, ŋ² = .032) with older adults showing lower baseline levels (young adults 82.3 ± 1.5 bpm vs. older adults 78.2 ± 1.3 bpm). ANOVA for repeated measurements revealed highly significant HR changes in response to the TSST in young and older adults (time effect: $F_{2.8,341.3}$ = 4.8, p = .003, ŋ² = .038). Furthermore, we found a significant age group by time effect ($F_{2.8,341.3}$ = 5.9, p = .001, ŋ² = .046), with older adults showing an attenuated HR stress response (Figure 3A). No time by sex or time by group by sex interaction was found. Univariate ANOVA of delta scores revealed only this previously described main effect of

group ($F_{1,127}$ = 16.2, p < .001, η² = .113) with older adults showing a lower acute HR increase (Figure 7.3B). Same was true for AUC_g (p < .001), while there was no effect of group or gender on AUC_i. Pearson correlations between indices of stress levels (CSSS, PSS) and summary indices (delta response, AUC_i, AUC_g) revealed no association between the parameters.

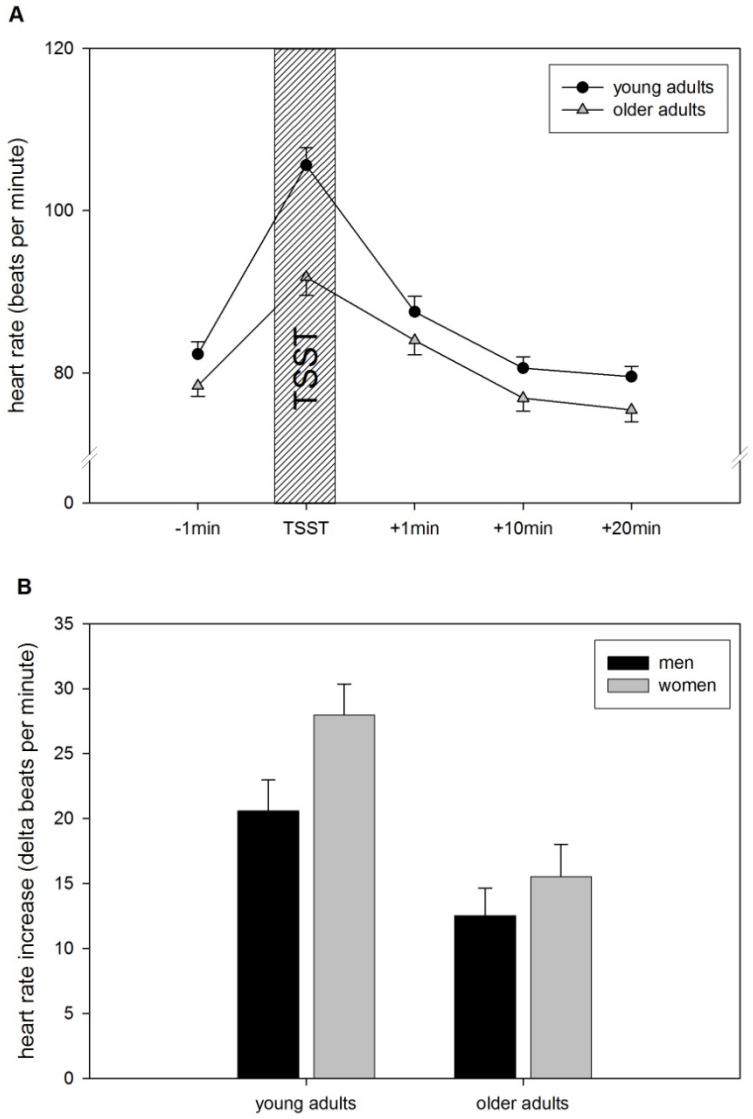

Figure 7.3 (A) mean heart rate levels (± standard error) after the Trier Social Stress Test (TSST) young adults and older adults, (B) mean heart rate increase (± standard error) after the Trier Social Stress Test (TSST) in children, young adults and older adults.

7.3.3.4. Heart rate variability

HRV measurements were obtained only from the two adult groups. The baseline RMSSD differed significantly between the groups ($F_{1,150}$ = 7.6, p = .007, η² = .048), with older adults showing lower RMSSD scores at baseline. ANOVA for repeated measurements revealed a significant decrease of RMSSD in response to the TSST in young and older adults ($F_{3.7,\ 460.2}$ = 23.8, p < .001, η² = .159). Furthermore, we found a significant group by time effect ($F_{3.7,\ 460.2}$ = 8.0, p < .001, η² = .060), with older adults showing an attenuated

stress-induced decrease in RMSSD (Figure 7.4A), in contrast to younger adults. Analyses revealed no time by sex or time by group by sex interaction. Univariate ANOVA of delta scores revealed only this previously described main effect of group for the stress-induced RMSSD ($F_{1,125}$ = 5.6, p = .019, η^2 = .043) delta scores, with older adults showing a lower acute decrease in RMSSD (Figure 7.4B). Furthermore, a significant main effect of group (p < .001) as well as sex (p = .037) on AUC_g was found, while there was no effect of group or gender on AUC_i. Older adults and women showed a lower total HRV, while there were no differences according to the reactivity of the HRV parameter RMSSD. Pearson correlations between indices of stress levels (CSSS, PSS) and summary indices (delta response, AUC_i, AUC_g) revealed no association between the parameters.

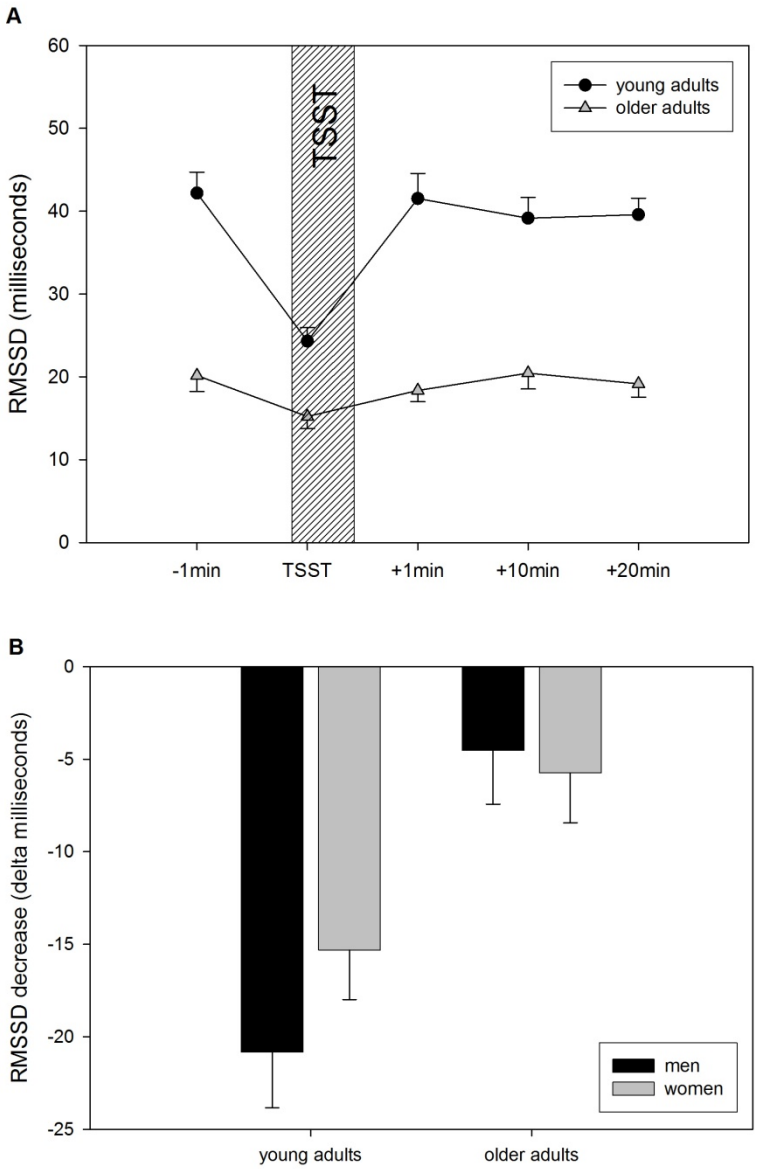

Figure 7.4 (A) mean square root of the mean squared difference of successive RR intervals (RMSSD) levels (± standard error) after the Trier Social Stress Test (TSST) in young adults and older adults, (B) mean square root of the mean squared difference of successive RR intervals (RMSSD) increase (± standard error) after the Trier Social Stress Test (TSST) in young adults and older adults.

7.3.3.5. Determinants of the salivary alpha-amylase stress response

To test whether indices of the sAA stress response (delta increase, AUC_g, AUC_i) were predicted by indices for cortisol, HR, and RMSSD responses, we calculated hierarchical linear regression controlling for age, BMI, and sex as well as subjective stress level indices (CSSS, PSS). The regression model for the two adult groups revealed age (delta: $\beta = .269$, $p = .029$; AUC_g: $\beta = .169$, $p = .270$; AUC_i: $\beta = .230$, $p = .055$) as the strongest predictor, whereas BMI, PSS, CSSS as well as cortisol, HR, and RMSSD response indices failed to predict stress-induced sAA. Including the group of children revealed similar results with one exception. Now, BMI was an even stronger predictor of stress induced alteration of sAA (delta: $\beta = .448$, $p < .001$; AUC_g: $\beta = .203$, $p = .061$; AUC_i: $\beta = .260$, $p = .020$) than age (delta: $\beta = .218$, $p = .043$; AUC_g: $\beta = .089$, $p = .407$; AUC_i: $\beta = .125$, $p = .262$). Again, all other parameters failed to predict sAA response indices.

7.4. Discussion

This is the first study investigating the effects of a psychosocial stressor on human salivary alpha-amylase in a wider age range of 6 to 61 years and to compare this to other markers of the psychophysiological response to acute stress. As expected, the TSST induced significant increases in sAA, cortisol, HR, and significant decreases in HRV in all age groups. Furthermore, sAA, HR, and HRV responses were attenuated in the group of older adults. In contrast to our expectations, cortisol responses were increased only in older men, but not in women. Also in accordance with our hypotheses, we found lower sAA responses in children, and no age differences in mean cortisol increases. However, lower AUC_i values indicated a lower sensitivity of the HPA in children. Age by sex interactions were found only for cortisol responses, which increased with age only in male participants. In contrast to our hypotheses, no association between sAA and cardiovascular responses were found. In line with our hypothesis, same was true for association between sAA and salivary free cortisol. Analyses revealed age and BMI as the strongest predictors of sAA increases, whereas subjective stress levels as well as cortisol, HR, and RMSSD response indices failed to predict sAA stress responses. A further unexpected finding was that children had significantly higher baseline sAA concentrations than both groups of adult participants. Also in contrast to our hypothesis, subjective chronic stress levels had no influence on sAA as well as cortisol, HR, and HRV stress responses.

Thus, our results replicate previous studies regarding HRV and cortisol and extend work on sAA in several ways. In line with former studies (Kudielka et al., 2004b; Nater et al., 2006; Rohleder et al., 2004a), stress-induced responses in most parameters are found in both adult groups. Furthermore, attenuated reactivity of sAA, HR, and HRV in older adults corresponds to the literature regarding stress-related SNS activity with aging (Kudielka et al., 2004a; Lavi

et al., 2006; White et al., 1997). This finding argues for the validity of sAA since this attenuation of stress-reactivity with aging was also found for the other sympathetic markers. However, there was no association between sAA and HR/HRV responses.

Furthermore, we found significant sAA increases in older adults as well as in children. This finding contradicts previous results of Stroud et al. (2009), showing no sAA increase of children aged 7 to 12 years in response to a child version of the TSST. However, to a peer rejection task, significant sAA emerged. This finding indicates existing but task specific sAA stress-reactivity in children. To date, nothing is known about the impact of age related changes of acute sAA response profiles in children of different ages. In our study, children aged 6 to 10 were included. Preliminary data from our group indicates that children under the age of 7 show no or only slight increases of sAA in response to a standardized psychosocial stressor, whereas in children above the age of 8 it reaches adult levels (Rosenloecher & Strahler, in preparation). However, in this study there was only one child younger than 7 years and excluding this subject from our analyses did not change our results. Furthermore, it could be speculated that lower reactivity in children is the result of a ceiling effect because of higher baseline values. According to studies looking at older age groups' basal sAA activity (Pajukoski, Meurman, Snellman-Gröhn, & Sulkava, 1999), there was no baseline difference in younger and older adults. Since nothing is known about possible mechanisms accounting for this unexpected baseline difference in children, it could be speculated that pre-task stress experiences or anticipatory stress are causing the higher baseline sAA activity found here. In agreement with previous findings (Buske-Kirschbaum et al., 1997; Kudielka et al., 2004b), no mean cortisol increase differences between children and the adult groups could be found. However, children showed lower total cortisol output (defined as AUC_g) as well as lower AUC_i indicating lower reactivity of this system.

In line with previous reports (Kudielka et al., 2004b), age by sex interactions were found for cortisol responses, with older men showing the highest mean salivary free cortisol increase. However, regarding sAA, HR, and HRV responses, no age by sex interactions were found. This is in contrast to findings of Kudielka and colleagues (2004a), showing differences between young men and women, while the HR responses in older men and women were comparable, as also indicated by our results. According to the literature, it is difficult to draw conclusions concerning sex specific autonomic responses in people of different ages because of different kinds of stress tasks and autonomic measurements (Seals & Dinenno, 2004).

The lack of data-based support for an association between sAA and cortisol responses found in this present study is interesting in light of the hypothesized relationship of SNS and HPA stress responsiveness discussed by Granger, Kivlighan, El-Sheikh, Gordis, and Stroud (2007b). Previously, correlations between sAA and cortisol were rarely reported (Nater et al.,

2005, 2006). Granger et al. (2007) attributed this lack of correlation to the differences in response kinetics and sensitivity to psychosocial stress of the two stress-response systems. Furthermore, we did not find any associations between sAA and cardiovascular measures. This lack of data-based support for the hypothesized association between sAA and cardiovascular autonomic measures may hint to different sympathetic effects on different organ systems, i.e., different mechanisms governing the stress-induced stimulation of salivary glands with the release of sAA on the one hand, and stress-induced cardiovascular alterations on the other. While salivary protein secretion is largely under sympathetic control and evoked by beta-adrenergic stimulation in particular (Busch & Borda, 2002; Garrett, 1987; Proctor & Carpenter, 2007), cardiovascular stress-reactivity seems to be modulated via either hormonal control mediated by the adrenal medulla or by increasing sympathetic or decreasing parasympathetic stimulation on alpha-adrenergic receptors (Hjemdahl, Fagius, Freyschuss,Wallin, Daleskog, et al., 1989; Thayer & Siegle, 2002). Thus, sAA might be an indirect indicator of the central sympathetic system (Ehlert et al., 2006) which is not necessarily associated with peripheral sympathetic effects.

Although it is known that chronic stress alters HPA stress responses (for a review, see Chida & Hamer, 2008), we found no correlation between subjective stress levels and cortisol stress responses. Same was true for sAA as well as HR and HRV. These results are in line with the phenomenon that subjective measures of stress often failed to be correlated with physiological stress markers.

A key finding is that we were able to induce increases in sAA in response to stress across a wide age range. Despite the lack of associations between sAA and cardiovascular responses, attenuated responses in the older adults' group were found for HR and HRV as well as sAA. This pattern closely fits the literature concerning sympathetic reactivity with aging and strengthens the role of sAA as a marker of psychophysiological stress responses. It needs to be excluded, however, that age-related differences are caused by changes of salivary flow and composition (Dodds, Johnson, & Yeh, 2005). In fact, there was no baseline difference between both adult groups, but significantly higher baseline values of sAA were found in the group of children. Nevertheless, we found significant increases of sAA activity in children aged 6 to 10 years in response to a standardized psychosocial stressor.

Some limitations should be considered. In our study, saliva was collected using salivettes, and participants were instructed to chew on the cotton roll. We thus collected stimulated whole saliva, and it might be argued that active chewing could alter sAA activity (DeCaro, 2008). However, stress-induced increases of sAA activity have been shown to be independent of salivary flow rate (Rohleder et al., 2006). Furthermore, it has been reported that stimulation or chewing changes the pH value of saliva (Polland et al., 2003). It is well known that enzymatic activity is affected by changes in pH. So, it could be suggested that

chewing on swabs with subsequent stimulation of salivary flow leads to alterations of pH value and thus to changes of enzymatic activity. However, unpublished data from our laboratory[4] shows that there is no alteration of sAA activity level after increasing or decreasing sample pH value with hydrochloride or sodium hydroxide. Another limitation might be that we cannot exclude the possibility that the slightly different stress protocols for children and adults caused stressor-specific responses of physiological parameters. This and the effectiveness of the TSST in producing stress response in children of different ages needs to be further investigated. Furthermore, there are constraints regarding cardiovascular parameters and the validity of data collected with commercially available inter-beat (RR) interval recorders (Polar S810i). However, adequate agreement between the Polar S810 and a classical 12-lead electrocardiogram (ECG) was shown (Nunan, Jakovljevic, Donovan, Hodges, Sandercock, & Brodie, 2008).

7.5. Conclusion

The present study illustrates age- and sex-related changes of endocrine and autonomic responses to a psychosocial stressor. The findings regarding cortisol and cardiovascular parameters are consistent with most previous studies. Results concerning salivary alpha-amylase draw attention to its usefulness as a sympathetic activity marker, since we found rapid stress-induced increases of this salivary enzyme above all age groups. These age- and sex related changes of stress reactivity might be important determinants of health and disease. Whether these changes are associated to vulnerability to disease and disease prevalence-patterns needs to be investigated in long-term studies.

[4] see 5.1.3.

8. Aging diurnal rhythms and chronic stress: Distinct alteration of diurnal rhythmicity of salivary alpha-amylase and cortisol[5]

8.1. Introduction

Organisms not only regulate their physiological functioning in response to acute environmental demands, but also rely on biological rhythms to continuously adapt to the environment. Thus, disruption of biological rhythms can impair the health and well-being. In humans, disturbed rhythmicity has been associated with a variety of mental and physical disorders (Koch, Nagtegaal, Kerkhof, & ter Wee, 2009; Tan, Bao, Tao, Liu, & Zhou, 2007; Wessa, Rohleder, Kirschbaum, & Flor, 2006). The suprachiasmatic nucleus (SCN) in the hypothalamus is discussed as the main circadian pacemaker, integrating endogenous and exogenous information (Gillette & Tischkau, 1999). Almost every physiological system has some degree of circadian rhythm that can be influenced not only by light exposure and the typical sleep–wake cycle but also by factors like age, sex, and stress. Of particular interest in the research of normal and abnormal physiology is the contribution of age. During the process of aging, cumulative exposure to psychological and biological stressors can, over time, result in a distinct and stable pattern of dysregulations, particularly in older individuals who experienced more stressors throughout their life spans. Most research on dysregulations of daily rhythms has focused on the hypothalamus–pituitary–adrenal (HPA) axis. Whether similar dysregulations also occur in the autonomic nervous system (ANS) is less well known. Therefore, this study was designed to examine the effects of age and stress on a non-invasive marker of autonomic-functioning salivary alpha-amylase (sAA).

The ANS is one of the main stress-sensitive systems in humans, and shows a distinct circadian rhythm, with sympathetic activity increasing significantly during the day and decreasing during the night, while parasympathetic activity decreases during the day and increases during the night (Yamasaki et al., 1996). Seals and Dinenno (2004) conclude in their review that the primary influence of aging on the human sympathetic nervous system (SNS) is an elevation of tonic activity. Another age-associated change of peripheral sympathetic function is an impairment of the baroreflex, and reduced neuronal reuptake of noradrenaline (NA) leading to higher NA as well as elevated sympathetic muscle nerve activity (Lakatta, 1993; Lavi et al., 2006). In contrast, adrenaline (A), an adrenomedullary sympathetic marker, remains unchanged (Leenen, Coletta, Fornuey, & White, 2005; Pfeiffer et al., 1983). Overall, there seems to be an age-related increase in systemic adrenergic

[5] **Acknowledgment**

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‘‘drive’’. In addition to age, other factors that need to be taken into account are those that activate the SNS, such as mental and physical stresses. So far, studies investigating chronic stress effects on various autonomic markers usually focus on topics such as job stress, racial discrimination, or burden of caregiving. The letter chronic stress experience has been associated, for example, with reduced HRV (Lampert, Ickovics, Horwitz, & Lee, 2005; Schubert et al., 2009). In a recent study conducted by Mausbach et al. (2008), caregiving stress was associated with decreased β_2-adrenergic receptor sensitivity, which may contribute to the development of cardiovascular illness among caregivers. This desensitization may occur by way of a chronic stress-induced greater release of adrenaline and noradrenaline from the adrenal medulla (Mausbach et al., 2008). In summary, studies investigating chronic stress effects on autonomic functions showed an increase of sympathetic activity and a concomitant decrease of parasympathetic function (Pickering, 2006).

Recently, salivary alpha-amylase (sAA) has gained interest as an indicator of autonomic activity. Since pharmacological studies were able to show a direct link between sAA and sympathetic nervous system (SNS) activity (Ehlert et al., 2006; van Stegeren et al., 2006), determination of sAA activity under both physically and psychologically stressful conditions is used more and more in biobehavioral research. Summarizing the existing literature, early studies suggested sAA as non-invasive measure of plasma noradrenaline (NA) concentration in humans (Chatterton et al., 1996; Skosnik, Chatterton, Swisher, & Park, 2000). Recently, a distinct diurnal rhythm of sAA was described in younger adults and children (Nater et al., 2007; Rohleder et al., 2004a; Wolf et al., 2008). However, only little is known about possible confounders of basal sAA levels (for a review Rohleder & Nater, 2009). Nearly all our previous knowledge concerning diurnal changes of basal sAA activity stems from studies with adult participants. Earlier studies showed lower morning and higher afternoon values in young adults (Jenzano et al., 1987; Rantonen & Meurman, 2000; Li & Gleeson, 2004). Yamaguchi et al. (2006) on the contrary, found no diurnal rhythm of sAA in students using a newly developed handheld electronic device that allows immediate amylase measurement (Yamaguchi et al., 2006). More recent studies revealed a pronounced diurnal rhythm characterized by a strong decrease after awakening and steadily increasing values during the day with peaks in the late afternoon in adults (Nater et al., 2007; Rohleder et al., 2004a). Just recently, the same diurnal profile was shown in children and adolescents (Wolf et al., 2008). Previous studies investigating basal sAA activity in healthy elderly subjects have led to the conclusion that sAA activity does not change with age (Aguirre et al., 1987; Ben-Aryeh et al., 1986; Salvolini et al., 1999). However, in all of the latter studies sAA activity was measured only once per participant, but to the best of our knowledge, no studies have assessed full diurnal profiles in elderly individuals. Furthermore, studies investigating the

cumulative effects of repeated or chronic stress on sAA rhythmicity are rare. One study reported an association between chronic stress and increases in daytime sAA levels (Nater et al., 2007). In a more recent study, Rohleder, Marin, Ma, & Miller (2009) reported a flattening of sAA diurnal profiles in cancer caregivers, which normalized at the end of the observation period. To address these gaps, our study was designed to investigate the association of aging and chronic stress experiences with diurnal rhythms of sAA.

The concept of Allostatic Load (AL) has served as a framework for a large body of research on the understanding of stress–health relationships. It is based on the hypothesis that there is a physiological accumulation of the effects of chronic stress - most notably seen during the process of aging. This concept refers to the cumulative biological wear and tear that can result from repeated stress system activation, leading to a chronic dysregulation of these systems, which finally causes damage to dependent tissues and organ systems (McEwen, 1998). For instance, age-associated elevations in sympathetic neuronal outflow result in higher concentrations of catecholamines and thus increase cardiac work. The resulting imbalance between oxygen supply and demand might lead to, e.g. cardiovascular hypertrophy, angina pectoris, or hypertension (Schulkin, 2004). Other hypothesized consequences are HPA axis dysregulations, i.e. impaired negative feedback and altered diurnal rhythmicity (for a review McEwen & Seeman, 1999), with its own set of hypothesized consequential dysregulations in for example the cardiovascular, immune, and the metabolic systems. In addition to the investigation of HPA axis dysregulations as a possible early indicator of AL (Gunnar & Vazquez, 2001), determination of basal diurnal sAA activity seems to be a unique possibility to extend our knowledge of age and chronic stress effects on autonomic/sympathetic nervous system.

In this study, competitive ballroom dancing was chosen as a model for chronic, non-habituating psychosocial stress. We have previously shown that competitive ballroom dancing induced substantial increases in cortisol, which did not habituate across competitions, was independent of individual experience, and was not a result of the physiological exercise component (Rohleder, Beulen, Chen, Wolf, & Kirschbaum 2007). Theoretically, repeated, non-habituating responses to social-evaluative conditions, which are characteristic for the lives of competitive ballroom dancers, should be associated with stress system dysregulations. Thus, we hypothesized that ballroom dancers would show altered diurnal patterns of salivary alpha-amylase and cortisol secretion in comparison with a control group of non dancers. The second purpose of our study was to investigate age related changes of basal amylase and cortisol diurnal rhythms, and potential stress-related alterations of these differences. Because of the potentially longer accumulation of wear and tear, we expected to see more pronounced alterations in older dancers in comparison with younger dancers and controls. In addition, we expected to see a sympathetic drive

associated higher overall alpha-amylase activity in older adults. For cortisol profiles it was shown, that chronic stress situations characterized by uncontrollability, emotions like shame, and threats to the social self are associated with flatter rhythms and higher daily output (Miller, Chen, & Zhou, 2007). Salivary cortisol profiles were obtained from the same samples as amylase for a comparison of the effects of chronic stress and age on diurnal profiles and the interaction of both stress-sensitive systems.

8.2. Methods

8.2.1. Participants

Ballroom dancers aged 15–30 as well as older ballroom dancers aged 49–75 were recruited with the help of the competitive dancing association of Nordrhein-Westfalen, Germany. They contacted dancing couples and asked them for participation in our study. We have included couples performing modern dancing (slow waltz, tango, Viennese waltz, slow foxtrot, and quickstep), performing latin dancing (cha-cha-cha, samba, rumba, paso doble, and jive), or both. During competitions, each couple has to perform five dances lasting 1min or 90 s, with minimal breaks in between. The quality of each couple's dancing is evaluated by five judges in relation to the other couples. Among other things, competitions are characterized by uncontrollability since judging in a performance oriented sport is inevitable subjective in nature. Except one couple all dancers were lifetime competitors. To be included, dancers had two pursue dancing at least two times per week and participate in one competition per month. Data for the control group of our study came from young adults aged 20–29 who were recruited via a notice posted on campus of the Technische Universität Dresden, Germany, via an advertisement in a local newspaper, and via personal contact. Older adults of the control group aged 51–75 years were recruited with the help of a general practitioner, who contacted criteria fitting patients without any illness, and asked for participation. A total of 26 young adults (14 men and 12 women), 33 older adults (19 men and 14 women), 27 younger ballroom dancers (12 men and 15 women) and 31 participants in the group of older ballroom dancers (18 men and 13 women) fulfilled the inclusion criteria. We included participants with a body mass index (BMI) above 17 and below 30 kg/m^2 in young adults and below 35 kg/m^2 in older adults. Smokers and individuals who reported excessive alcohol consumption (> three times a week) were excluded, also people under asthma medication, taking anti-rheumatics, psychotropic substances and excessive use of sleeping pills or painkillers. Participants were free of psychiatric and severe somatic diseases as evaluated by interview by one of the authors (J.S.). Use of other medication in the presence of changes of cardiovascular functioning (adrenergic active antihypertensive medication, anticoagulants), fat metabolism (antilipemics) and thyroid functioning (thyroid therapeutics), and the use of vitamins and natural therapeutics were allowed. Participants

were instructed to take their prescribed drugs either after the completion of the first two samples in the morning or at least one hour before the following samples. In order to investigate possible influences of the use of oral contraceptives on basal salivary alpha-amylase, women taking oral contraceptives were included. All young women were investigated in the luteal phase of their menstrual cycle. All women in the groups of older adults and older ballroom dancers were postmenopausal and free of any hormonal replacement therapy. Participants were not allowed to brush their teeth, drink (except water), and eat 30 min before sampling since these factors can also modify the activity of salivary alpha-amylase (Rohleder & Nater, 2009).

8.2.2. Study protocol

In the control group, young adults were invited to our laboratory while older adults were invited to the doctor's practice. After a short introduction, both were told about the study, and the procedure how to collect a diurnal profile of biochemical parameters with the help of saliva samples was shown. Young as well as older adults were given a package with all the necessary study materials and asked to send it back to our laboratory upon completion. Dancers were contacted via telephone. After a short introduction, they were asked to collect saliva samples over the course of one day. After declaring their consent, a package with all the study materials and again a written instruction was sent to all dancers. Furthermore, all participants were asked to fill in questionnaires concerning their experience of stress in the last 4 weeks (Perceived Stress Scale, PSS; Cohenet al., 1983), questions regarding their trait anxiety (State-Trait-Anxiety-Inventory, STAI-T; Laux et al., 1981) and short questions concerning awakening time, length of sleep, and possible stressful events on the sampling day. The study protocol was approved by the Ethics Committee of the Deutsche Forschungsgemeinschaft (DFG).

8.2.3. Measures

8.2.3.1. Saliva sampling

To determine salivary alpha-amylase and cortisol levels, five saliva samples were collected immediately after awakening, 30 min after awakening, 11 am, 3 pm, and 8 pm with the help of cotton swabs (Salivettes, Sarstedt, Nümbrecht, Germany). Participants were instructed to gently chew on the swab for 0.5 - 1 min. A common problem when collecting saliva is that older individuals do not secrete enough saliva without stimulation. We therefore decided to instruct all participants to chew on the cotton rolls for one min to stimulate saliva flow. Cotton swabs were then transferred to the plastic containers and stored at 20°C until analysis. MEMS6 TrackCap Monitors (Aardex Ltd., Switzerland) were used to test compliance, defined as collecting the first saliva sample within 10min after awakening and the second sample 30 ± 7 min after awakening. In addition, compliance for the remaining

three samples was defined as ± 1 h of the intended time (based on the recommendations by Kudielka, Broderick, & Kirschbaum, 2003). We found compliance to be 88.2% for all samples. Non-compliant samples were also included into following analyses and compliance was included as covariate where appropriate.

8.2.3.2. Biochemical parameters

After thawing, saliva samples were centrifuged at 3000 rpm for 3 min. The concentration of salivary alpha-amylase was measured by a quantitative enzyme kinetic method described elsewhere (Strahler, Mueller, Rosenloecher, Kirschbaum, & Rohleder, 2010b). In short, 20 µl of diluted saliva (1:625) and standard were incubated with 80 µl of substrate reagent (Alpha-Amylase EPS Sys; Roche Diagnostics) and then warmed in an incubator at 43°C for 90 s. After a first interference measurement at 405 nm, the plate was incubated for another 5 min and the second measurement was done. Increases of absorbance of samples were transformed to alpha-amylase activity using a linear regression calculated for the standard curve on each microplate (GraphPad Prism 4.0c for MacOSX, GraphPad Software, San Diego, USA). Intra- and inter-assay precision expressed as percent coefficient of variation was below 10%. Concentrations of salivary free cortisol were measured using a commercially available chemiluminescence-immuno-assay (CLIA; IBL, Hamburg, Germany) with intra- and inter-assay precision of 2.5% and 4.7%, respectively.

8.2.3.3. Psychological parameters

In order to control for possible influences of perceived stress on basal alpha-amylase activity all groups filled in the German version of the Perceived Stress Scale (PSS; Cohen et al., 1983). This 14-item scale assesses the frequency of experiencing a situation as unpredictable, uncontrollable, or overloading during the past month; its internal consistency in this sample was alpha = 0.63. Furthermore, trait anxiety was measured with the help of the trait version of the State-Trait-Anxiety-Inventory (STAI-S; Laux et al., 1981) consisting of 20 items; internal consistency in this sample was alpha = 0.98.

8.2.4. Statistical analyses

8.2.4.1. Preliminary analyses

Data were tested for normal distribution and homogeneity of variance using a Kolmogorov–Smirnov and Levene's test before statistical procedures were applied. These analyses revealed significant deviations from normality of some absolute alpha-amylase and cortisol values. Amylase and cortisol values were therefore log-transformed prior to analyses, which restored normality of distribution. These log-transformed data were then used to calculate the changes of salivary alpha-amylase (amylase awakening response, AAR) and cortisol (cortisol awakening response, CAR) after awakening, indexed as delta score

between levels immediately after awakening and levels 30 min after awakening. Furthermore, the area-under-the curves (AUC) were calculated according to Pruessner et al. (2003) as well as the slope of the regression line. To test for possible influences of using oral contraceptives (OC) in the groups of younger adults or drugs associated with high blood pressure (BP) and lipid lowering medication (LLM) in older adults, repeated measure (rm) ANOVAs were computed to test for time (5 sampling times) by group (young adults: OC vs. non-OC vs. men; older adults: BP vs. non-BP and LLM vs. non-LLM) effects on sAA as well as cortisol. Univariate ANOVAs were computed for comparisons of the groups concerning summary indices of sAA (AAR, AUC_{sAA}, and sAA slope) and cortisol (CAR, $AUC_{cortisol}$, and cortisol slope).

8.2.4.2. Diurnal course of salivary alpha-amylase

With regard to salivary alpha-amylase and cortisol diurnal profiles, rmANOVAs were used to analyze possible time (5 sampling times), age (young adults vs. older adults), group (ballroom dancers vs. controls), sex (male vs. female) effects, and their interactions. All results than were corrected by the Greenhouse-Geisser procedure where appropriate (Greenhouse & Geisser, 1959; Vasey & Thayer, 1987). Furthermore, because of significant differences between the groups with respect to BMI and awakening time, both variables were included as covariates. Concerning summary variables (AAR, CAR, AUC_{sAA}, $AUC_{cortisol}$, sAA slope, and cortisol slope), univariate ANOVAs with the factors age (young adults vs. older adults), group (dancers vs. controls), and sex (female vs. male) were computed for comparisons of the groups, and partial correlations including the above-mentioned covariates were computed to test for associations. Furthermore, bivariate correlations were computed to test for correlations of summary variables with the amount of physical activity in hours per week (h/wk). Partial correlations with the covariates age group, study group, sex, BMI, and awakening time were computed to test for association between summary scores of sAA (AAR, AUC_{sAA}, and sAA slope) and cortisol (CAR, $AUC_{cortisol}$, and cortisol slope). Post hoc analyses using the LSD method were conducted to determine subgroup differences. Finally, hierarchical linear regression equations were used to predict the three different indices for the sAA response (AAR, AUC_{sAA}, and sAA slope) by indices for cortisol, controlling for age, BMI, and sex, as well as the amount of physical activity, perceived stress and trait anxiety. For significant results we report partial eta squared (η^2) as a measure for effect size. For all analyses, the significance level was alpha = 5%. All results shown are the mean ± standard error of mean (SEM).

8.3. Results

8.3.1. Sample characteristic

Main characteristics of the groups are shown in Table 8.1. Of the 27 younger women, 16 female participants took oral contraceptives (10 younger dancers and 6 younger controls). No participant of the two young adult groups was under blood pressure medication, but 18 older adults of the control group (5 women and 13 men) and 8 older ballroom dancers (3 women and 5 men) were under such medication. Twelve older adults (2 dancers and 9 controls) were under lipid lowering medication. Younger dancers reported the highest self-perceived stress and trait anxiety what might be due to the time point of data collection. Data were collected after a two-week training camp followed by the decision of staying in the team. However, participants were within the normal range according to normative criteria (normative data not shown).

Table 8.1 Characteristics of study participants

	younger dancers (n=27)	younger controls (n=26)	older dancers (n=31)	older controls (n=33)	two-way ANOVA*			Post-hoc
Variable	**means (SD*)**	**mean (SD)**	**means (SD)**	**mean (SD)**	p_{age}	p_{group}	p_{a*g}	
age (years)	21.1 (4.4)	24.6 (2.0)	60.2 (6.8)	62.2 (6.7)	< .001	.008	.700	y = yd < o = od
BMI (kg/m²)	20.0 (1.6)	21.0 (1.8)	23.3 (2.2)	26.4 (2.9)	< .001	< .001	.008	y = yd < od < o
PSS	24.2 (7.1)	22.4 (5.6)	18.6 (5.5)	18.9 (5.8)	< .001	.477	.449	y = yd > o = od
STAI	42.1 (8.7)	34.0 (7.2)	31.0 (6.0)	32.9 (6.7)	< .001	.013	< .001	yd > y = o = od
awakening time (hh:mm)	08:11 (01:24)	07:05 (01:18)	07:24 (01:03)	06:16 (00:49)	< .001	< .001	.984	yd > y = od > o
sleep quantity (min)	498 (68)	448 (107)	468 (67)	439 (60)	.106	.003	.201	yd > y = o = od
activity (h/week)	14.7 (5.7)	5.1 (3.1)	8.0 (3.6)	1.1 (2.0)	< .001	< .001	.396	

* p_{age}, main effect of age; p_{group}, main effect of group; p_{a*g}, interaction effect of age and group
SD, standard deviation; PSS, Perceived Stress Scale; STAI, State-Trait-Anxiety-Inventory
y, young controls; yd, younger dancers; o, older controls; od, older dancers
=, no significant difference; <, >, significant difference and direction

8.3.2. Preliminary analyses: impact of oral contraceptives, blood pressure, and lipid lowering medication on diurnal profiles

In this chapter we want to explore whether amylase and cortisol levels are affected by oral contraceptive and antihypertensive or lipid lowering medication use since available data seems inconclusive. ANOVA for repeated measurements including the data of young adults

revealed significant changes of sAA (time: $F_{2.8,117.7}$ = 17.5, p < .001; ŋ² = .294) as well as cortisol (time: $F_{3.5,148.5}$ = 143.5, p < .001; ŋ² = .769) over the day. For sAA, no time by OC interaction ($F_{5.6,117.7}$ = 1.113, p = .359, ŋ² = .050) appeared, indicating no differences between young women using OCs, young women without and young men in their daily rhythm of sAA. Same was true for the AAR, AUC_{sAA} and sAA slope showing no main effect of OCs (AAR: $F_{2,45}$ = 1.6, p = .222, ŋ² = .065; AUC_{sAA}: $F_{2,39}$ = 0.3, p = .718, ŋ² = .017; sAA slope: $F_{2,40}$ = 1.2, p = .312, ŋ² = .057). On the contrary, a highly significant time by OC interaction was found for cortisol ($F_{6.9,148.5}$ = 2.8, p = .009, ŋ² = .116) with women taking OCs showing a flattened diurnal profile. This result was confirmed by a significant main effect of OCs on cortisol slope ($F_{2,41}$ = 6.3, p = .004, ŋ² = .234). However, univariate ANOVA of the CAR and $AUC_{cortisol}$ revealed no main effect of OCs (CAR: $F_{2,46}$ = 0.533, p = .590, ŋ² = .023; $AUC_{cortisol}$: $F_{2,40}$ = 0.508, p = .606, ŋ² = .025). Including the data of older ballroom dancers and age and sex-matched older adults, rmANOVA revealed significant changes of sAA (time: $F_{3.6,197.9}$ = 79.8, p < .001; ŋ² = .592) as well as cortisol (time: $F_{3.5,197.6}$ = 279.4, p < .001; ŋ² = .831) over the day. No time by BP interaction was found (sAA: $F_{3.6,197.9}$ = 1.5, p = .211, ŋ² = .026, cortisol: $F_{3.5,197.6}$ = 1.3, p = .289, ŋ² = .022) indicating no differences between participants taking BP medication and those without according to their daily rhythm of sAA and cortisol. Univariate ANOVA of the responses to awakening, AUCs and slopes revealed no main effect of BPs (all p > .10). ANOVA for repeated measurements revealed no time by LLM interaction (sAA: $F_{3.6,199.9}$ = 0.172, p = .941, ŋ² = .003; cortisol: $F_{3.5,195.7}$ = 0.427, p = .516, ŋ² = .011) and univariate ANOVA of summary scores revealed no main effect of LLM (all p > .10).

8.3.3. Diurnal course of salivary alpha-amylase

According to the results of our preliminary analyses, participants taking oral contraceptives, drugs associated with high blood pressure or lipid lowering medication were included into following analyses.

8.3.3.1. Salivary alpha-amylase over the day

Controlling for BMI and awakening time, rmANOVA using the log-transformed sAA values revealed a significant main effect of age group ($F_{1,92}$ = 6.0, p = .016, ŋ² = .061) as well as significant changes over time ($F_{3.3,300.9}$ = 2.6, p = .049, ŋ² = .027). Furthermore, a significant time by sex interaction ($F_{3.3,300.9}$ = 4.0, p = .006, ŋ² = .042) and a time by age group by study group interaction ($F_{3.3,300.9}$ = 2.7, p = .041, ŋ² = .029) appeared with especially older men of the control group and younger male dancers showing an altered diurnal profile of sAA (Figure 8.1). No other interaction effects were found. Univariate ANOVA of AUC_{sAA} and sAA slope with age group, study group, and sex as factors and the covariates BMI and awakening time revealed no main effect of study group, sex or any interaction effects on AUC_{sAA} (all p >

.10). However, there was a main effect of age group on AUC_{sAA} ($F_{1,89}$ = 5.2, p = .025, η^2 = .055) with higher values in older participants. Analyses of the slope revealed a significant age group by study group interaction ($F_{2,94}$ = 6.1, p = .016, η^2 = .061) with older controls and younger dancers showing the flattest slope. No other main effect or interactions were significant on this measure.

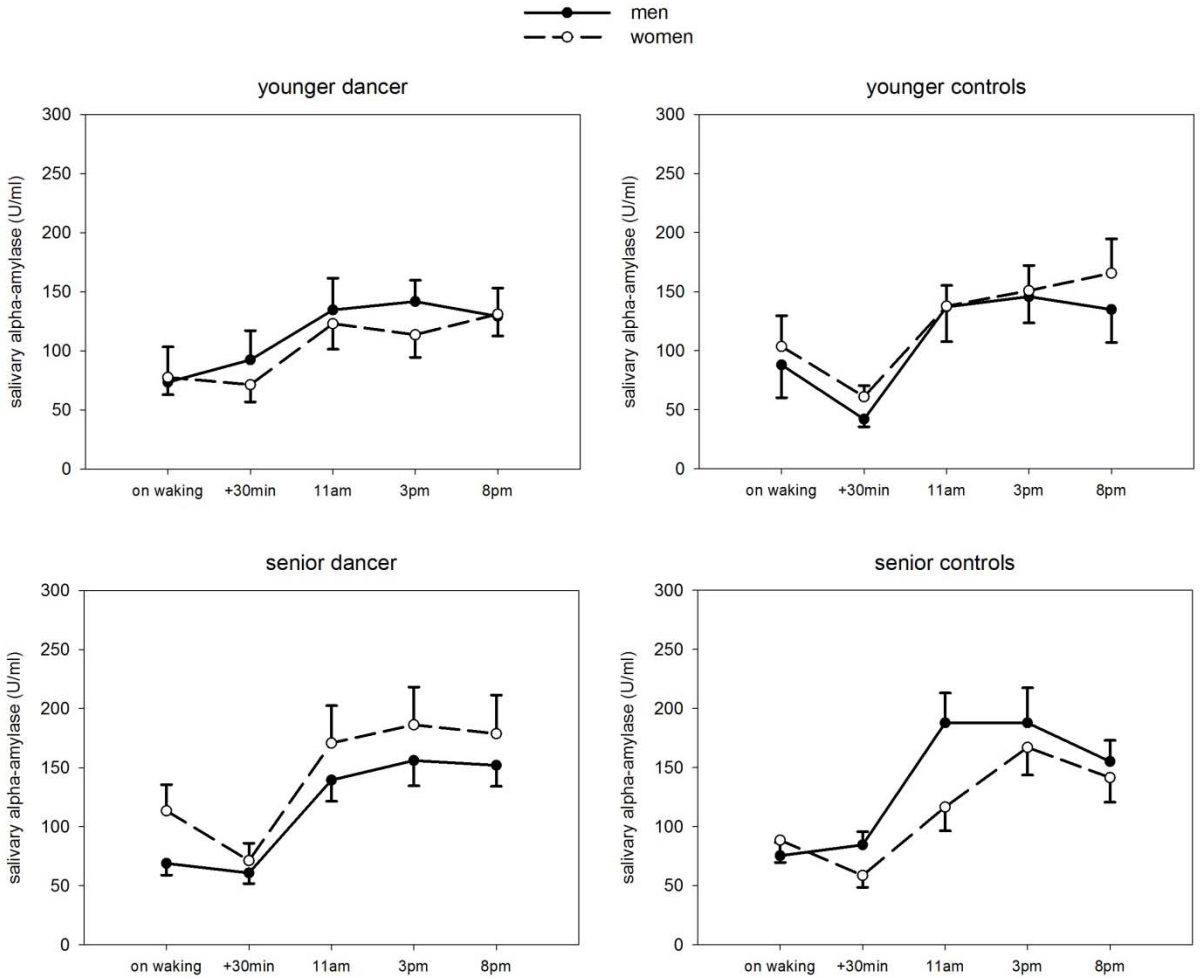

Figure 8.1 Salivary alpha-amylase profiles in younger dancers (n=27), younger controls (n=26), older dancers (n=31) and older controls (n=33) according to sex. Graph shows means and standard errors.

8.3.3.2. Salivary alpha-amylase after awakening

Controlling for BMI and awakening time, rmANOVA with age group, study group and sex as factors revealed no alpha-amylase changes after awakening in the whole group (time effect: $F_{1.0,95.0}$ = 0.3, p = .606, η^2 = .003). However, we found a significant time by age group by study group ($F_{2.0,95.0}$ = 6.9, p = .010, η^2 = .068), as well as a time by sex interaction ($F_{1.0,95.0}$ = 8.4, p = .005, η^2 = .081) with all female participants showing a well-defined sAA decrease in the first 30 min after awakening whereas, except younger male controls, all male participants showed only slight changes or even an increase (Figures 8.1 and 8.2). Following univariate ANOVA of the AAR with age group, study group and sex as factors and the

covariates BMI and awakening time confirmed this significant main effect of sex ($F_{sex\ 1,95}$ = 8.4, p = .005, ŋ² = .081) and age group by study group interaction ($F_{age\ x\ group\ 2,95}$ = 6.9, p = .010, ŋ² = .068) with younger male dancers and older male controls showing the lowest sAA response to awakening. No other main effects or interactions reached statistical significance.

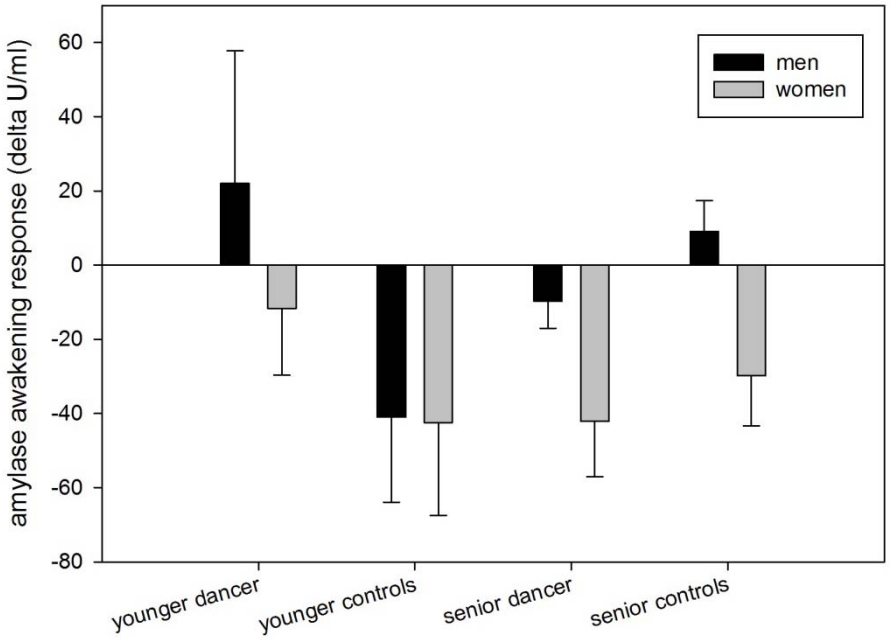

Figure 8.2 Mean salivary alpha-amylase response to awakening (± standard error) in younger dancers (n=27), younger controls (n=26), older dancers (n=31) and older controls (n=33) according to sex, indexed as delta score between levels immediately after awakening and levels 30 minutes after awakening. Graph shows means and standard errors.

8.3.4. Diurnal course of salivary cortisol

According to the results of our preliminary analyses, participants taking drugs associated with high blood pressure or lipid lowering medication were included into following analyses. Despite significant differences of women taking oral contraceptives and those without, all young women were included in following analyses. Younger female controls and younger female dancers were matched for this factor.

8.3.4.1. Salivary cortisol over the day

Controlling for BMI and awakening time, rmANOVA using the log-transformed cortisol values revealed significant changes over time ($F_{3.5,336.0}$ = 9.9, p < .001, ŋ² = .095), whereas there was no time by age group ($F_{3.5,336.0}$ = 0.9, p = .444, ŋ² = .010) or time by sex interaction ($F_{3.5,336.0}$ = 1.8, p = .139, ŋ² = .018). However, we found a time by study group and a time by age group by sex interaction ($F_{time\ x\ group\ 3.5,336.0}$ = 2.7, p = .037, ŋ² = .028; $F_{time\ x\ group\ x\ sex\ 3.5,336.0}$ = 3.0, p = .024, ŋ² = .050) with female older dancers showing a blunted diurnal variation (Figure 8.3). Univariate ANOVA of $AUC_{cortisol}$ and cortisol slope with age group, study group and sex as factors and the covariates BMI and awakening time revealed no main effect of

age group or study group (all p > .10), but a significant main effect of sex ($AUC_{cortisol}$: $F_{1,91}$ = 4.2, p = .043, η² = .044; cortisol slope: $F_{1,93}$ = 4.4, p = .040, η² = .045) with female participants showing a lower overall output of salivary cortisol as well as flatter cortisol slopes. Furthermore, a study group by sex interaction appeared ($AUC_{cortisol}$: $F_{1,91}$ = 3.4, p = .069, η² = .036; cortisol slope: $F_{1,93}$ = 8.0, p = .006, η² = .079) with especially younger female dancers showing the flattest slope.

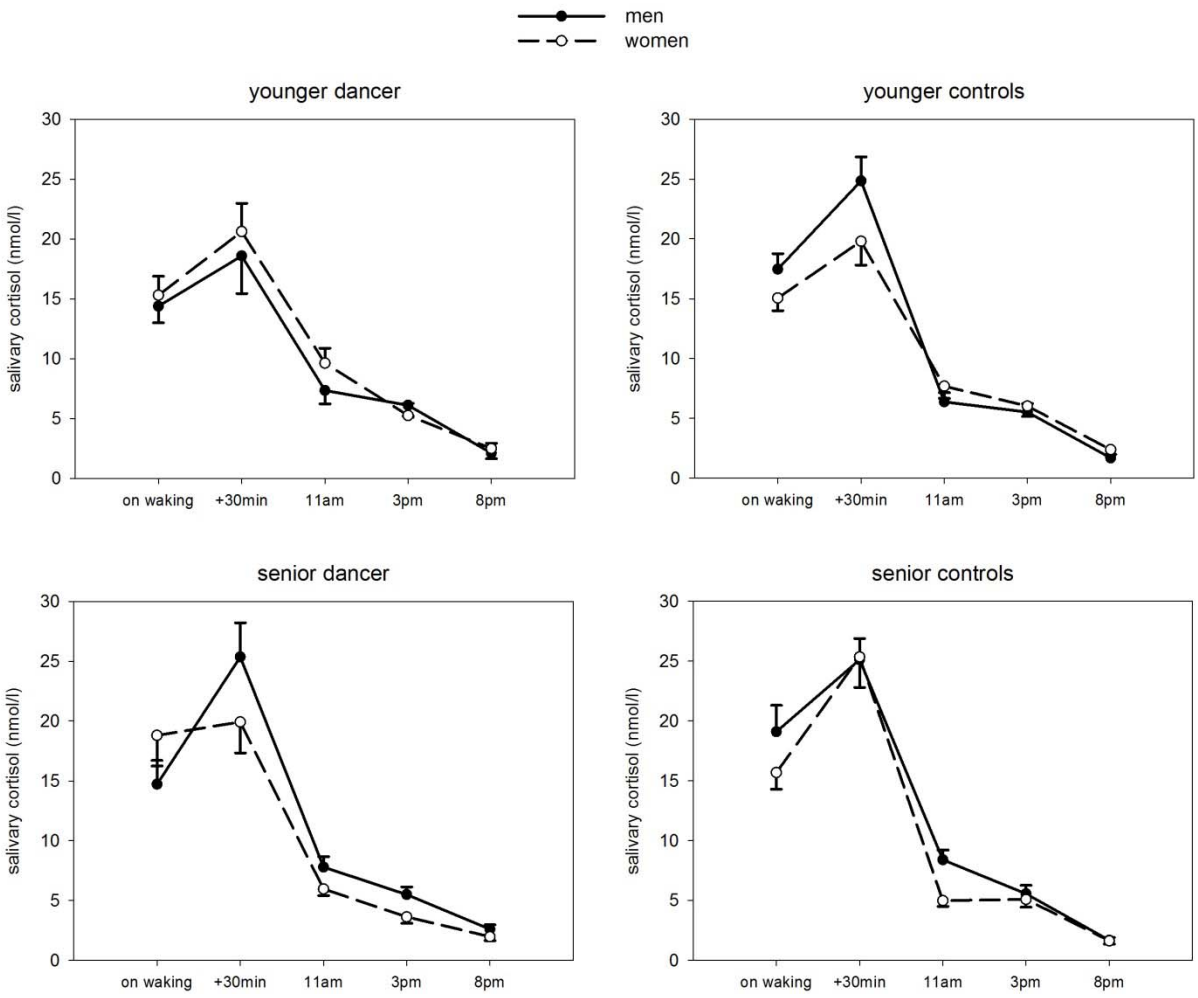

Figure 8.3 Salivary cortisol profiles in younger dancers (n=27), younger controls (n=26), older dancers (n=31) and older controls (n=33) according to sex. Graph shows means and standard errors.

8.3.4.2. Salivary cortisol after awakening

Controlling for BMI and awakening time, rmANOVA with age group, study group and sex as factors revealed no significant cortisol changes after awakening in the whole group (time effect: $F_{1.0,101.0}$ = 1.2, p = .273, η² = .012) as well as no effect of age group, study group, sex or any interactions (all p > .10). Following univariate ANOVA of the CAR with age group, study group and sex as factors and the covariates BMI and awakening time confirmed these results (all p > .10). As shown in Figure 8.4 younger dancers and female older dancers

showed the lowest mean increase of cortisol after awakening. However, post hoc tests revealed no significant differences between older dancers and both control groups as well as no difference between younger dancers and younger control (all p > .10). There was a trend towards a difference between younger dancers and older controls (p = .075).

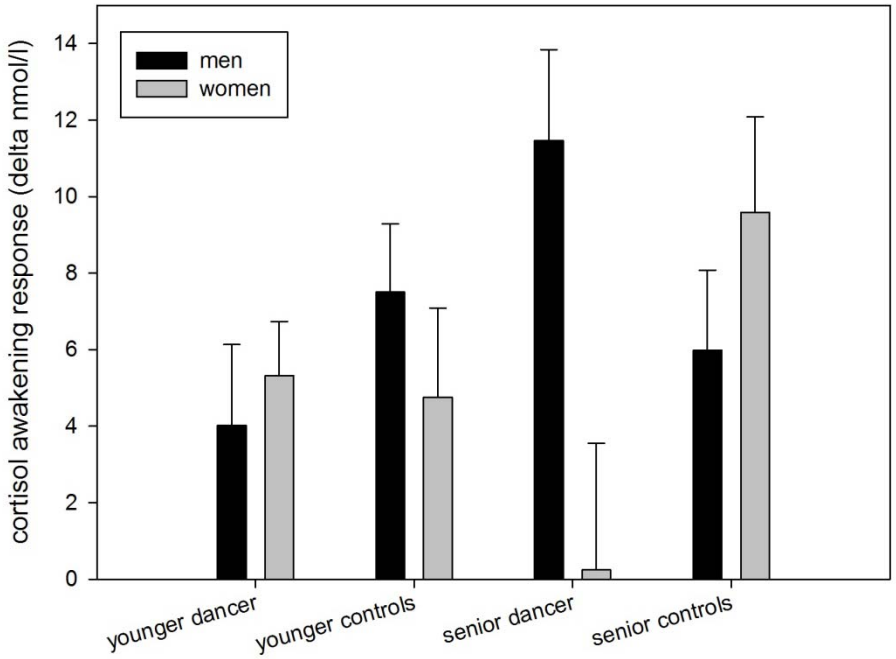

Figure 8.4 Mean salivary cortisol response to awakening (± standard error) in younger dancers (n=27), younger controls (n=26), older dancers (n=31) and older controls (n=33) according to sex, indexed as delta score between levels immediately after awakening and levels 30 minutes after awakening. Graph shows means and standard errors.

8.3.5. Diurnal course of salivary biomarkers: associations and determinants

Correlation and regression analysis allowed us to examine relationships between basal sAA as well as cortisol activity and variables possibly associated with these parameters. Furthermore, a hypothesized association between sAA and cortisol as markers of different but interacting stress systems was examined.

Testing for correlations of summary variables with the level of physical activity measured in hours per week (h/wk), bivariate correlations revealed no significant correlation between responses to awakening, slopes and level of physical activity (all p > .05). However, AUC_{sAA} as well as $AUC_{cortisol}$ showed a highly significant negative correlation (r = -.259, p = .008 and r = -.366, p < .001, respectively) indicating lower overall output of salivary biomarkers in more active individuals. Looking at the correlation between summary variables of sAA and cortisol, we found no correlations for AUC, slopes, and awakening responses (partial correlations controlling for age group, study group, sex, BMI, and awakening time: AUC: r = .165, p = .127; slope: r = .167, p = .122; awakening response: r = -.043, p = .689). To test whether indices of basal sAA activity (AAR, slope, AUC) were predicted by indices for cortisol, we

calculated hierarchical linear regression controlling for age, sex, and BMI as well as level of physical activity and subjective stress and anxiety level indices (PSS, STAI-T). The regression model for the whole group revealed sex as the strongest predictor of AAR (β = -.286, p = .010) as well as physical activity, $AUC_{cortisol}$ and age as the strongest predictors of AUC_{sAA} values (physical activity: β = -.288, p = .018; $AUC_{cortisol}$: β = .239, p = .028; age: β = .234, p = .097). Body-Mass-Index, PSS, STAI-T, as well as other basal cortisol indices failed to predict basal sAA activity.

8.4 Discussion

To our knowledge, this is the first study assessing full diurnal amylase profiles in older adults. In line with our hypothesis, daily output of sAA was elevated in older adults. In contrast, there was no effect of age on mean cortisol levels. In line with our hypothesis, younger dancers, especially younger male dancers, showed a blunted diurnal variation of sAA, which might be an effect of repeated stressful experiences. However, same was also true for older male controls but not older dancers, which contradicts our hypothesis of a stress-induced accumulating wear and tear of the body. While all women as well as older male dancers and young male controls displayed the previously shown decrease after awakening (Nater et al., 2007), younger male dancers and older male controls showed an increase of sAA in the morning. There was no effect of being a ballroom dancer on mean sAA levels and slopes. For the daily rhythm of cortisol we found female older dancers having the flattest diurnal profile. Furthermore, female participants showed a lower overall output of salivary cortisol as well as flatter slopes, which was most pronounced in younger female dancers. In line with these results, younger dancers and female older dancers showed the lowest mean increase of cortisol after awakening. Furthermore, we found no effect of using OCs, antihypertensive drugs or lipid lowering medication on the daily rhythm and output of sAA. There was no effect of antihypertensive drugs or lipid lowering medication on salivary free cortisol profiles and summary variables. However, women using OCs showed an attenuated diurnal profile and a flatter slope. In line with former stress-related (Nater et al., 2005, 2006; Granger et al., 2006) and basal findings (Nater et al., 2007; Wolf et al., 2008), no significant correlations between summary variables of sAA and cortisol were found. This finding suggests a clear distinction between sAA and cortisol representing different stress-sensitive systems, e.g. the SNS and the HPA axis, respectively. Investigating other determinants of the diurnal course of salivary biomarkers, results showed a significant negative correlation between AUC values and the amount of physical activity indicating lower overall output of sAA as well as cortisol in more active participants. Hierarchical linear regression revealed sex as the strongest predictor for the sAA response to awakening. Physical activity, age and overall cortisol output were the strongest predictors for AUC_{sAA},

while BMI, perceived stress and trait anxiety failed to predict basal sAA activity. To summarize, our hypothesized alterations of diurnal rhythms in dancers compared to controls were only seen in younger male dancers (for sAA) and female older dancers (for cortisol). Furthermore, we have to reject the hypothesis of a longer accumulation of chronic stress in older dancers. Older dancers did not show the hypothesized alterations of their daily sAA patterns and only female older dancers showed changes of their cortisol rhythms.

Our results showing higher overall amylase output in older adults are in line with previous studies indicating a higher sympathetic ‘‘drive’’ with aging (White et al., 1997). It was shown that there is an age-associated baroreflex impairment and reduced neuronal reuptake of NA (Lakatta, 1993; Lavi et al., 2006) leading to higher NA levels and elevated muscle sympathetic nerve activity, two markers of sympathetic activity. Interestingly, one study investigating circadian profiles of HRV showed an overall decrease, indicating a less effective responsiveness to possible stressors, as well as an attenuation of the morning peak (Bonnemeier et al., 2003). The same was true in our study, with especially older male controls showing an attenuated amylase peak in the morning. This finding of a less pronounced diurnal rhythm on a higher level could be an indicator of future illness, since it was similarly shown that higher levels and blunted diurnal variation of salivary cortisol are cross-sectionally associated with frailty burden (Varadhan et al., 2008). In breast cancer patients, cortisol slopes were a significant predictor of survival time with flattened slopes being linked to earlier mortality (Sephton, Sapolsky, Kraemer, & Spiegel, 2000). Furthermore, differences in gonadal steroid hormone secretion between males and females and a sexual dimorphism in brain structures modulating HPA axis activity have been shown to influence basal HPA axis functioning (Viau, 2002). In agreement with our results of a lower CAR, lower overall output and flatter cortisol slopes in especially female dancers, Ranjit, Young, and Kaplan (2005) found lowered cortisol levels in chronically stressed women. Therefore it could be suggested that the HPA axis adapts to chronic stress by downregulation with blunting of the morning rise and decline. In contrast, Traustadottir (2003) found no age- or fitness-related differences in diurnal cortisol variability in women. The author attributed this result to the excellent health of the older participants regardless of fitness level.

Examining possible influences of use of oral contraceptives, antihypertensive drugs and lipid lowering medication, we found no effect of OCs on sAA profiles. This was in line with previous reports (Laine et al., 1991). In contrast, antihypertensive drugs have been shown to decrease sAA activity (Nederfors et al., 1994; Nederfors & Dahlöf, 1996). In our study, no such an effect on diurnal rhythms was found. Since it was recognized that sAA can also be found in high-density lipoprotein (HDL; Karlsson, Leanderson, Tagesson, & Lindahl, 2005), a circulating complex of lipids and proteins, we further looked at the effects of LLM on sAA profiles. Again, there was no effect of a typical age-associated medication on diurnal sAA

activity. Regarding the effect of OCs on cortisol diurnal profiles, women using OCs showed an attenuated CAR compared to men. Previous results concerning the cortisol awakening response are inconsistent (Pruessner et al., 1997; Wüst et al., 2000) and it was concluded that the effects of OCs on the CAR are virtually negligible (Fries, Dettenborn, & Kirschbaum, 2009). Nevertheless, 10% of variability in the CAR was explained by the use of OCs in this study. Furthermore, it is known that hypertension is significantly associated with impaired glucocorticoid feedback control (Gold et al., 2005) leading to a relative attenuation in the CAR and HPA axis feedback sensitivity in hypertensive individuals (Wirtz et al., 2007). But to our knowledge, there are virtually no studies investigating the effects of antihypertensive drugs on basal HPA activity in older adults. We found no effect of BPs and LLM on cortisol profiles and the CAR of older adults. However, nothing is known about differences between normotensive unmedicated older adults, normotensive medicated and hypertensive medicated older adults. Although we found differences with regard to actual blood pressure, there was no difference between the above-mentioned groups according to their cortisol profiles and CAR. Future studies are needed to clarify this issue in more detail.

Investigating a possible relationship between regular physical activity, indicated as amount of exercise in hours per week, and basal autonomic and HPA axis activity, analyses revealed higher overall output of sAA as well as cortisol in participants who are less active. Furthermore, the age effect on sAA responses to awakening in older male controls was less pronounced in older male dancers indicating an attenuation of age-related changes of basal autonomic activity in participants being more active and having a higher fitness level. Thus, we might expect that regular physical activity could reduce sympathetic and HPA axis hyperactivity in older adults, as indicated by lower overall amylase activity and cortisol output in more active subjects. Previous studies showing this exercise-induced decrease of heightened sympathetic activity (Cornelissen & Fagard, 2005) support this assumption. In line with our result of a maintained diurnal variation of amylase activity, it was hypothesized that physical activity produces plasticity within neural networks that regulate autonomic activity, i.e. exercise training reduces the activation of neuron within cardiovascular regions of the brain (Mueller, 2007). However, results concerning HRV as another marker of SNS functioning remain inconclusive. While some studies found no effect of longterm exercise training on basal HRV (Uusitalo, Laitinen, Väisänen, Länsimies, & Rauramaa, 2004), others reported an increase of HRV in normal older adults (Levy et al., 1998; Stein, Ehsani, Domitrovich, Kleiger, & Rottman, 1999). The authors concluded that exercise training increases parasympathetic tone at rest (Levy et al., 1998) which could contribute to the reduction in mortality seen with increased HRV (Tsuji et al., 1996) and thus support the relevance of physical activity for healthy aging.

Overall, results of this present study are not in line with our hypothesis of a cumulative wear and tear of the body in ballroom dancers, because the matter seems to be more complex than expected. Although we found differences in agreement with this hypothesis in younger age, this effect disappeared in older ballroom dancers. This might be indicative of a compensating or healthy effect of regular physical activity and dancing competitions, respectively. One might suggest that ballroom dancing is a potent stressor according to the self-preservation theory (Dickerson & Kemeny, 2004), and threat to the social self in younger dancers, while older dancers might evaluate competitions in a more positive manner, i.e. as a challenge rather than threat. Thus, future studies need to clarify the issue of evaluating dancing competitions as challenge, threat or thrill with the help of questionnaires. In addition to the effects of regular physical activity and positive stress, as discussed above, another aspect of ballroom dancing in older ages needs to be addressed - the positive effects of social relationship experiences and social integration affecting psychological and physical health as discussed elsewhere (Seeman, Singer, Ryff, Dienberg Love, & Levy-Storms, 2002). Ballroom dancers are a tight-knit community, meeting regularly for competing and networking. Beyond this, it would be interesting to look at acute stress reactivity in dancers of different ages. After having investigated the effects of chronic stress and age on basal activity of different stress systems, this could be a valuable addition to our knowledge of integrated stress system functioning.

Some limitations of our study should be considered. Saliva for the assessment of biomarkers was collected using salivettes and participants were instructed to chew on the cotton roll. Thus, it might be argued that active chewing could alter sAA activity per se (DeCaro, 2008). However, stress-induced increases of sAA activity have been shown to be independent of salivary flow rate (Rohleder et al., 2006). Another limitation might be that we cannot exclude age-related differences of basal sAA activity caused by changes of salivary flow and composition (Dodds et al., 2005). Furthermore, only little is known about the impact of regular physical activity on salivary gland function. Acute exercise induces changes of saliva composition (Chicharro et al., 1998), but nothing is known about chronic changes. In our study, the only indicator of physical activity was the amount of exercise quantified as hours per week. There was no other marker of fitness. Future studies assessing the impact of regular physical activity should include measures of actual fitness level (e.g. maximal oxygen consumption).

8.4. Conclusion

The present study illustrates changes of endocrine and autonomic basal activity related to the process of aging as well as chronic psychosocial stress and regular physical activity, respectively. The findings regarding cortisol are consistent with most previous studies and

highlight the usefulness of this salivary marker in stress research. In a recent review, the CAR has been suggested as having the most significance in linking psychosocial factors and physiological functioning (Clow, Thorn, Evans, & Hucklebridge 2004). Results concerning salivary alpha-amylase draw attention to its usefulness as a sympathetic activity marker since we found higher overall output of this salivary enzyme in older adults which might be associated with the so-called sympathetic ‘‘drive’’ with increasing age. Furthermore, a lower output of sAA in people who are more physical active is in line with the hypothesis of an exercise-induced decrease of sympathetic activity. Overall, our study does not support the Allostatic Load hypothesis of a cumulative wear and tear of the body through repeated ballroom dancing competitions, but rather highlights the importance of regular physical activity, challenging events and social relationships in older ages.

9. Impact of blood pressure and antihypertensive drugs on diurnal alpha-amylase activity: A novel marker of sympathetic drive[6]

9.1. Introduction

The autonomic nervous system (ANS) plays a pivotal role in everyday human life. Nearly every tissue of the body is innervated by autonomic nerve fibers providing regulatory input. The sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS) form two subsystems regulating the activity of cardiac muscle, smooth muscle, and glandular tissue, for example. Due to this subdivision and dual innervation of tissues by nerve fibers from both branches, the ANS is able to communicate excitatory or inhibitory regulatory input.

Even in the absence of disease, tonic sympathetic activity increases with age (Seals & Dinenno, 2004). In the long run, inappropriately high SNS activity contributes to the pathogenesis of different forms of hypertension and hypertension-induced target organ damage (Grisk & Rettig, 2004). As a result, this sympathetic drive with aging is associated with higher blood pressure (BP). Interestingly, there seems to be a difference between the sexes, with women showing a more marked sympathetic overdrive and age-related increase of blood pressure, independent of menopausal status (Narkiewicz et al., 2005). Since it has been shown that the level of sympathetic activation parallels the degree of BP, antihypertensive treatments should be aimed at reducing BP as well as sympathetic overactivity. So far, studies that have been conducted on the impact of antihypertensive drugs (AD) on sympathetic drive remain inconclusive and show controversial results (Del Colle et al., 2007).

There are several methods to measure central nervous and peripheral activity of the SNS. Traditionally and predominantly used markers of sympathetic activity are heart rate (HR) and urinary or plasma noradrenaline (NA; Goldstein, McCarty, Polinsky, & Kopin, 1983). More direct and sensitive approaches include microneurography recordings, providing direct assessment of efferent postganglionic muscle sympathetic nerve activity, radiolabeled NA to measure regional NA spillover, and the analysis of heart rate variability (HRV; Grassi & Esler, 1999; Grassi, 2009). Finally, imaging techniques have been used to study and visualize autonomic innervations of human organs (Goldstein, 1995). These refined methods conclusively showed that an adrenergic overdrive occurs in hypertension (Grassi, 2009).

Within this field, the development of new biomarkers that assess sympathetic activity is of great interest. Especially salivary biomarkers have gained interest since saliva sampling is non-invasive, easy and inexpensive. The salivary enzyme alpha-amylase has been

[6] I would like to thank Sarah Brand for her excellent technical assistance in saliva sample processing and analyzing. Furthermore, I would gratefully acknowledge the help of Dr. Kerstin York who contacted criteria-fitting patients, asked for participation, and gave her expert assistance during blood pressure monitoring. I also thank all our participants for their cooperation during this study and their ongoing interest in this work.

suggested to reflect stress-related sympathetic activity (Chatterton et al., 1996; Granger et al., 2007; Nater et al., 2004; Rohleder et al., 2004a). Psychological as well as physiological stressors result in beta-adrenergic mediated autonomic activation (e.g. Willemsen et al., 1998), also involving beta-adrenoceptors of the parotid glands, which upon such stimulation release salivary alpha-amylase (sAA; Ehlert et al., 2006; van Stegeren et al., 2006). Given this fact, adrenergic agonists and antagonists may change sAA activity due to their impact on adrenoceptor mediated protein secretion (Busch & Borda, 2002; Garrett, 1987; Proctor & Carpenter, 2007) and salivary flow rate (Parvinen et al., 1984).Only two studies investigated the effects of AD on sAA activity showing a diminishing effect (Nederfors et al., 1994; Nederfors & Dahlöf, 1996). Nederfors & Dahlöf (1996) reported that the metoprolol-induced decrease of sAA disappeared on drug withdrawal and that drug re-exposure again decreased sAA.. However, samples were collected only once per assessment time point. Indeed, sAA has been shown to display a pronounced diurnal rhythm characterized by lowest values in the morning and steadily increasing values during the day with peaks in the late afternoon in younger and older adults, and children (Nater et al., 2007; Rohleder et al., 2004a; Strahler, Berndt, Kirschbaum, & Rohleder, 2010a; Wolf et al., 2008). To the best of our knowledge, there are no studies conducted on the impact of AD on diurnal sAA rhythmicity in older adults. Furthermore, nothing is known about differences between normotensive and hypertensive older adults. Therefore, this study aimed at investigating the effects of chronic hypertension and administration of AD on diurnal sAA in older adults. According to the literature we hypothesize to find higher levels of sAA activity in older adults suffering from high BP and not taking AD.

9.2. Methods

9.2.1. Participants

Older non-smoking and otherwise healthy adults aged 50–65 years were recruited with the help of a general practitioner, who contacted criteria-fitting patients and asked for participation. Participants were free of any psychiatric or physical disease except for high blood pressure. Parts of this data set (n = 33) are already published in Strahler et al. (2010a). Overall, 78 adults fulfilled the inclusion criteria. Individuals having a body mass index (BMI) below 17 and above 35 kg/m^2, reporting excessive alcohol consumption (> three times a week), or people taking anti-rheumatics, asthma medication, psychotropic substances and use of sleeping pills or painkillers within the last two weeks were excluded. Use of other medication (adrenergic active antihypertensive medication, anticoagulants, antilipemics, thyroid medications), and the use of vitamins and natural therapeutics were allowed. Participants were asked to withhold taking their prescribed medication until completion of two morning samples, and during one hour before the following samples. All women were

postmenopausal and free of any hormonal replacement therapy. At least 30 min before sampling participants were asked to abstain from eating, drinking (except water), or brushing their teeth according to the recommendation by Rohleder and Nater (2009).

9.2.2. Study protocol

Participants were invited to the doctor's practice where they were informed about the study, and the procedure of saliva sampling was shown. After obtaining written informed consent, participants received a package including study materials and were asked to return these by regular mail after completion. It was recently shown that sAA activity was stable for up to 5 days at room temperature (O'Donnell, Kammerer, O'Reilly, Taylor, & Glover, 2009). Furthermore, all participants were asked to fill in questionnaires concerning subjective chronic stress levels (Perceived Stress Scale, PSS; Cohen et al., 1983) and trait anxiety (State-Trait-Anxiety-Inventory, STAI-T; Laux et al., 1981). Furthermore awakening time, length of sleep, and sleep quality was assessed. The study protocol was approved by the Ethics Committee of the Deutsche Forschungsgemeinschaft (DFG).

9.2.3. Measures

9.2.3.1. Saliva sampling

Saliva samples were collected using Salivettes (Sarstedt, Nümbrecht, Germany). Participants were instructed to gently chew on the cotton roll for up to one minute or until it is saturated with saliva. To determine diurnal sAA rhythmicity, five saliva samples were collected immediately after awakening, 30 min after awakening, 11 am, 3 pm, and 8 pm. After sampling, cotton rolls were transferred to the plastic containers and stored at -20 °C until analysis. To objectively control compliance, MEMS6 TrackCap Monitors (Aardex Ltd., Switzerland) were used. Based on the recommendation by Kudielka and colleagues (2003), compliance was defined as collecting the first saliva sample within 10min after awakening, the second sample 30 ± 7 min after awakening and as ± 1 h of the intended time for the remaining samples. Compliance was found to be 91.7%. Samples taken outside the defined compliance period were included in further analyses, but compliance was included as covariate where appropriate.

9.2.3.2. Biochemical parameters

For analyses, saliva samples were thawed and centrifuged at 3000 rpm for 3 min. The concentration of salivary alpha-amylase was measured by a quantitative enzyme kinetic method described elsewhere (Strahler et al., 2010b). Intra- and inter-assay precision expressed as percent coefficient of variation was below 10%.

9.2.3.3. Blood pressure assessment

Systolic and diastolic blood pressure was monitored at the left arm of the participants while sitting in an upright position. Assessment was done by the general practitioner according to the Riva-Rocci method (Boso sphygmomanometer, Germany). According to actual criteria (WHO, 1999), subjects were classified as hypertensive if displaying either systolic BP ≥ 140 mmHg or diastolic BP ≥ 90 mmHg.

9.2.4. Statistical analyses

Kolmogorov–Smirnov and Levene's test showed normal distribution and homogeneity of variance of raw alpha-amylase values. Therefore, absolute sAA values were used to calculate the change of sAA (amylase awakening response, AAR) after awakening, indexed as delta score between levels immediately after awakening and levels 30 min after awakening. Furthermore, the slope of the regression line as well as the area-under-the curves (AUC) as a measure of diurnal overall output was calculated (Pruessner et al., 2003). Repeated measurement (rm) ANOVA was used to test for time (5 sampling times), BP (normotensive vs. hypertensive), AD (using AD vs. no AD), sex (male vs. female) effects, and their interactions. Greenhouse-Geisser procedure was applied to correct results if necessary (Greenhouse & Geisser, 1959; Vasey & Thayer, 1987). Univariate ANOVAs were computed for comparisons of the groups concerning summary indices of sAA (AAR, AUC, and sAA slope). To determine subgroup differences, post hoc analyses using the LSD method were conducted. For significant results we report partial eta squared (η^2) as a measure for effect size. For all analyses, the significance level was alpha = 5%. All results shown are the mean ± standard error of mean (SEM).

9.3. Results

9.3.1. Participants

Table 9.1 shows main characteristics of the sample. Fourteen adults (6 men and 8 women) were under lipid lowering medication. Participants were within the normal range of self-reported chronic stress within the last 4 weeks and state anxiety according to normative criteria (see Table 9.1).

Table 9.1 Main characteristics of study participants

Variable[1]	Normotensives participants	Hypertensive participants	Statistics[2]
n (females)	33 (20)	45 (19)	p_{X^2} = .169
AD (n/females)	16/9	34/13	*p_{X^2} = .018*
age (years)	60.6 ± 7.1	61.8 ± 7.9	p_t = .806
BMI (kg/m²)	25.4 ± 2.6	27.2 ± 2.8	*p_t = .006*
awakening time (hh:mm)	06:22 ± 00:50	06:27 ± 01:00	p_t = .709
sleep quantity (min)	443 ± 53	459 ± 67	p_t = .329
sleep quality[3]	median: 3.5	median: 4.0	p_z = .105
PSS	20.7 ± 5.9	18.8 ± 6.6	p_t = .192
STAI	38.1 ± 8.6	37.2 ± 7.8	p_t = .659

[1] results shown are mean ± standard deviation;
[2] X^2 Chi-square test, t Student´s t-test, Z Wilcoxon-Mann-Whitney test
[3] 5-point scale: from *very bad* to *very good*
AD, antihypertensive drugs; PSS, Perceived Stress Scale, age-specific norm 18.3 ± 8.1; STAI, State-Trait-Anxiety-Inventory, norm 37.3 ± 9.8

9.3.2. Salivary alpha-amylase

9.3.2.1. Salivary alpha-amylase over the day

Repeated measurements ANOVA revealed a significant main effect of BP group ($F_{1,62}$ = 4.2, p = .046, η² = .063) as well as significant changes over time ($F_{3.3,201.9}$ = 33.9, p < .001, η² = .354). Furthermore, a significant time by AD interaction appeared ($F_{3.3,201.9}$ = 3.3, p = .019, η² = .050) with subjects not using antihypertensives showing a heightened diurnal profile (Figure 9.1A). A time by BP interaction failed to reach significance ($F_{3.3,201.9}$ = 0.7, p = .581, η² = .011). However, hypertensive subjects displayed descriptively more pronounced sAA alterations throughout the day (Figure 9.1B). A high significant time by sex interaction was found ($F_{3.3,\ 201.9}$ = 5.1, p = .002, η² = .075) with men showing more pronounced daily sAA profiles (Figure 9.2). However, there was no significant time by BP by sex or time by AD by sex interaction (all p > .20). Univariate ANOVA of AUC with BP group, AD group, and sex as factors revealed a significant main effect of AD ($F_{1,60}$ = 4.1, p = .047, η² = .064) and trend towards a main effect of BP ($F_{1,60}$ = 3.0, p = .091, η² = .047) and a AD by BP interaction ($F_{1,60}$ = 3.4, p = .071, η² = .053) with hypertensive subjects and those not using antihypertensives showing a higher diurnal output of sAA (Figure 9.3). In contrast to the above mentioned results, there was no main effect of sex or a BP by sex interaction (all p > .15). However, a AD by sex interaction ($F_{1,89}$ = 5.2, p = .025, η² = .055) indicated higher values in male participants not using AD (Figure 9.3A). Analyses of the slope revealed a significant AD by BP group interaction ($F_{1,67}$ = 4.1, p = .048, η² = .057) with hypertensive subjects not taking AD showing the steepest slope. No other main effects or interactions were significant on this measure.

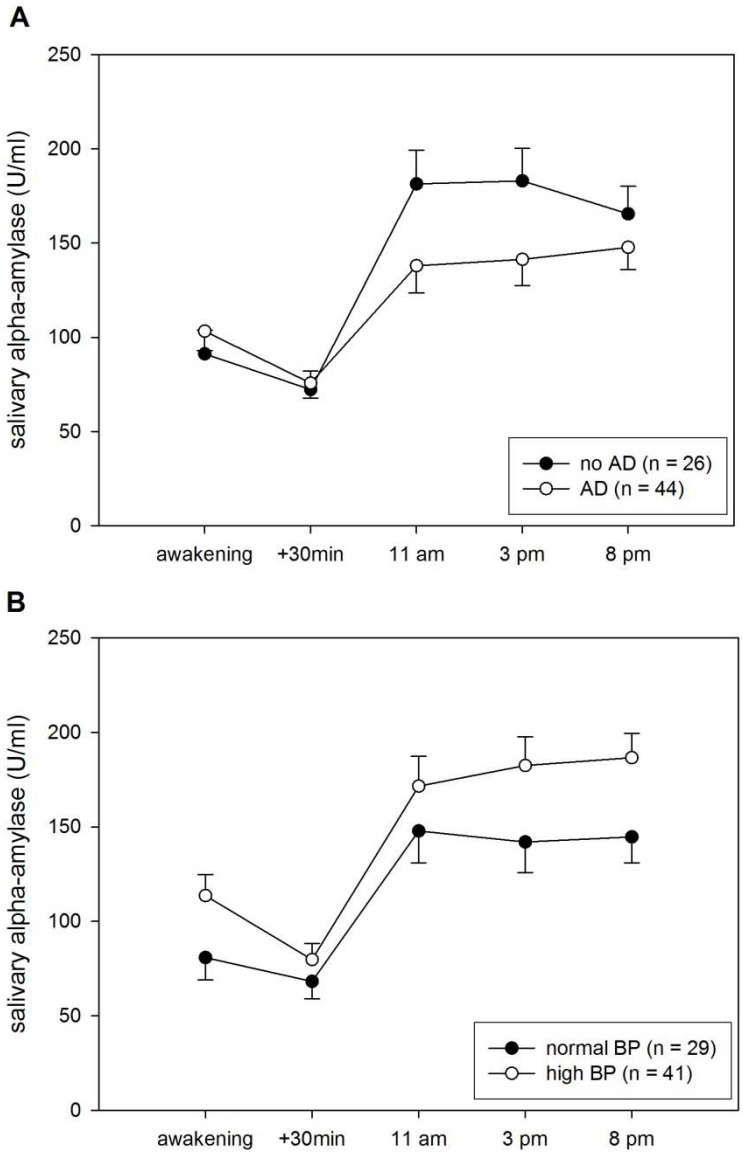

Figure 9.1 Salivary alpha-amylase profiles in (A) subjects taking antihypertensive drugs (AD) versus non users, and (B) subjects suffering from high blood pressure (BP) versus normotensive participants. Graph shows means and standard errors.

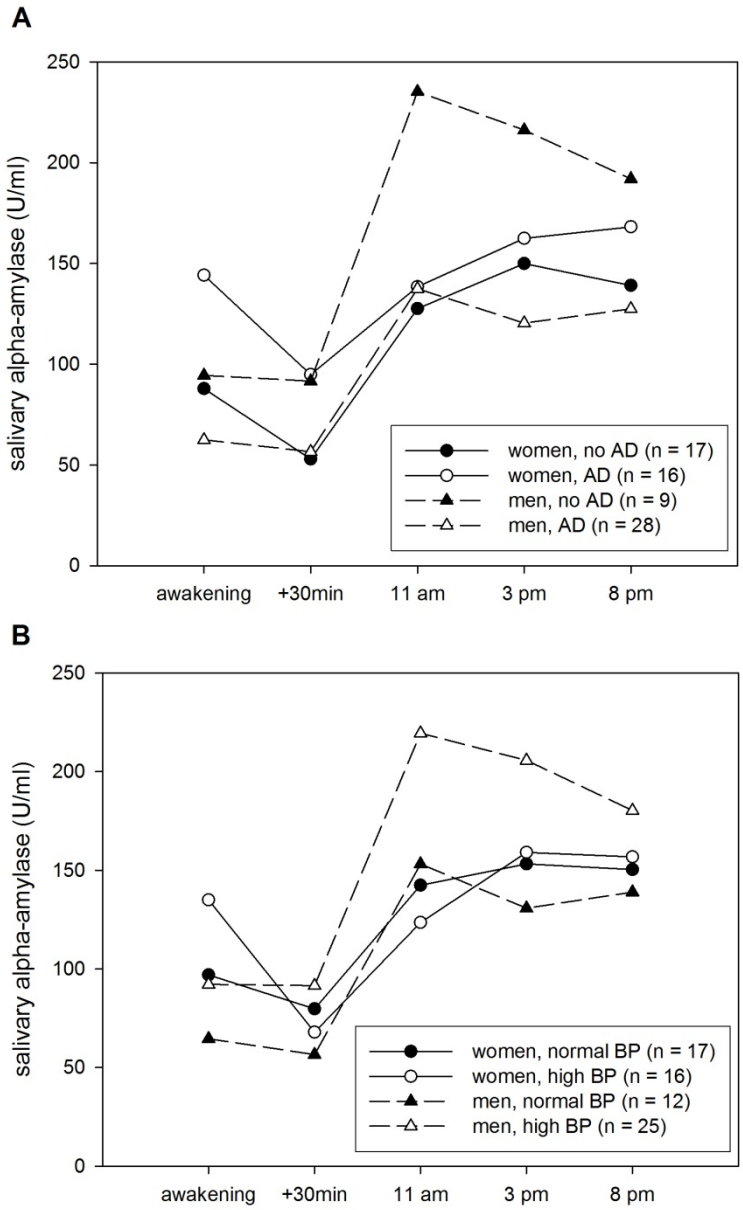

Figure 9.2 Salivary alpha-amylase profiles in (A) subjects taking antihypertensive drugs (AD) versus non users, and (B) subjects suffering from high blood pressure (BP) versus normotensive participants according to sex. Graph shows means (for the sake of clarity error bars are omitted).

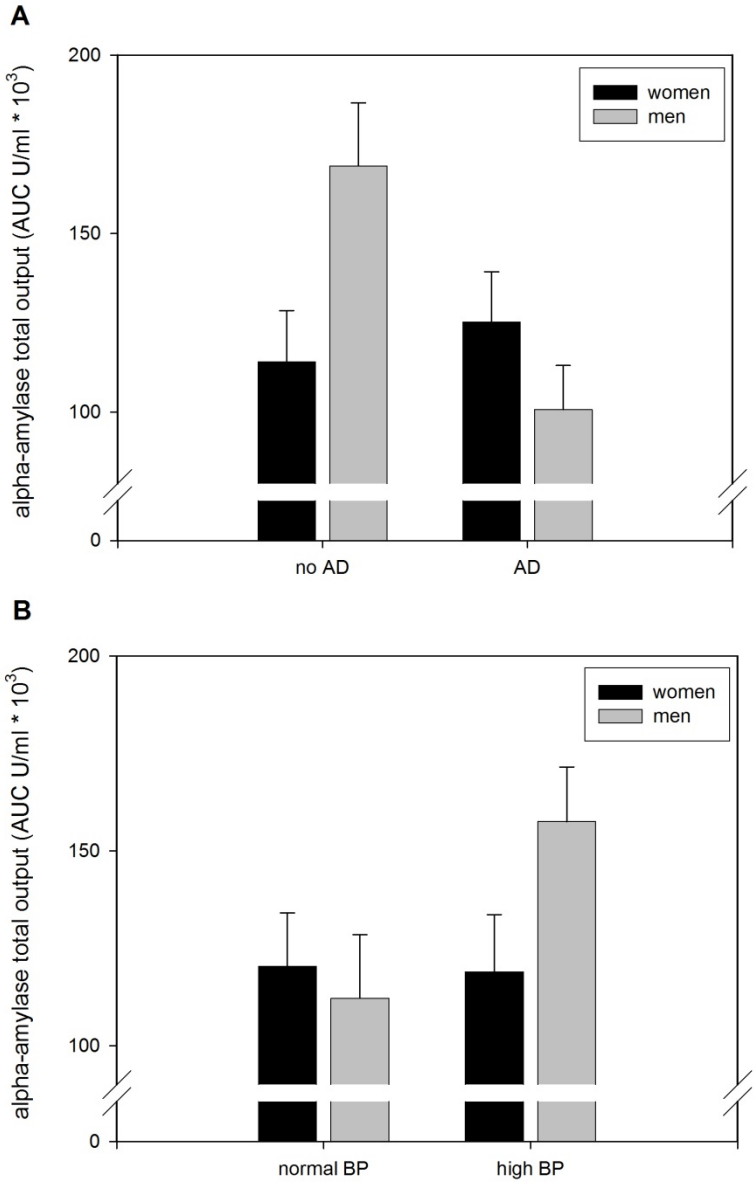

Figure 9.3 Mean diurnal amylase output (± standard error) indexed as area under the curve values (AUC) in (A) subjects taking antihypertensive drugs (AD) versus non users, and (B) subjects suffering from high blood pressure (BP) versus normotensive participants according to sex. Graph shows means and standard errors.

9.3.2.2. Salivary alpha-amylase after awakening

ANOVA for repeated measures with BP group, AD group and sex as factors revealed significant sAA changes after awakening ($F_{1.0,67.0}$ = 6.7, p = .012, η² = .090) with women showing more pronounced changes in the first 30 min after awakening ($F_{1.0,67.0}$ = 4.8, p = .031, η² = .067; Figure 9.2). No other interaction reached statistical significance. Following univariate ANOVA of the AAR confirmed these results ($F_{sex\ 1,67}$ = 4.8, p = .031, η² = .067) with men lacking the typical decrease after awakening.

9.4. Discussion

The main aim of this study was to assess diurnal sAA rhythmicity in a sample of older adults and to investigate a possible impact of blood pressure and the use of antihypertensive drugs on sAA profiles. In line with earlier findings in young adults and children we found significant changes of sAA activity with steadily increasing values throughout the day (Nater et al., 2007; Rohleder et al., 2004a, 2008; Wolf et al., 2008). In addition, we were able to replicate findings in a part of this data set with women showing much more pronounced changes in the first 30 minutes after awakening (Strahler et al., 2010a). Furthermore, we hypothesized to find higher levels of sAA activity in older adults suffering from high BP and not taking AD. In this study, subjects not using antihypertensive agents showed a heightened diurnal profile. Descriptively, this was also true for hypertensive older adults. These results were confirmed by analyses of the overall diurnal output indicating a higher total output of sAA in unmedicated adults which was especially pronounced in those being hypertensive. Furthermore, hypertensive subjects not taking AD showed the steepest slope. Our results therefore provide strong evidence for a possible influence of blood pressure and antihypertensive drugs on sAA activity. In sum, higher diurnal sAA output in unmedicated older adults and the highest diurnal output in unmedicated and hypertensive men indicate higher sympathetic activity in these groups.

Early findings of resting heart rate and cardiac output in hypertensives raise the hypothesis of an elevation in sympathetic activity in these subjects (Somers, Mark, & Abboud, 1988). Following studies confirmed this via the assessment of urinary adrenergic metabolites and plasma noradrenaline (Esler, 1997; Goldstein, 1995). To date, sympathetic nerve activity is widely accepted as an aetiological factor in human essential hypertension (Guyenet, 2006). However, it has to be discussed whether the increase in SNS activity affects all circulatory districts or is rather organ specific. Studies using NA spillover technique reveal substantial regional heterogeneity of sympathetic nerve activity in hypertension subgroups (Grassi & Esler, 1999, 2002).In addition, it needs to be established whether this adrenergic imbalance affects the peripheral, the central, or both branches of the SNS. Studies using microneurography technique imply that the hypertension-induced NA increase occurs due to increased central sympathetic neural outflow (for a recent review see Grassi, Seravalle, Quarti-Trevano, 2010). Hypertension in humans is characterized by impaired NA reuptake from sympathetic nerve terminals (Esler, 1997; Grassi & Esler, 1999) and a downregulation of peripheral alpha-and beta-adrenoceptors due to chronic stimulation (Michel, Brodde, Insel, 1990). Furthermore, it is known that adrenaline and noradrenaline clearance is mainly mediated via beta-adrenergic rather than alpha-adrenergic mechanism (Cryer, Rizza, Haymond, & Gerich, 1980). Together with the fact that salivary protein secretion in the acinar cells is beta-adrenoceptor mediated (Busch & Borda, 2002; Garrett,

1987; Proctor & Carpenter, 2007) it might be hypothesized that chronic sympathetic activation also occurs in salivary glands. In addition, it might be of importance to look at endogenous rhythmicity that might be influenced by hypertension. Patients with essential hypertension showed reduced levels of three important transmitters in the suprachiasmatic nucleus (SCN) - the “biological clock” which controls circadian rhythms (Goncharuk, Van Heerikhuize, Dau, Swaab, & Buijy, 2001). The authors also provided anatomical support for an altered SCN output to the SNS and the hypothalamic-pituitary-adrenal-axis in primary hypertension (Goncharuk, Van Heerikhuize, Swaab, & Buijs, 2002). Furthermore, Nater (2004) reported a relationship between chronotype and amylase activity with morning types showing higher values of sAA activity. The author therefore suggested to conduct saliva sampling at the same time of day. To the best of our knowledge, there are virtually no studies investigating the impact of sunlight, one of the major *zeitgeber* of circadian endogenous rhythms, on diurnal sAA activity. In sum, these results suggest anatomical evidence for the involvement of hypertension in diurnal sAA activity, possibly mediated via altered beta-adrenoceptor density on parotid glands or altered SCN output to the SNS.

There are some limitations that should be considered. Collecting saliva samples with the help of salivettes and instructing participants to chew is argued as altering sAA activity per se (DeCaro, 2008). However, sAA activity has been shown to be independent of salivary flow rate in an acute stress paradigm (Rohleder et al., 2006). Furthermore, this study fails to provide information on to what extent the sympathetic overdrive, as reflected in higher sAA levels, is a feature of the hypertensive disease or rather a characteristic of specific hypertensive states. Future studies that are conducted on this issue will have to distinguish between different kinds of hypertension. Same was true for different kinds of antihypertensive drugs. In this study, there was no differentiation between substance classes such as alpha- or beta-adrenergic active substances, calcium channel blockers, ACE inhibitors, or antidiuretic drugs.

9.5. Perspectives

Results of this study provide supportive evidence that the characteristics of diurnal sAA activity differ between normotensive and hypertensive subjects. Furthermore, these data indicate an impact of antihypertensive medication on sAA activity. The overall accepted increase in sympathetic drive with aging is associated with an increased diurnal output of sAA, what was most pronounced in older men not taking antihypertensive drugs. Findings are of particular interest for the increasing research area of aging and also highlight the usefulness of sAA as an index of adrenergic activity even in specific pathological states. Furthermore, we suggest to carefully control for blood pressure and the use of adrenergic active drugs, i.e. antihypertensives, in studies examining sAA as a measure of autonomic functioning.

10. Light affects morning salivary cortisol, but not salivary alpha-amylase[7]

10.1. Introduction

Many aspects of human life underlie distinct alterations throughout the 24 hour cycle. Different parameters such as morphological, biochemical, and physiological functioning are thought to be regulated by environmental factors and generated by an endogenous pacemaker. In humans, disturbed rhythmicity can impair health and well-being since disruption in circadian rhythms has been associated with pathology (e.g. Koch et al., 2009). The suprachiasmatic nucleus (SCN), located in the ventral hypothalamus, is proposed as the main circadian pacemaker and a central autonomic clock (Gillette & Tischkau, 1999; Ueyama et al., 1999). The SCN as the "master oscillator" integrates endogenous and exogenous information and thus modulates autonomic and endocrine functions to prepare for environmental changes. Hence, at the end of the sleep period, the SCN prepares the body for activity, e.g. a peak in blood glucose (la Fleur, 2003). The molecular core of this system is a transcription-translation feedback loop. This loop comprises clock genes such as *Clock*, *Per* and *Cry*, and their protein products (King & Takahashi, 2000) that are relevant for human health and disease (Lamont, James, Boivin, & Cermakian, 2007).

Almost every physiological system relies on rhythmic alterations throughout the day. One of those parameters that is characterized by a pronounced circadian rhythmicity is the glucocorticoid cortisol. Cortisol is the final effector of the hypothalamic-pituitary-adrenal (HPA) axis, one of the major physiological response systems (Sapolsky et al., 2000). In humans, the process of morning awakening is associated with a marked increase of cortisol over the first 30 - 45 minutes afterwards: the so called cortisol awakening response (CAR; Pruessner et al., 1997). The CAR as a main feature of the HPA axis has gained increasing interest as parameter having most significance in linking psychosocial factors and physiological functioning (Clow et al., 2004; Fries et al., 2009). Kuehner, Holzhauer, and Huffziger (2007) showed an association between a decreased CAR and self-focused rumination - a cognitive vulnerability marker for depression. It was often demonstrated that the CAR can be influenced by light exposure following awakening (Scheer & Buijs, 1999; Thorn, Hucklebridge, Esgate, Evans, & Clow, 2004). Results of these studies revealed an enhancement of the CAR due to light exposure (Scheer & Buijs, 1999) or dawn simulation (Thorn et al., 2004). The effect of light on HPA functioning is thought to be mediated by the SCN. The SCN receives input from the retina where melanopsin, a photopigment, is present in a subpopulation of retinal ganglion cells which are light sensitive and project directly to the SCN (Berson, 2007; Hankins, Peirson, & Foster, 2008). Via nucleus paraventricularis

[7] I would like to thank Sarah Brand and Gabriele Arnold for their laboratory work. A special thank goes to Anja Gerischer for her contribution of time and energy in recruiting participants and collecting the experimental data.

pathways, the SCN connects with the adrenal cortex (Bear et al., 2006; Buijs & Kalsbeek, 2001; Saper, Lu, Chou, & Gooley, 2005) what provides the anatomical basis for the effects of light exposition on cortisol release.

In addition, the autonomic nervous system (ANS) constitutes a second physiological response system affecting almost every organ. As well, marked circadian rhythmicity has been described for various functions controlled by the ANS, e.g. body core temperature (Weinert & Waterhouse, 2007) and cardiovascular functioning (Guo & Stein, 2003). Furthermore, distinct alterations of different ANS components are reported with sympathetic activity increasing throughout the day and decreasing during night, and parasympathetic activity showing an inverse rhythm (Furlan et al., 1990). It was suggested that these changes will enable the organism to cope with demands while being awake. For heart rate and blood pressure a marked surge in the period soon after awakening has been reported, the so called morning surge (White, 2001). Consistent with the diminished sympathetic activity during the night, plasma catecholamine levels fall during sleep (Rasch, Dodt, Molle, & Born, 2007). This was most pronounced for adrenaline (A) while noradrenaline (NA) showed only slight changes. Furthermore, A but not NA concentrations gradually increase after morning awakening. In contrast, standing up led to a sharp rise in NA levels and only small increases in A (Dodt, Breckling, Derad, Fehm, & Born, 1997). Lesion studies in rats suggested the involvement of the SCN in the regulation of autonomic functions, e.g. the heart (Scheer, Horst, van der Vliet, & Buijs, 2001). In humans, light at night results in an increase of autonomic activity (Scheer, van Doornen, & Buijs, 1999). This study also demonstrated the presence of multisynaptic autonomic connections arising from the SCN and projecting to multiple organs of the body. Buijs and colleagues (2003) demonstrated a functional specialization of preautonomic neurons within the hypothalamus that either project to the sympathetic or the parasympathetic branch of the ANS (Buijs et al., 2003). In contrast to their previous suggestion of a uniform day/night signal to autonomic branches (Scheer & Buijs et al., 1999), the authors now suggest a parasympathetic-sympathetic differentiation of efferent SCN signals to the periphery, by means of the activity of separate autonomic branches driven by different SCN neurons.

In the last few years, evidence is accumulating that the enzyme salivary alpha-amylase (sAA) may serve as a surrogate marker of autonomic and sympathetic functioning (for a review see Nater & Rohleder, 2009). This enzyme is mainly produced by the parotid glands that are innervated by sympathetic and parasympathetic nerve fibers. Using blocking or stimulating pharmacological agents, different studies indicated sAA as a product of an interacting sympathetic and parasympathetic stimulation via central nervous noradrenergic input (Ehlert et al., 2006; Spears et al., 1974). Animal studies demonstrated that rats chronically exposed to constant light showed an increase of sympathetic activity in salivary

glands (Bellavía & Gallará, 2000). Furthermore, there is a diurnal variation in salivary adrenaline with higher levels during darkness (Wurtman & Axelrod, 1966) resulting from an inhibitory effect of light on sympathetic efferent activity innervating salivary glands (Wurtman, Axelrod, Sedvall, & Moore, 1967). In contrast, there is a stimulating effect of light on adrenal catecholamine secretion (Goldstein et al., 1983). Bellavia and Gallar concluded in their review that light-induced alterations of saliva protein secretion are mediated by circulating catecholamines while dark-induced responses are controlled by sympathetic efferents to salivary glands (Bellavia & Gallar, 1998). In humans, resting parotid saliva secretion can also be influenced by light-darkness shifts (Shannon & Suddick, 1973). While there is evidence for a marked circadian rhythmicity of sAA with decreasing values after awakening and increasing values throughout the day in different age groups (Nater et al., 2007; Rohleder et al., 2004a; Rohleder et al., 2008; Strahler et al., 2010a), nothing is known about the impact of sunlight on responses of sAA to awakening in humans. According to the literature summarized above, we would expect that sAA, as a product of an interacting sympathetic and parasympathetic stimulation, is sensitive to light. This might be due to the observation that adrenaline, mainly released by the adrenal medulla, shows a sharp increase after awakening and the suggestion that light induced changes of salivary protein secretion are mediated by circulating catecholamines. Thus, we set out to examine how light influences sAA responses to awakening. Since there is evidence for a sex difference in human circadian phase responses to light (Kripke, Elliott, Youngstedt, & Rex, 2007), we also tested the hypothesis that men and women differ markedly in aspects of sAA morning changes. Furthermore, we tested whether sAA and cortisol responses are associated since awakening is associated with simultaneous changes of both parameters, representing different physiological systems influenced by external stimuli.

10.2. Methods

10.2.1. Participants

Subjects aged 18 to 55 years were recruited via a notice posted on campus of the Technische Universität Dresden, via an advertisement in a local newspaper, and via personal contact. Finally, 22 subjects (6 men, 16 women, mean age: 35.2 ± 12.6 years) fulfilled the inclusion criteria. We included participants with a body mass index (BMI) below 30 kg/m^2 (mean BMI: 23.5 ± 2.3 kg/m²). Heavy smokers (> 5 cigarettes a day) and individuals who reported excessive alcohol consumption (> 3 times a week) as well as individuals under anti-hypertensive medication, asthma medication, anti-rheumatic medication, using psychotropic substances, sleeping pills, or painkillers were excluded. Participants were free of psychiatric and severe somatic diseases as evaluated by interview by one of the authors (J.S.). Overall, two participants reported to smoke < 5 cigarettes a day. Those participants

were asked to abstain from smoking until completion of sampling. Six participating women reported the intake of oral contraceptives (OC). Since data concerning the impact of OC´s on sAA is inconclusive (Rohleder & Nater, 2009) and there is evidence for an impact on cortisol (Fries et al., 2009), this variable was also examined in statistical analyses. Participants were not allowed to drink (anything but water) and eat during sampling, since these factors have been shown to modify the activity of sAA (Rohleder & Nater, 2009).

10.2.2. Study protocol

Subjects were contacted via telephone and a short interview clarified a possible inclusion into this study. After a short introduction, participants who declared their consent to collect saliva samples on two consecutive mornings were asked to either come to our laboratory or an appointment for a home visit was made. At this visit, the procedure of collecting a morning profile of biochemical parameters with the help of saliva samples was shown. After obtaining written informed consent, participants received a package with all the necessary study materials and were asked to return it to our laboratory upon completion. Furthermore, all participants were asked to fill in short questionnaires concerning awakening time, length of sleep, quality of sleep, possible stressful events on the day before sampling, and anticipated stress on the sampling day. The study protocol was approved by the Ethics Committee of the Technische Universität Dresden.

10.2.3. Measures

10.2.3.1. Saliva sampling

To determine salivary alpha-amylase and cortisol levels, two saliva samples were collected immediately after awakening and 30 min afterwards with the help of cotton swabs (Salivettes, Sarstedt, Nümbrecht, Germany) on two consecutive mornings. All subjects were tested in both lighting conditions in a counterbalanced within-subjects design. In condition "*bright*" participants should leave their shutters open and thus waking up with sunlight and take both samples under bright conditions. In condition "*dark*" participants should close their shutters and leave them closed until both samples in the morning were taken. Under both conditions, participants should wake up autonomously without the use of an alarm clock.

To collect saliva, participants were instructed to gently chew on the swab for 0.5 - 1 min. To test for compliance of sampling MEMS6 TrackCap Monitors (Aardex Ltd., Switzerland) were used. Compliance was defined as collecting the first saliva sample within 10min after awakening and the second sample 30 ± 7 min after awakening based on the recommendations by Kudielka and colleagues (2003). Non-compliant samples were excluded from further analyses resulting in the inclusion of n = 22 data sets. Furthermore, participants were asked to collect their morning profiles on working days since there is

evidence for a difference of the CAR between working days and weekend (Kunz-Ebrecht, Kirschbaum, Marmot, & Steptoe, 2004)

10.2.3.2. Biochemical parameters

After thawing, saliva samples were centrifuged at 3000 rpm for 3 min. The concentration of salivary alpha-amylase was measured by a quantitative enzyme kinetic method described elsewhere (Strahler et al., 2010b). Intra- and inter-assay precision expressed as percent coefficient of variation was below 10%. Concentrations of salivary free cortisol were measured using a commercially available chemiluminescence-immuno-assay (CLIA; IBL, Hamburg, Germany) with intra- and inter-assay precision of 2.5% and 4.7%, respectively.

10.2.4. Statistical analyses

Kolmogorov–Smirnov and Levene's test showed normal distribution and homogeneity of variance of raw alpha-amylase and cortisol values. Therefore, absolute amylase and cortisol values were used to calculate the changes of salivary alpha-amylase (amylase awakening response, AAR) and cortisol (cortisol awakening response, CAR) after awakening, indexed as delta score between levels immediately after awakening and levels 30 min after awakening. To test for possible influences of using OC´s repeated measure (rm) ANOVAs were computed to test for time (2 sampling times) by group (OC vs. non-OC) effects on sAA as well as cortisol. Analyses revealed no difference between women taking OC´s and those without according to their amylase profiles on both testing days (all $p > .10$). Same was true for cortisol (all $p > .10$). Thus, all women were included into following analyses.

Using the dependent Student´s t-test for paired samples differences between the conditions with respect to awakening values were examined. With regard to sAA and cortisol morning profiles, rmANOVAs were used to analyze possible time (2 sampling times), condition (bright vs. dark), sex (male vs. female) effects, and their interactions. Univariate ANOVAs were computed for comparisons of both sexes (female vs. male) concerning awakening values and responses of sAA and cortisol (AAR, CAR). Results were corrected by the Greenhouse-Geisser procedure when necessary (Greenhouse & Geisser, 1959; Vasey & Thayer, 1987). Furthermore, there was a significant difference between the testing conditions with respect to awakening time. This variable was therefore included as covariate where appropriate. Partial correlations including the above-mentioned covariate were computed to test for associations between salivary parameters. For significant results we report partial eta squared (η^2) as effect size. For all analyses, the significance level was alpha = 5%. All results shown are the mean ± standard error of mean (SEM).

10.3. Results

10.3.1. Sociodemographics

Main characteristics of the study group are shown in Table 10.1 differentiated between men and women. Six out of 16 female participants took oral contraceptives. One woman and one man reported to smoke cigarettes. Table 10.2 describes sampling related factors on both days showing a difference with regard to awakening time and sleep quantity with participants sleeping longer and waking up earlier in the *dark* condition.

Table 10.1 Characteristics of study participants

Variable	men (n=6) mean (SD*)	women (n=16) mean (SD)	student´s t-test
age (years)	32.2 (6.1)	33.6 (13.7)	p = .806
BMI (kg/m²)	23.7 (1.6)	22.7 (2.4)	p = .368

* SD, standard deviation

Table 10.2 Characteristics of study days

Variable	bright mean (SD[1])	dark mean (SD)	statistics[3]
awakening time (hh:mm)	07:45 (00:49)	06:16 (00:46)	$p_t < .001$
sleep quantity (min)	422 (52)	470 (63)	$p_t = .005$
sleep quality[2]	median: 4	median: 4	$p_Z = .930$

[1] SD, standard deviation
[2] 6-point scale: from *very bad* to *very good*
[3] t = student´s t-test; Z = Wilcoxon matched pairs test

10.3.2. Salivary alpha-amylase

With respect to awakening values, Student´s t-test for paired samples revealed no difference between both conditions (t_{21} = 1.2, p = .244) and univariate ANOVAs revealed no effect of sex (*dark*: $F_{1,20}$ = 0.1, p = .765, η² = .005; *bright*: $F_{1,20}$ = 0.5, p = .501, η² = .023).

Repeated measurements ANOVA revealed significant alpha-amylase changes after awakening in the whole group (time effect: $F_{1.0,19.0}$ = 10.2, p = .005, η² = .348) without a difference between men and women (time by sex: $F_{1.0,19.0}$ = 1.0, p = .331, η² = .050). Furthermore, there was no difference between sAA morning profiles during both conditions (time by condition: $F_{1.0,19.0}$ = 1.9, p = .185, η² = .090; Figure 10.1A), again no interaction with sex was found (time by condition by sex: $F_{1.0,19.0}$ = 0.8, p = .381, η² = .041). Following Student´s t-test for paired samples confirmed these results showing no difference between

the conditions with respect to AAR (t_{20} = -1.1, p = .284). Univariate ANOVAs revealed no significant difference between men and women (*dark*: $F_{1,19}$ = 0.2, p = .638, ŋ² = .012; *bright*: $F_{1,20}$ = 2.0, p = .178, ŋ² = .089). However, descriptively men showed lower responses during the bright condition (Figure 10.1B).

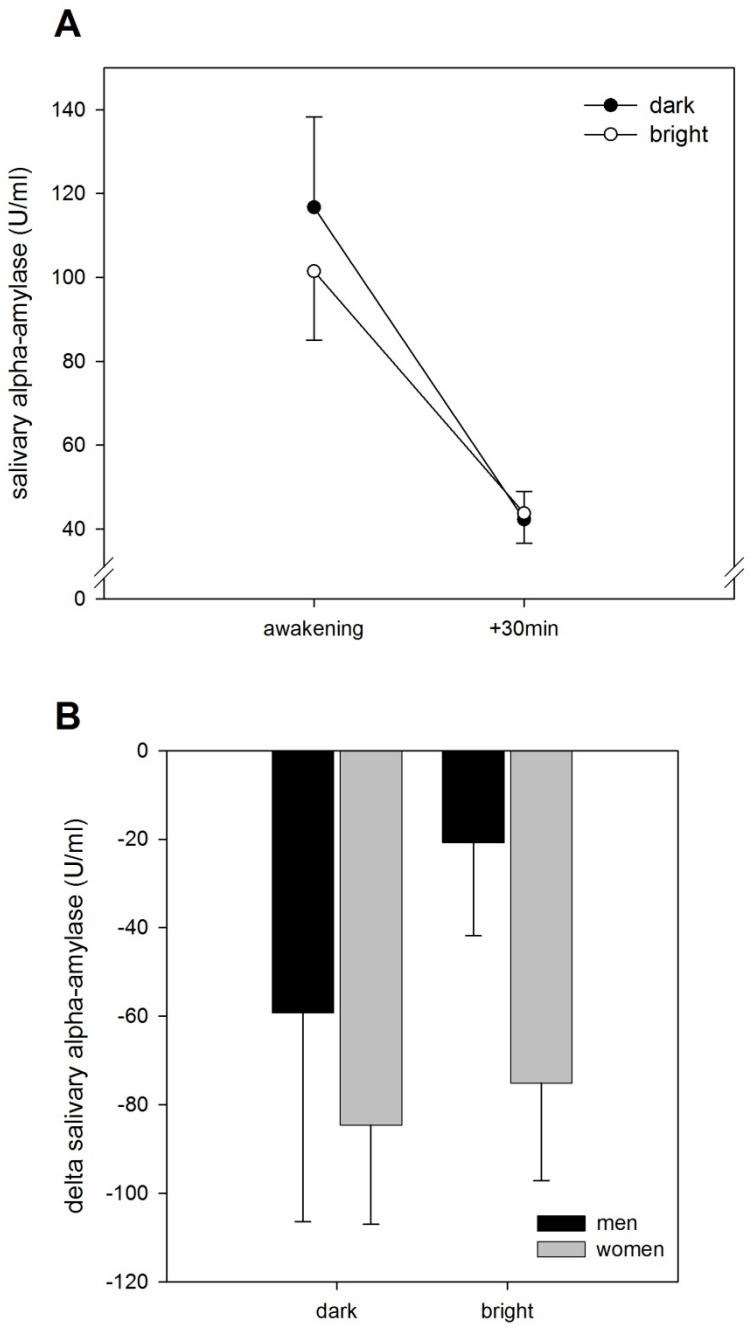

Figure 10.1 (A) Salivary alpha-amylase morning profiles under both lighting conditions, and (B) mean amylase decrease (± standard error) indexed as delta score between awakening and +30 min values according to sex. Graph shows means and standard errors.

10.3.3. Salivary cortisol

With respect to awakening values, Student´s t-test for paired samples revealed no difference between both conditions (t_{21} = -0.2, p = .871) and univariate ANOVAs revealed no effect of sex (*dark*: $F_{1,20}$ = 1.1, p = .313, η² = .051; *bright*: $F_{1,20}$ = 0.5, p = .479, η² = .025). Repeated measurements ANOVA revealed significant cortisol changes after awakening in the whole group (time effect: $F_{1.0,20.0}$ = 19.6, p = < .001, η² = .496) and significant differences between men and women (time by sex: $F_{1.0,20.0}$ = 10.1, p = .005, η² = .335) with women displaying more pronounced changes. Furthermore, there was a significant time by condition interaction ($F_{1.0,20.0}$ = 4.8, p = .040, η² = .195; Figure 10.2A) with cortisol values showing sharper increases when awakening in bright light independent of sex (time by condition by sex: $F_{1.0,20.0}$ = 1.4, p = .248, η² = .066). Student´s t-test for paired samples confirmed these results and revealed larger mean increases during the bright condition (t_{21} = -3.0, p = .006). Univariate ANOVAs showed significant differences between men and women (*dark*: $F_{1,20}$ = 6.1, p = .023, η² = .233; *bright*: $F_{1,20}$ = 8.5, p = .008, η² = .299) with women showing the well known increase of cortisol after awakening during both conditions and men showing only slight changes (Figure 10.2B).

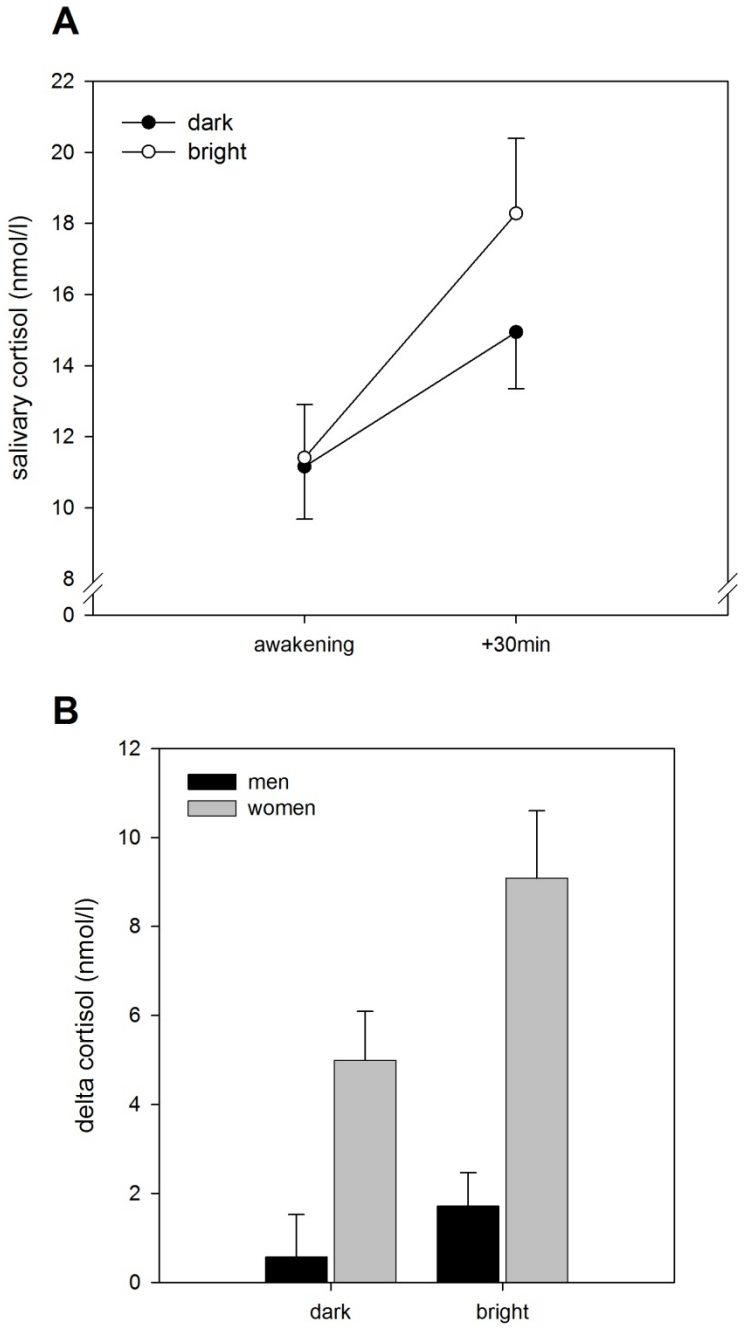

Figure 10.2 (A) Salivary cortisol morning profiles under both lighting conditions, and (B) mean cortisol increase (± standard error) indexed as delta score between awakening and +30 min values according to sex. Graph shows means and standard errors.

10.3.4. Associations between biochemical parameters

Partial correlations including awakening time as covariate revealed a significant correlation between AAR and CAR values during the *dark* condition (r = -0.524, p = .018) and by trend during the *bright* condition (r = -0.396, p = .076). These results indicate sharper decreases of sAA in the morning being associated with more pronounced increases of cortisol.

10.4. Discussion

To our knowledge, this is the first study assessing sAA responses to awakening under different sunlight conditions. In line with earlier findings, we found significant changes of sAA as well as cortisol in the first 30 minutes after awakening with decreasing sAA values (Nater et al., 2007, Rohleder et al., 2004a, 2008; Strahler et al., 2010a) and increasing cortisol values (Pruessner et al., 1997, Wüst et al., 2000). In contrast to our hypothesis, analyses revealed neither a difference between the *dark* and the *bright* condition nor a difference between men and women with regard to sAA awakening responses. Important to note is that men descriptively showed a difference between the conditions with much lower decreases when waking up with sunlight. With regard to the response of cortisol to awakening we found larger mean increases when participants had to wake up with leaving their shutters open. In addition, women showed much higher CAR values, independent of condition. Furthermore, there was no effect of using OC´s on the morning profiles of sAA as well as cortisol. In contrast to previous results (Nater et al., 2007; Strahler et al., 2010a), a significant correlation between sAA and cortisol awakening responses was found. This finding suggests not only parallels of temporal awakening response patterns but may also indicate a functional relationship. An activating role has been hypothesized for the CAR, providing the organism with energy to cope with upcoming demands of diurnal activity (Adam, Hawkley, Kudielka, & Cacioppo, 2006; Pruessner et al., 1997). Consistent with this notion are findings of a positive association between the CAR and state arousal (Stalder, Evans, Hucklebridge, & Clow, 2010; Thorn et al., 2004). Furthermore, in vivo studies could show that glucocorticoids are able to phase shift circadian rhythms in cells and tissue (Balsalobre et al., 2000) and are able to oppose resetting actions of other cues, e.g. food intake (Le Minh, Damiola, Tronche, Schütz, & Schibler, 2001). These results indicate a mechanism for a possible relationship between cortisol and sAA and are in line with the hypothesis that the HPA axis and the ANS buffer one another as a negative feedback loop (Nicolson, 2007). Similarly, there is a shift in sympatho-vagal balance towards increased sympathetic activity in the morning (Dodt et al., 1997; Furlan et al., 1990). Dodt and colleagues (1997) found a decline in plasma NA levels on waking while A levels increased. Furthermore, Hansen and colleagues (2001) reported a morning dip of urinary NA (Hansen, Garde, Skovgaard, & Christensen, 2001). Given the fact that circadian rhythmicity of autonomic balance is mainly a product of parasympathetic activity (Scheer, van Doornen, & Buijy, 2004), it might be hypothesized that sAA changes after awakening reflect this shift in autonomic balance. This possibility is yet to be investigated in pharmacological blocking studies, targeting the parasympathetic system. However, since sAA secretion is mainly mediated via sympathetic efferences (Witt, 2006), this hypothesis is not that convincing. Another interesting hypothesis arises from the finding of increasing concentrations of metabolic parameters insulin and glucose around the time of

awakening (Shea, Hilton, Orlova, Ayers, & Mantzoros, 2005) that are also influenced by light (Kalsbeek et al., 2008). Furthermore, Harthoorn (2008) found systematically increasing sAA activity upon food intake and satiation suggesting this salivary enzyme as an objective measure of satiety. This implies a possible association between diurnal sAA activity and metabolic functioning and it might be hypothesized that sAA alterations in the first 30 minutes after awakening reflect increases of hunger. This hypothesis needs to be examined.

Our result showing no difference between both lighting conditions is in contrast to our hypothesis that sAA might be sensitive to light due to the suggestion that light induced changes of salivary protein secretion are mediated by circulating catecholamines (Bellavia & Gallar, 1998). This result might therefore highlight the relevance of peripheral circadian rhythms independent of the "master oscillator" SCN. Vollrath (2002) provides a review of those independent rhythms, e.g. islets of Langerhans producing insulin or cells of the anterior pituitary producing luteinizing hormone. Furthermore, there is evidence for so called clock-genes and clock-proteins that may also be active in a circadian manner in peripheral tissue (Balsalobre, Damiola, & Schibler, 1998). Animal studies revealed the rhythmic expression of clock genes and Amylase 1 Gene in salivary glands of rats, influenced by feeding (Furukawa et al., 2005). In 2001, Bjarnasen and colleagues were the first who reported clock gene expression across the 24-h cycle in human oral mucosa (Bjarnasen et al., 2001). Traditionally, these cycles were seen as passively driven by efferent SCN signals. More modern concepts favor the view of a hierarchical circadian axis with autonomous cellular oscillators being synchronized by SCN-dependent cues (Hastings, O´Neill, & Maywood, 2007). Furthermore, diurnal variation of amylase levels is coupled with density of parotid beta-adrenoceptors in rats (Ishikawa, Amano, Chen, & Ishida, 1992). This might be controlled at the level of gene expression since the diurnal variation of amylase coincides with alterations of the phosphorylation of parotid nuclear non-histone proteins (Ishikawa et al., 1992). In sum, recent evidence suggest that instead of "master" (SCN) and "slave" (peripheral tissue), peripheral oscillators may act more like integrators of multiple different time cues (e.g. light, food intake).

Our finding of descriptively lower sAA decreases when waking up with sunlight in men is in line with previous preliminary evidence for sex differences in human circadian phase responses to light (Kripke et al., 2007). However, since only 6 male adults were included into our study, no further conclusions could be drawn. Higher cortisol awakening responses when waking up with sunlight are in line with previous findings concerning a stimulating effect of light on cortisol secretion (Cermakian & Bovin, 2009; Scheer & Buijs, 1999; Thorn et al., 2004). Circadian cortisol may thus be described as a central clock driven rhythm (Cermakian & Bovin, 2009). Furthermore, the result of higher CAR values in women is in line with previous results (Pruessner et al., 1997, Wüst et al., 2000; Wright & Steptoe, 2005; Kunz-

Ebrecht et al., 2004). In contrast to former reports of a smaller CAR in women using OC´s (Pruessner et al., 1997; Pruessner, Hellhammer, & Kirschbaum, 1999), this study could not find an effect of OC´s on morning profiles of cortisol. However, the effect size for OC´s on the CAR was very small in those studies, explaining about 4% of variability. Thus, the effect of OC´s seems to be too small to be meaningful.

Several important limitations of our study need to be mentioned. The most important limitations are the relative small sample size and an unbalanced gender distribution where over- or underestimated results may be reported. Furthermore, including also men and women above the age of 50 years have to be discussed. Just recently we could show that older men displayed damped diurnal sAA profiles compared to younger men and older women (Strahler et al., 2010a). Aging effects on cortisol morning profiles are not entirely clear. Whereas it seems that age does not have a strong impact with data having led to mixed results (for a review see Fries et al., 2009), longitudinal studies in older adults showed a substantial interindividual variety. Thus, possible effects of postmenopausal status can not be ruled out. However, these factors should not have affected our results since all subjects were exposed to every condition in a counterbalanced within-subjects experimental design. Another aspect to be discussed is the use of salivettes and that participants were instructed to gently chew on the cotton rolls. DeCaro (2008) argued that actively chewing might change sAA activity. However, Rohleder and colleagues (2006) could show that sAA activity was independent of salivary flow rate in a psychosocial stress paradigm. So far, no study has been conducted on the impact of salivary flow rate on diurnal profiles of sAA activity. However, no significant changes of flow rates over the course of a day could be found for stimulated whole saliva (Rantonen & Meurman, 2000). We therefore instructed all participants to chew up to one minute or until cotton rolls are saturated with saliva to minimize possible interindividual alterations. Given the overall natural setting of the study design, there was no objective control of light intensity, measured e.g. in lux meters illuminance, what might have influenced the results. In 1996, Boivin and colleagues reported light-induced phase shifts of the circadian rhythm of plasma cortisol in an intensity-dependent manner (Boivin, Duffy, Kronauer, & Czeisler, 1996). They could also show that even normal indoor room light (~50 - 300 lux) was able to advance the circadian cortisol rhythms. Thus, it could be concluded that our design was reliable enough to assess the impact of light on salivary parameters. Another limitation might be that there was no assessment of chronotype what might have influenced our results. Thus, we can not exclude possible effects of extremely early or extremely late chronotypes within our sample.

10.5. Conclusion

The aim of this study was to illustrate changes of endocrine and autonomic basal activity after awakening related to the impact of sunlight. The findings regarding cortisol are consistent with most previous reports showing cortisol rhythmicity being sensitive to sunlight. This effect seems to be mediated via efferences from the suprachiasmatic nucleus that receives photic input from retinal glands via the retinohypothalamic pathway and thereby regulates endogenous circadian rhythms. Furthermore, multisynaptic autonomic connections arise from the SCN projecting to multiple organs of the body. However, we could not find an effect of sunlight on sAA morning profiles. Since there is evidence for circadian clock gene expression in human oral mucosa (Bjarnasen et al., 2001), one might conclude that with regard to sAA peripheral oscillators may act more like integrators of multiple different time cues, e.g. light, food intake, instead of a “master” oscillator (SCN). Proper functioning of this integration is of great interest since disturbances in the normal relationship between light cycle and food intake can predispose to disease as seen e.g. in shift workers (Knutsson, 2003).

11. General discussion

The aim of this thesis was to advance our knowledge of confounders of alpha-amylase activity in saliva under basal and acute stress conditions and to further strengthen the validity of this enzyme as an easy obtainable alternative parameter for adrenergic activity testing. Although this assumption was already made in the mid seventies, scientific verification not began until the mid nineties. Since then, more and more empirical evidence is accumulating for this putative role. A number of studies have found pronounced changes of sAA in response to physiological and psychological stressful stimuli. Furthermore, a pronounced circadian rhythmicity was shown. Given the fact that the integration of sAA into developmental and aging research is a relative recent phenomenon, there are several gaps of knowledge that need to be to be addressed before a final conclusion could be drawn. Therefore, we conducted a series of studies incorporating these considerations regarding behavioral correlates of inter- and intraindividual differences in sAA activity with a special emphasis on older adults. In this chapter, findings from four studies presented within this thesis are summarized. After an integration of main findings and the discussion of the biological meaning and possible methodological consequences, the last part of this chapter will provide a reflection of possible applications.

11.1. Summary of the results

Within four studies we investigated possible confounders of alpha-amylase activity in a psychosocial stress paradigm and under basal condition in order to examine to what extent sAA could serve as surrogate marker of adrenergic activity in different scientific research areas. Special attention has been paid on the inclusion of elderly subjects, an age group that has been neglected until now. Results therefore might be of particular interest for the increasingly important research area of aging.

11.1.1. Salivary alpha-amylase stress reactivity across different age groups

In the first study, we set out to investigate sAA responses to acute psychosocial stress, i.e. the Trier Social Stress Test, in three age groups representing relevant stages of human development - healthy children, young adults, and older adults of both sexes. Our goal was to test for associations of sAA with more established stress system markers, i.e., salivary cortisol as outcome measurement of HPA reactivity, HR and HRV as markers for autonomic reactivity, and to directly compare these responses between different age groups across the life span.

Secretion of sAA and cortisol was repeatedly assessed in 62 children, 78 young adults, and 74 older adults after exposure to the Trier Social Stress Test. Additionally, cardiovascular activity was measured in both adult groups. Older adults showed attenuated sAA, HR, and HRV responses. Furthermore, we found higher sAA but lower cortisol at baseline as well as lower sAA and cortisol responses in children. Age by sex interactions were observed only for cortisol with higher responses in older male participants. No associations between the parameters were found.

To the best of our knowledge, this is the first study to directly compare children, young adults, and older adults with regard to their physiological response to a standardized psychosocial stressor. Results in children and young adults confirm previous results. Overall, findings implicate sAA as an alternative or additional sympathetic stress marker throughout the life span, with marked and rapid responsiveness to stress in three relevant age groups.

11.1.2. Aging diurnal rhythms and chronic stress: Distinct alteration of diurnal rhythmicity of salivary alpha-amylase and cortisol

In the second study we assessed diurnal profiles of sAA and salivary cortisol in competitive ballroom dancers as well as age- and sex-matched controls to investigate age-related changes of basal activity and potential chronic psychosocial stress-related alterations. Theoretically, repeated, non-habituating responses to social-evaluative conditions, which characterize the lives of competitive ballroom dancers, should be associated with stress system dysregulations. We expected to see an increased sympathetic drive associated higher overall alpha-amylase activity in older adults. According to the Allostatic Load concept of a cumulative wear and tear of the body and a hypothesized physiological accumulation of the effects of stress and age, this should be especially pronounced in older dancers. Salivary cortisol profiles were obtained from the same samples as amylase for a comparison of the effects of chronic stress and age on diurnal profiles and the interaction of both stress-sensitive systems.

Twenty-seven younger dancers, 26 younger controls, 31 older dancers, and 33 older controls collected five saliva samples throughout the day. Daily overall output of sAA was

elevated in older adults while there was no effect of age on mean cortisol levels. Alterations of diurnal rhythms were only seen in younger male dancers showing a flattened diurnal profile of sAA and younger dancers and female older dancers showing a blunted diurnal rhythmicity of cortisol. Furthermore, we found a negative correlation between summary indices of basal sAA and the amount of physical activity.

In conclusion, higher overall output of sAA in older adults is in line with the phenomenon of a “sympathetic overdrive” with increasing age. Furthermore, a lower output of sAA in people who are more physically active is in line with the hypothesis of an exercise-induced decrease of sympathetic activity.

11.1.3. Impact of blood pressure and antihypertensive drugs on diurnal alpha-amylase activity: A novel marker of sympathetic drive

The goal of the third study was to evaluate the impact of adrenergic active substances, i.e. antihypertensive drugs (AD), on diurnal sAA activity. Furthermore, nothing is known about differences between normotensive and hypertensive older adults. Since chronic administration of AD and high blood pressure are both factors often present in older adults, investigating their confounding effects on diurnal sAA is of great interest.

To determine sAA basal rhythms, five saliva samples were collected immediately after awakening, 30 minutes after awakening, 11am, 3pm, and 8pm in 79 older adults (33 normotensive adults, 16 medicated vs. 45 hypertensive adults, 34 medicated). Results showed a pronounced rhythm of sAA in all groups. Diurnal profiles differed significantly between men and women with men lacking the typical decrease of sAA in the morning and showing more pronounced alterations throughout the day. We found an effect of AD on sAA profiles with subjects not using antihypertensive drugs showing a heightened diurnal profile. Descriptively, this was also true for hypertensive older adults. The main effect of AD was also shown for area under the curve (AUC) values, indicating a higher total output of sAA in unmedicated adults. Hypertensive subjects and those not using AD showed the highest diurnal output of sAA and the steepest slope.

These data indicate an impact of antihypertensive medication on characteristics of diurnal sAA activity and a difference between normotensive and hypertensive subjects. We therefore suggest to carefully control for blood pressure and use of adrenergic active drugs, i.e. antihypertensives, in studies investigating sAA rhythms. Findings highlight the usefulness of sAA as an index of adrenergic activity even in specific pathological states.

11.1.4. Light affects salivary morning cortisol, but not salivary alpha-amylase

The aim of the fourth study was to illustrate changes of endocrine and autonomic basal activity after awakening related to the impact of sunlight, i.e. waking up in the dark versus waking up with daylight.

Data were obtained from six men and 16 women aged 18 to 55 years who were tested in both lighting conditions in a counterbalanced within-subjects design. Saliva samples were collected immediately after waking up and 30 minutes later on two consecutive days (*dark* = shutters closed versus *bright* = shutters open). Compliance with the sampling protocol was assured with the help of electronic monitoring devices. Data on sleep quantity, time of awakening, and mood were also obtained. The cortisol awakening response differed between *dark* and *bright* day, with higher responses during the *bright* condition. On either day, women showed larger cortisol increases than men. We found no effect of sunlight or gender on sAA awakening responses. Furthermore, a significant correlation between sAA and cortisol awakening responses appeared what might not only be discussed as a parallel of temporal awakening response patterns but may also hint to a functional relationship, i.e. providing energy.

In line with previous reports, cortisol rhythmicity seems to be sensitive to sunlight mediated via efferences from the suprachiasmatic nucleus that receives photic input from retinal glands via the retinohypothalamic pathway. Despite multisynaptic autonomic connections arising from the SCN projecting to multiple organs of the body, we could not find an effect of sunlight on sAA morning profiles. Evidence for circadian clock gene expression in human oral mucosa might indicate that peripheral oscillators may act more like integrators of multiple different time cues, e.g. light, food intake, instead of a “master” oscillator (SCN).

11.2. Integration of main findings

This thesis investigated sAA as surrogate marker of adrenergic activity and paid special attention on the inclusion of elderly subjects, an age group that has been neglected until now. Study 1 (“*Salivary alpha-amylase stress reactivity across different age groups”*) was conducted in order to assess the usefulness of sAA for investigating acute stress responses throughout the life span. Findings of age- and sex-related changes of stress reactivity might be important determinants of health and disease. Long-term studies are now needed to examine whether these changes increase vulnerability to disease and are associated with disease prevalence-patterns. In addition, the process of aging is known to not only have an impact on physiological stress responses but also on circadian rhythmicity of different body systems. Distinct and stable pattern of dysregulations have been shown to be associated with a variety of mental and physical disorders (Koch et al., 2009; Tan et al., 2007; Wessa et

al., 2006). Thus, Study 2 ("*Aging diurnal rhythms and chronic stress: Distinct alteration of diurnal rhythmicity of salivary alpha-amylase and cortisol*") was designed to assess full diurnal profiles in elderly individuals and to investigate cumulative effects of repeated or chronic stress on sAA rhythmicity. Findings were in contrast to our hypothesis of a cumulative wear and tear (*Allostatic Load*) through repeated/chronic psychological (ba*llroom dancing*) and biological (*aging*) stressors, but rather highlight the importance of regular physical activity and social relationships in older ages. To the best of our knowledge, this was also the first study showing the well known age-associated "sympathetic overdrive" in a saliva-based biomarker. One problem when integrating sAA into developmental and aging research is the use of adrenergic agonists and antagonists due to their impact on adrenoceptors-mediated protein secretion (Busch & Borda, 2002; Garrett, 1987; Proctor & Carpenter, 2007). Furthermore, tonic sympathetic overactivity that occurs with normal aging is associated with higher blood pressure (Grisk & Rettig, 2004). We therefore conducted Study 3 ("*Impact of blood pressure and antihypertensive drugs on diurnal alpha-amylase activity: A novel marker of sympathetic drive*") on hypertensive and normotensive subjects that either used antihypertensive drugs or were not on medication to determine differences in diurnal sAA rhythms. Overall, our findings showed an altered diurnal profile and higher salivary alpha-amylase output in hypertensive subjects and subjects not being on antihypertensive medication indicating higher sympathetic activity in these groups. This was especially pronounced in male participants what might hint to a possible mechanism of an increased susceptibility to cardiovascular events in older men. These results strengthen the validity of sAA as a sympathetic marker as it was shown to represent sympathetic activity in a specific pathological state, i.e. hypertension. Hence, findings are of particular interest in research using sAA as a prognostic indicator of pathological processes. Hypertension was also shown to be associated with substantial changes of transmitters within the suprachiasmatic nucleus (SCN) - the "biological clock" (Goncharuk et al., 2001), and an altered output from the SCN to the sympathetic nervous system (Goncharuk et al., 2002). Since there is also evidence for a relationship between chronotype and amylase activity (Nater, 2004), it is of great interest to investigate the impact of sunlight, one of the major *zeitgeber* of circadian endogenous rhythms and mediated via the SCN, on sAA rhythmicity. In Study 4 ("*Light affects morning salivary cortisol, but not salivary alpha-amylase*") we therefore investigated subject´s morning profiles on two consecutive days with leaving their shutters closed on the one day and open their shutters on the other day. In this natural setting, we replicated earlier findings of light-induced changes of salivary cortisol but could not find an effect of sunlight on morning sAA. There is evidence for circadian clock gene expression in human oral mucosa (Bjarnasen et al., 2001). This points towards the importance of peripheral oscillators in the integration of different time cues. Disturbances in

the normal relationship between time cues (light cycle and food intake) weaken proper functioning of this integration and might therefore lead to an increased susceptibility to disease as seen in shift workers (Knutsson, 2003).

In sum, our studies provided clear evidence that sAA is heightened in states of autonomic arousal, i.e. stress, aging and hypertension, and that its circadian rhythm seems to be regulated rather integrative than directly via efferent input from hypothalamic SCN neurons.

11.3. Stress-induced amylase activity, basal rhythm, and its biological meaning

Human salivary alpha-amylase is fundamental for the oral processing of food and for a healthy oral environment. Within a food related context sAA is a calcium-containing metalloenzyme and can therefore be regulated via pH value. Thus, the salivary enzyme sAA is, in the absence of calcium, completely unable to function. Amylase hydrolyzes the alpha-1,4 linkages of starch to sugar molecules glucose and maltose. Furthermore, sAA binds to oral streptococci and might therefore contribute to bacterial clearance (Scannapieco et al., 1993). Noradrenaline released from sympathetic nerve terminals binds to alpha- and beta-adrenoceptors on the acinar cell. This leads to an elevation of intracellular calcium (alpha-adrenoceptor mediated) and cAMP levels (beta-adrenoceptor mediated). The latter is linked to the secretion of salivary proteins from membrane-bound secretory granules (Castle & Castle, 1998). The two branches of the ANS work together in a harmonious manner to evoke saliva secretion (Proctor & Carpenter, 2007). While sympathetic input mainly stimulates protein secretion, the PNS stimulates saliva flow. Therefore, sAA activity has been proposed to be mainly regulated via central sympathetic efferences (Chatterton et al., 1996; Ehlert et al., 2006; van Stegeren et al., 2006). However, there is only preliminary and little evidence that clarifies if sympathetic or parasympathetic nerve fibers are predominant in increasing sAA during psychological stress. While Nater and colleagues (2006) reported a positive association between sAA and HRV parameters as makers of sympathetic tone during stress, we could not find such a relation (Study 1). Under psychological stress there seems to be a predominant SNS together with parasympathetic withdrawal in the secretion of sAA (Nater & Rohleder, 2009).

In addition, van Stegeren and colleagues (2006) concluded that sAA is a more sensitive indicator for sympathetic drive than e.g. heart rate. In line, our results therefore suggest that high diurnal levels of sAA activity in older adults (Study 2) reflect more strongly increased sympathetic than parasympathetic drive. This withdrawal of parasympathetic function might indicate a loss of vagal efferent activity, possibly due to disturbances of the cortical-subcortical pathways modulating ANS activity. A physiological state that seems to be related to these processes is hypertension. Since human hypertension is characterized by

downregulation of alpha-adrenoceptors and impairment in neuronal reuptake of NA from sympathetic nerve terminals (Michel et al., 1990; Esler, 1997; Grassi & Esler, 1999), higher sAA levels found in hypertensive subjects (Study 3) might reflect an increase in peripheral sympathetic tone. Given the assumption that differences might be more likely observed during challenge, future studies should examine whether there is also abnormal sAA responsiveness to stressful stimuli in subjects suffering from hypertension. To assess functioning and integrity of the ANS is of major interest to scientists and investigators, especially in the elderly.

Moreover, there is evidence that parasympathetic drive is the main cause for circadian rhythmicity of autonomic balance (Scheer et al., 2004). Thus, it might be hypothesized that the endogenous rhythm in sAA is mainly caused by a circadian rhythm in parasympathetic activity and that sAA changes after awakening reflect shifts in autonomic balance, what needs to be examined in future studies. Furthermore, there is preliminary evidence that the assumption of counter-phased diurnal profiles of sAA and cortisol might be revised. There is preliminary evidence that the two rhythms are not precisely counter-phased. Figueiro and Rea (in press) demonstrated that sAA peaks in the middle of the day. Furthermore, sAA was lowest during the middle of the night what led the authors conclude that sAA is more counter-phased with melatonin than cortisol. However, they also found that sAA varies with a symmetric, cosine-like waveform over the 24-hour-day what therefore questions the relation to melatonin. Furthermore, our studies provided first evidence for an age-associated change in the dynamic of diurnal rhythmicity with older men lacking the typical decrease of sAA that was repeatedly shown for younger adults (Nater et al., 2007; Rohleder et al., 2004a, 2008). This finding might be interpreted as an altered autonomic balance during the aging process which was most pronounced in male participants. Disturbances in circadian timing are already recognized as a relevant factor in the genesis and progress of pathological states. Hence, more studies are needed to examine a possible relation between altered sAA rhythmicity/ heightened sAA activity and disease as well as detrimental effects of chronic elevated concentrations, in terms of Allostatic Load (McEwen, 1998, 2000), on e.g. the oral cavity.

One of the questions that can not be answered by any of the studies presented within this thesis concerns the biological meaning of those rapid increases and decreases consistently shown during acute stress on the one hand (Davis & Granger, 2009; Filaire et al., 2009; Gordis et al., 2006, 2008; Nater et al., 2005, 2006; Nierop et al., 2006; Räikkönen et al., 2010; Rohleder et al., 2004a, 2006, 2008; Schoofs et al., 2008; Stroud et al., 2009; Takai et al., 2007; van Stegeren et al., 2008) and the pronounced daily rhythm on the other. According to this enzyme´s anti-bacterial and digestive action, short term changes might not have a biological meaning itself but rather reflect just a small part of multiple coordinated

body responses to stressful stimuli. As already mentioned, diurnal profiles of sAA might reflect circadian changes in autonomic balance. So far, no study has looked at a possible relationship between sAA morning changes and other autonomic markers such as HR, HRV, skin conductance, or plasma (nor)adrenaline. Indeed, endogenous rhythms of all principal body functions such as cardiovascular activity, renal filtration, and the mobilization of nutrient are coordinated via endocrine signals and the ANS (Buijs & Kalsbeek, 2001). Circadian rhythms are of great advantage since they enable individuals to anticipate. This pre-adaptation enables the individual to cope with upcoming demands and challenges (Hastings, Reddy, & Maywood, 2003). Since the discovery of clock gene expression in peripheral tissue, endogenous cycles are no longer seen as passively driven by efferent SCN signals but rather as autonomous cellular oscillators being synchronized by the SCN (Hasting et al., 2007). Important to note is our finding of a relationship between sAA and salivary cortisol in Study 4. This result strengthens the relevance of glucocorticoids that were shown to be able to phase shift circadian rhythms in cells and tissue (Balsalobre et al., 2000) and to oppose actions of other cues (Le Minh et al., 2001). Within a food-related context, sAA seems to be associated with the state of satiety in humans. Harthoorn and colleagues (2008) found sAA systematically increasing during food consumption and with the subjective state of satiety. Thus, decreasing levels of sAA in the morning might consequently reflect increases of feeling hungry. In line with this assumption, leptin, known to inhibit appetite, is also present in human salivary glands (De Matteis, Puxeddu, Riva, & Cinti, 2002) and shows a pronounced circadian rhythm with a mid-morning nadir (Ribero, Busnello, Wong, & Licínio, 2006). In this way, alpha-amylase is co-ordinated in time with other satiety-associated measures and matched to the demands of a daily rest/activity cycle. Consequently, autonomic activity might be an important satiety system that helps to regulate food intake behavior and energy expenditure. So far, much more research is needed to identify underlying physiological mechanisms of circadian sAA rhythmicity.

11.4. Methodological consequences

As discussed above, there are multiple confounders of salivary alpha-amylase activity. It is therefore important to consider these interindividual differences that might influence variation within studies. Therefore, this paragraph provides a discussion of possible methodological consequences of the findings presented within this thesis.

11.4.1. Circadian variation

As soon as in the early 1970´s it was shown that salivary flow rate differs throughout the day. Same was true for salivary electrolytes and proteins (Dawes & Chebib, 1972). Previous data as well as data presented here provide clear evidence for a well pronounced daily

rhythm of sAA activity. Therefore, investigators should follow the practice of collecting saliva samples only during a defined time period when applying sAA in acute stress paradigms.

11.4.2. Longitudinal variation

Intra-individual variation is of great importance when investigating subjects cross-sectionally. A number of studies suggest that salivary protein concentrations tend to remain stable and strong correlations over time have been reported (Jenzano et al., 1987; Rudney, Krig, & Neuvar, 1993). In contrast, Keller and El-Sheikh (2009) could not find any association and therefore stability of sAA in children who were examined when being in their 3rd grade and two years later. However, results must be carefully considered since basal sAA activity was assessed only once on each assessment time point in this study. Just recently, especially overall diurnal output and daily slopes were shown to be of high stability within a two year sampling period in young women (Rohleder, Jungmann, Kirschbaum, & Miller, 2010). Evidence thus suggests that sAA tends to remain stable over at least two years in young female adults.

11.4.3. Short-term variation and stability

While longitudinal data suggests only limited variability of sAA, short-term changes might occur for multiple reasons (for reviews see Rohleder & Nater, 2009 and chapter 5). In sum, acute caffeine intake and tobacco smoking, eating and drinking (except water), and physical exercise should be avoided prior sampling. Another conclusion of significance in this context is that it would be useful to implement a higher sampling rate to reveal and better understand the impact of those factors, particularly on diurnal profiles. The use of oral contraceptives seems to have no impact on either stress-induced sAA activity (Schoofs et al., 2008) or basal diurnal rhythmicity (Study 2 and 4). In contrast, pregnancy had a profound effect on sAA responses to psychosocial stress (Nierop et al., 2006). So far, there are no studies examining variations of sAA activity over the course of the menstrual cycle. For this reason, future studies should control for menstrual cycle and possible effects of variation in sex hormones.

Furthermore, sAA was shown to directly response to oral inflammatory diseases (Da Rós Gonçalves et al., 2010; Henskens et al., 1996). Altered protein secretion has also been shown in respiratory infection (Cockle & Harkness, 1983). Additionally, systemic diseases seem to affect salivary glands (see 5.3.5.7.). To the best of our knowledge, there are no studies investigating sAA activity in response to acute systemic diseases, e.g. common cold. Studies should therefore control for acute disease states.

Moreover, previous studies support the idea that protein concentration in saliva is at least partially under genetic control (Rudney, Michalowicz, Krig, Kane, & Pihlstrom, 1994). Amylase gene copy numbers were positively correlated with sAA levels in such a way that

populations characterized by high-starch dietary habits had more gene copies (Perry et al., 2007). Possible genetic variations should be considered when investigating subjects of different ethnical backgrounds.

11.4.4. Long-term change

Not only with regard to its antibacterial activity it is of great interest whether salivary alpha-amylase changes with age. As described in detail in chapter 5, basal sAA activity is pretty low in newborns and continually increases reaching adult levels in older toddlers. Afterwards, sAA does not substantially change over the life span and remains stable even in older adulthood. However, methodological concerns weaken this conclusion since in most studies cited only single time-point measures were applied. Hence, one of the studies presented here was conducted on this issue and revealed substantial differences between young and older adults with much higher amylase levels in older participants (Study 2). With regard to acute stress-induced sAA activity, previous studies reported low or absent responses in newborns, significant responses in children that reach adult levels in adolescence. Our results (Study 1) provided first evidence for acute stress responses above a wider age range with older adults showing attenuated responses.

In addition, medication may be another source of altered salivary functioning not only in older ages. Drugs that may have an impact due to their alpha- and beta-adrenergic-mediated diminishing effect on salivary flow rate and protein composition are anticholinergics, diuretics and antihypertensive agents, and psychopharmaceutics (Parvinen et al., 1984). With regard to sAA activity this seems to be especially true for nonselective antidepressive drugs (von Knorring & Mörnstad, 1986), antipsychotics (Inagaki et al., 2010), and antihypertensive drugs (Study 3), and, to a lesser degree, selective serotonin reuptake inhibitors and benzodiazepines (de Almeida et al., 2008). Another contributor to long-term changes of sAA is systemic disease itself as already discussed (5.3.5.7.). Therefore, future studies should carefully control for or even exclude the use of adrenergic-active substances as well as specific disease states.

11.5. Outlook

As already noted by Goldstein (2003) and Schulkin (2004) ideas and research about stress must go beyond the investigation of only one effector system and only one examined variable such as the HPA axis and cortisol. Therefore, salivary alpha-amylase comes with the potential to be incorporated into multisystem approaches investigating multiple homeostatic systems that are regulated in parallel. In the past two decades, knowledge about the usefulness of sAA as a marker of sympathetic activity under acute stress conditions has accumulated and supports this idea. As well, research regarding possible confounders and behavioral concomitants is starting and provides insights into possible areas of application. Taking the next step, future studies will have to focus on the integration of sAA assessment into longitudinal studies and different disease states to prove its applicability as a marker of sympathetic neural functioning in the genesis and prognosis of disease.

One of those new areas is the use of sAA activity as an alternative and more objective measure of satiety, instead of self-reported subjective ratings (Haarthorn et al., 2008). Hence, sAA might be applied in studies investigating different types of food and diet products, nutritional factors and eating disorders in particular. Another promising finding is a positive association between sAA activity and psychiatric symptoms in schizophrenia (Inagaki et al., 2010). This suggests that elevated sAA responses may be an indicator of psychiatric symptoms. Clearly, more studies in a variety of clinical conditions are needed to exame sAA and its relationship to underlying mechanisms of interindividual variability.

It was often assumed that differences between individuals might be more likely observed under challenging conditions and that these responses are associated with a wide variety of disorders (for a review see Otte et al., 2005). However, there are no long-term studies looking at this relation between stress reactivity and pathology. With regard to sAA, preliminary findings support another view. Hill-Soderlund and colleagues (2008) could show that overall sAA level rather than sAA reactivity is differential correlated with behavior encompassed in attachment status in the strange situation paradigm. Keller and El-Sheikh (2008) reported a u-shaped relation between basal sAA and externalizing symptoms with both low and high sAA levels predicting more symptoms over the course of two years. In sum, individual differences in sAA are associated with children´s behavior and affective states what strengthens the usefulness of sAA in developmental research.

The existence of temporal dynamics and their possible underlying mechanisms, such as control via cycles of gene expression or hormonal input synchronizing these dynamics, raises a new perspective on the relation between temporal disorganization and disease. In a healthy organism, body functions follow coherent daily cycles within and between systems. Pre-existing pathologies or genetic vulnerabilities might therefore aggravate proper daily

variation and disturb internal integrity. This disruption can have detrimental effects on health, as e.g. seen in poor sleep patterns (shift work) being associated with metabolic and mental disease states (Knutsson et al., 2003; Van Cauter et al., 2007) or cancer (Schernhammer, Kroenke, Laden, & Hankinson, 2006). Investigating the mechanisms how disruption of circadian rhythms can underlie systemic illness helps to improve diagnosis and treatment. But not only circadian vulnerabilities should be considered. It is already recognized that basal and stress-induced alpha-amylase levels are associated with genetic polymorphisms, e.g. the catechol-O-methyltransferase (COMT) enzyme or the serotonin transporter promoter polymorphism (5-HTTLPR) (Frigerio et al., 2009). However, these results are only the beginning. It seems worthwhile to study genetic variations as vulnerability markers of disease states associated with abnormal autonomic functioning such as post-traumatic stress disorder (Krystal & Neumeister, 2009; Rohleder et al., 2004b), borderline personality (Weinberg, Klonsky, & Hajcak, 2009), phobia and anxiety (Coupland et al., 2003), hypertension (Malpas, 2010), or human immunodeficiency virus (Freeman, Roberts, Friedman, & Broadbridge, 1990). Especially in ambulatory settings, sAA would be an easy obtainable, inexpensive, and non-invasive alternative. The understanding of integrative autonomic physiology will not only ultimately provide insight into diagnosis but also into treatment. In the latter context, alpha-amylase may be examined as a marker of successfulness of implemented therapies (psychotherapy, medication), either its basal or stress-induced activity.

To summarize, the past provided convincing evidence for the usefulness of sAA as an autonomic markers in different research areas and the future will tell to what extend sAA will play a role within research of pathological mechanisms and treatment of stress-related disorders.

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