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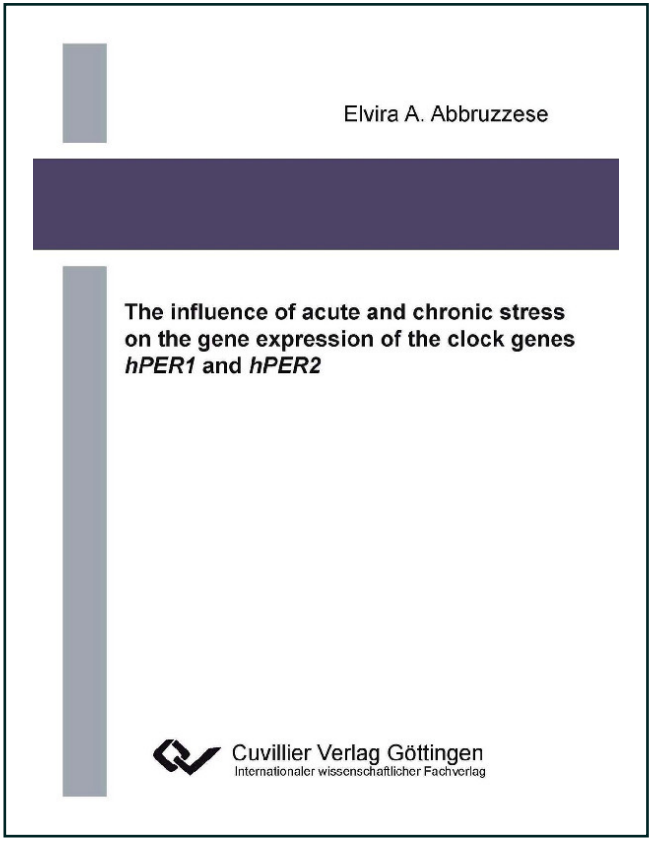
The influence of acute and chronic stress on the gene expression of the clock genes hPER1 and hPER2



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1. Introduction

It is only a little more than half a century ago that Aschoff postulated an endogenous 24-hour rhythm driven by physical oscillators and observed that light intensity is able to modulate this rhythm ("Aschoff's rule"; Aschoff & Meyer-Lohmann, 1954; Aschoff, 1958; Aschoff, 1959; Aschoff, 1965). Together with Bünning and Pittendrigh – also pioneers in the field of chronobiology – he organized the first "Cold Spring Harbor Symposium for Biological Clocks" in 1960 (the second was held in 2007), and with this, officially initiated the research of biological rhythms (Fischer, 2003). Menaker stated that all organisms dispose of a daily rhythm (Menaker, 1969). Some years later, the first clock gene *Per* was discovered in the fruit fly Drosophila melanogaster (Konopka & Benzer, 1971). It seems surprising that such a vital system as the circadian clock, with such a long evolution, was not discovered earlier, but at the same time, this indicates how naturally cycles of dark and light, of night and day, of sleep and wake are experienced by humankind. As self-evidently as we breathe, so too does the synchronisation of our internal clock and the environment occur without us noticing. Only at times – and mostly in association with modern phenomena such as jetlag – might we feel that our internal clock is not correctly set.

Some decades before Aschoff's postulations, Cannon observed an organism's behaviour in dangerous situations and described for the first time the "fight-or-flight" reaction (Cannon, 1915). This concept was expanded in the thirties, when Selye delineated "a syndrome produced by diverse nocuous agents" (Selye, 1936), which he later called the "general adaptation syndrome" (Selye, 1950). Furthermore, he was the first to coin the word "stress" as a medical scientific idea (Goldstein & Kopin, 2007).

It was 1985 when Saiki and colleagues revolutionized molecular biology with the almost incidental discovery of the polymerase chain reaction (PCR) (Saiki et al., 1985). This new method accelerated research in the field of genomics immensely and was constantly

improved upon (e.g. Saiki et al., 1988). Right up to the present day, PCR is considered as one of the most important methods in molecular biology (Mülhardt, 2006).

These three pioneering findings – the discovery of "stress", the "circadian clock" and the PCR – were basic preconditions for the presentation of this thesis. Nowadays, in 2009, the concept of stress-induced diseases is no longer questioned, and indeed has been proven in many cases (e.g. Ehlert et al., 2001; Gill et al., 2008). Equally, the association of a disrupted circadian clock with various physical and mental disorders now seems to be manifest (e.g. Gery et al., 2006; Grandin et al., 2006; Benedetti et al., 2008) and research in this field is just beginning to flourish. New findings on the circadian clock or related clock genes are published on an almost daily basis. However, despite the flood of presented animal studies, so far, few researchers have investigated the circadian clock in human subjects (e.g. Bjarnason et al., 2001; Boivin et al., 2003; Takimoto et al., 2005; Azama et al., 2007), which is partly due to considerable interindividual variability (Brown et al., 2005) and the limited possibility of investigating elements of the central circadian clock, such as the suprachiasmatic nucleus. Nevertheless, we planned to explore the association between chronic/ acute psychosocial stress (and therefore also the role of the glucocorticoid cortisol; c.f. Dickerson & Kemeny, 2004) and parameters of the circadian clock, the so-called clock genes "*hPer1*" and "*hPer2*".

This thesis is subdivided into three main parts: a theoretical background, the empirical investigation and a general discussion. The theoretical background is arranged into three main chapters. The first chapter introduces essential developments in stress research (chapter 2: "Stress"), followed by a chapter which describes the basic principles of gene expression (chapter 3: "Gene Expression"), thus providing a better comprehension of the subsequent chapter about the circadian clock (chapter 4: "Circadian Clock").

PART I THEORETICAL BACKGROUND

2. Stress

Although the term “stress” has been used since the 18th century (Oxford English Dictionary, 2005), the concept of stress as a medical scientific idea (Goldstein & Kopin, 2007) is somewhat more recent. In the course of World War I, Walter Cannon (1871-1945) explored the adaptation of animals to stress episodes, describing for the first time the fight-or-flight reaction (Cannon, 1915). In the 1930s, Cannon specified the concept of “homeostasis” (Cannon, 1932) and Selye published an article about the concept of a general adaptation syndrome and reintroduced the term “stress” in this context (Selye, 1936). Until that point, stress had been seen as uniform response pattern to “acute nonspecific nocuous agents” (Seyle, 1936), and stress research had been limited to animals only. Therefore, psychological factors had not yet been considered, and it was another thirty years before the first models began to consider stress as a psycho-biological phenomenon and accounted for the *meaning* of the stimulus to a stressed human (Lazarus, 1966; Mason, 1971; Lazarus & Cohen, 1977; Antonovsky, 1979; Lazarus & Folkman, 1984). Amongst the most pioneering concepts and models are “the transactional model of stress and coping” by Lazarus and Folkman (1984), the concepts of “salutogenesis” and the “sense of coherence” by Antonovsky (1972, 1979), and the concepts of “allostasis” and “allostatic load” by McEwan (1998). In the following pages, a brief overview of these milestones in stress research will be given.

2.1. Biological and psychological stress concepts

A principle of homeostasis, and therefore a cornerstone of the subsequent stress concepts, was Claude Bernard’s (1813-1878) description of the “milieu intérieur”. For the first time, Bernard described the physiological capacity of the extra-cellular fluid environment to

compensate for external variations with the purpose of maintaining and equilibrating the internal environment and hence maintaining the vital condition *("La fixité du milieu intérieur est la condition de la vie libre, independante."* Bernard, 1974; Gross, 1998). On the basis of Bernard's work, Cannon coined the term "homeostasis" (Cannon, 1932), describing in greater detail how the internal environment is regulated by an organism to equilibrate internal and external demands, maintaining stable conditions. Furthermore, Cannon suggested that any threats to homeostasis cause a sympathoadrenal activation (Goldstein & Kopin, 2007). He had already described the "emergency reaction", involving the activation of the sympathetic nervous system, in his concept of "fight-or-flight" (Cannon, 1914): Whenever an organism perceives a threatening stimulus, a pattern of physiological changes helps the organism to adapt quickly to the situation. The preparation of the organism for a fight-or-flight reaction involves, among other things, a rise in catecholamines, an increase in heart rate, breathing frequency and blood pressure as well as dilatation of the pupils, reduced secretion of saliva and vasoconstriction. This concept of an emergency reaction was extended by the findings of Selye, who described the "general adaptation syndrome" and distinguished an alarm, a resistance and an exhaustion phase (Selye, 1936). In the *alarm* phase, the body is prepared to deal with threatening situations. The homeostasis is disturbed, and therefore the sympathetic nervous system, and as a consequence the adrenal medulla, are activated and the emergency activation is induced. Additionally, along with the sympathoadrenergic system (SAM), another, slower running system is activated: the hypothalamic-pituitary-adrenal axis (HPA axis). The activation of the HPA axis leads to a steep increase of glucocorticoid (GC) levels; as a consequence of this process, immunosuppressive effects can be observed (Selye, 1950). *Resistance* is the phase in which adaptive reactions are intensified. However, if the stress situation endures, the parasympathetic nervous system is activated as a counter-steering process, diminishing the dominance of the sympathetic nervous system. Yet, catecholamine and GC levels remain high. In the *exhaustion* phase, the adaptive capacity is exhausted and the body encounters serious problems in allocating energy

(glucose as well as muscle energy), resulting in adaptation problems. When the storage of the adrenal cortex is emptied and this situation becomes chronic, the organism is no longer able to cope with stress, which implicates diseases and disturbances. Possible long-term consequences are the disturbance of processes of growth and reproduction, disruption of the immune defence, enlargement of the adrenal cortex, shrinkage of the thymus, disturbances of the bowel and weight loss. Although Selye recognized the impact of the stressor (*"... but with further continued treatment, after a period of one to three months (depending on the severity of the damaging agent), the animals lose their resistance and succumb with symptoms similar to those seen in the first stage, this phase of exhaustion being regarded as the third stage of the syndrome."* Selye, 1936, p. 32), he still defines stress as a uniform response pattern (Goldstein & Kopin, 2007).

Influenced by Selye's work, Antonovsky published the innovative idea of going beyond the concept of individual disease by asking why people stay healthy (Antonovsky, 1972; Antonovsky 1979). After what he had seen in the concentration camps of World War II, he asked himself the question of how some of these people managed to stay reasonably healthy despite everything they had suffered. Instead of following the common pathogenic approach, he developed the idea of "salutogenesis", exploring factors that promote or maintain health (Antonovsky, 1979). As a core concept of salutogenesis, Antonovsky defined the concept of the "sense of coherence" (SOC). He observed that the specific disposition of individuals to understand the world and what happens as comprehensible, manageable and meaningful, can be protective. In his own words, Antonovsky described the SOC as follows:

> *"A global orientation that expresses the extent to which one has a pervasive, enduring though dynamic feeling of confidence that*
>
> *1) the stimuli deriving from one's internal and external environments in the course of living are structured, predictable, and explicable;*

2) the resources are available to one to meet the demands posed by these stimuli; and 3) these demands are challenges, worthy of investment and engagement" (Antonovsky, 1987, p.19).

At about the same time, Lazarus and colleagues developed their "transactional model of stress and coping" (Lazarus, 1966; Lazarus & Cohen, 1977; Lazarus & Launier, 1978; Lazarus & Folkman, 1984), accentuating the importance of appraisal, coping and the perception of one's own resources. From the beginning, Lazarus was convinced – as was Antonovsky – that the perception of stress was to a great extent dependent on the individual evaluation of a situation (Lazarus, 1966). The model has been modified and extended over the years, and is based on two successive processes: primary appraisal and secondary appraisal. According to Lazarus and colleagues, primary appraisal depends on the attribution of relevance to what is happening. Therefore, a situation can be either irrelevant or – if the situation is relevant to the perceiver – benign or stressful. When a situation is regarded as stressful, the stress can be considered as a challenge, threat or harm/loss. The process of the secondary appraisal focuses on what coping possibilities the individual possesses: resources and coping options need to be evaluated and decisions need to be taken. The term "coping" is defined as "*constantly changing cognitive and behavioural efforts to manage specific external and/or internal demands that are appraised as taxing or exceeding the resources of the person*" (Lazarus & Folkman, 1984, p.141). Later versions of the model distinguish between problem-focused and emotion-focused coping, and add a third process: the reappraisal (Folkman, 1997). After the coping with a stressful situation, the outcome is evaluated and compared to the initial situation. If necessary, emotions and cognitions are altered in the reappraisal. Thirty years after the first publications about the transactional model of stress, Lazarus wrote retrospectively (Lazarus, 1998):

"...As I hope is clear to the reader at this point, my substantive theory of stress and emotion focuses on two main concepts, appraisal and coping. It proposes that

individual goals and beliefs interact with environmental demands, constraints, personal resources, and opportunities to shape the appraisal of what is happening, the stress reaction, and how the individual copes." (p.125)

Another important concept – presented in the late 1990s – is that of "allostasis" and "allostatic load" described by McEwen and colleagues (McEwen et al., 1998). Allostasis was first defined by Sterling and Eyer as maintaining stability (equal to homeostasis) through change, and they explored how the cardiovascular system adjusts to resting and active states (Sterling & Eyer, 1988). McEwen and co-workers applied this model to other physiological mediators such as cortisol or catecholamines and extended the concept with the suggestion that allostatic load could describe the sum of repeated cycles of allostasis. Furthermore, allostatic load refers to the inefficient turning-on or shutting-off of specific stress responses. Hence, they distinguish between short-term adaptive actions (allostasis), which can be protective, and long-term effects (allostatic load), which can induce damage to an organism (McEwen et al., 1998). The authors propose a concept of a cascade of cause and effect, beginning with the primary stress mediators (cortisol and catecholamines inducing primary effects such as direct cellular events or second-messenger-mediated effects), leading to secondary outcomes, reflecting the summarized outcome of primary effects (i.e. sustained elevation of glucose as a result of elevated cortisol levels), which finally induce tertiary outcomes in the form of actual diseases and disturbances. Although McEwan and colleagues highlighted the influence of stress on physiological processes on a molecular level, they also accounted for psychological factors. They describe four key features of the model:

(1) The brain as an integrative centre for the coordination of behavioural and neuroendocrine responses,

(2) The individual differentials regarding the coping with stressful situations (cf. Lazarus & Folkman, 1984: appraisal of the situation as a challenge, threat or harm/loss) on the basis of individual genetic, developmental and experiential differences,

(3) The general capacity of an individual to adapt, turning on and shutting off physiological responses efficiently, and

(4) The price of allostasis, meaning that number and character of stressful events as well as the inefficiency of physiological responses accumulate through life

(McEwen et al., 1993; Seeman et al., 1997; McEwen et al., 1998).

McEwen and colleagues propose an association between the macro-level of symptoms and diseases and the micro-level of single physiological activities. Therefore, signalling pathways are of major interest: e.g. the mechanisms of glucocorticoids influencing gene expression via glucocorticoid responsive elements (GRE) or via protein-protein interactions with other transcriptional regulators (Miner et al., 1991). Hence, the model of allostasis and allostatic load offers the possibility to integrate psychological, behavioural, genetic as well as (molecular) biological factors to explain short-term and long-term effects of "stress".

Although the activation after a stressor affects the sympathoadrenergic system (SAM) as well as the hypothalamic-pituitary-adrenal axis (HPA axis), in the following, we focus on the HPA axis, due to the fact that we measured cortisol levels (a parameter of the HPA axis), but not parameters of the SAM in our empirical study.

2.2. The hypothalamic-pituitary-adrenal axis

The hypothamalmic-pituitary-andrenal axis (HPA axis) is a regulatory system that interconnects the central nervous system (CNS) with the periphery via hormonal signalling (Nawroth & Ziegler, 2001; Spinas & Fischli, 2001). The system is regulated via the endocrine signalling and multiple feedback mechanisms (Klinke & Silbernagl, 2003). Corticotropin-releasing hormone (CRH) is released from the paraventricular nucleus (PVN) of the hypothalamus with a pulsatile, circadian rhythm and induces the release of adrenocorticotropin (ACTH) in the pituitary. The polypeptide hormone ACTH in turn stimulates the adrenal cortex, with the result of inducing the synthesis of glucocorticoids in the zona fasciculata of the adrenal cortex (Spinas & Fischli, 2001). The released

glucocorticoid is species-specific – cortisol in humans (Klinke & Silbernagl, 2003). Hormones affect further physiological processes by binding to specific receptors (Horn et al., 2005). The regulation of the HPA axis is effected partly by several feedback machanisms: there is a "short" feedback from the pituitary to the PVN of the hypothalamus (ACTH signalling back inhibiting further release of CRH) and two "long" feedbacks from the adrenal cortex to the pituitary (cortisol inhibiting further release of ACTH) as well as to the PVN (cortisol inhibiting further release of CRH) (Fritsch & Kühnel, 2005). Of course, this is a simplified delineation due to the fact that there are numerous further affecting factors, such as signalling from the limbic system, the immune system or the influence of the sympathoadrenergic system in the adrenals (Nawroth & Ziegler, 2001). There is abundant evidence that the HPA axis is strongly activated in situations of psychosocial stress, and therefore cortisol levels increase considerably after stress (Kudielka et al., 2003). Moreover, there are other parameters, such as the genetic disposition, age, gender, intake of exogenous hormones (e.g. oral contraceptives), endogenous hormones (e.g. sex steroids), the phase of the menstrual cycle, pregnancy, consumption of nicotine etc, which affect the endocrine levels of the HPA axis (Kirschbaum & Hellhammer, 1999; Kudielka et al., 2009). In the face of all of these influencing factors, a clear circadian periodicity underlying each of the hormones CRH, ACTH and cortisol was soon found (e.g. Horrocks et al., 1990; Kirschbaum & Hellhammer, 1994). However, various clinical populations can differ significantly from this circadian rhythm of HPA axis-related hormones – in particular persons with stress-related disorders (Heim et al., 2000; Ehlert et al., 2001). Especially well explored are the blunted levels of plasma cortisol and urinary cortisol of patients with posttraumatic stress disorder (Mason et al., 1986; Yehuda et al., 1995; Boscarino, 1996; Yehuda et al., 2001; Gill et al., 2008) and increased cortisol levels in patients with major depression (overview in: Holsboer, 2001; Jokinen & Nordström, 2008). To scrutinize the feedback sensitivity of the HPA axis, there are well validated feedback tests, such as the dexamethasone (synthetic glucocorticoid) suppression test, which should induce a suppression of ACTH and cortisol in an intact HPA axis (Heim &

Ehlert, 1999; Baghai et al., 2002). A lacking suppression as well as an excessive suppression indicate an unbalanced functioning of the HPA axis. Nevertheless, it should be considered that about 9% of the normal population are non-responders (Kirschbaum & Hellhammer, 1999).

2.2.1. Cortisol – a well measurable parameter of the HPA axis

Cortisol is a lipophilic glucocorticoid and its secretion varies with a circadian rhythm, peaking in the morning and decreasing over the day. The synthesis of the hormone cortisol is induced by the activation of the HPA axis (see chapter 2.2). When released from the adrenal cortex, it reaches the bloodstream, where most of the cortisol binds to transport proteins. Two thirds of the released cortisol in a healthy adult binds via high-affinity receptors to the corticosteroid-binding globulin (CBG; saturated at a concentration of 400 to 500 nmol/l; Nawroth & Ziegler, 2001; Spinas & Fischli, 2001). Another 15 to 20 per cent binds via low-affinity receptors to albumin, while a further 5 per cent binds to erythrocytes. Hence, only 5 to 10 per cent of unbound, free cortisol circulates and is therefore biologically active (Kirschbaum & Hellhammer, 1999). This unbound fraction of cortisol can be assessed in the urine (not recommended for the measurements of single time point levels), the blood, and in the saliva – with measurements in the blood and in the saliva being highly correlated (Vining & McGinley, 1987; Kirschbaum & Hellhammer, 1994; Pruessner et al., 1997; Calixto et al., 2002). While the collection of cortisol from the plasma necessitates a minimally invasive blood collection, the collection from the saliva is effected non-invasively by chewing a cotton swab (e.g. Salivette©, Sarstedt, Sevelen, Switzerland). Therefore – in particular when exploring the stress reactivity – the non-invasive method is expedient. The levels of cortisol from the saliva (as well as from blood and urine) are analyzed with radioimmunoassay (Vining et al., 1983; Kirschbaum & Hellhammer, 1994).

Glucocorticoids play a decisive role in numerous processes of the metabolism as well as in the interaction with the immune system, the circulation and the regulation of electrolytes.

Amongst other things, cortisol in particular is responsible for the gluconeogenesis in the liver and is thus fundamental for the allocation of energy. In high doses, cortisol dramatically inhibits the build-up and activation of lymphatic tissue, which leads to a reduced number of lymphocytes, eosinophil granulocytes and antibodies. Thus, defence against infection is weakened. This effect is utilized in immunosuppressive therapies (e.g. in organ transplantation). Moreover, cortisol features anti-inflammatory as well as antiallergic effects (Horn et al., 2005).

Dramatic dysregulations of glucocorticoid levels can lead to hypo- or hypercortisolism. As a consequence, blunted or excessive levels of cortisol can induce an atrophy or hypertrophy, respectively, of the adrenal cortex, which in turn hugely affects the functioning of the adrenal cortex. A primary adrenal insufficiency – better known as Addison's disease – is characterized by a strongly reduced secretion of hormones, and therefore a deficiency of cortisol (morning cortisol after dexamethasone suppression test <0.4 nmol/l; KMI Diagnostics, 2006). Another disease based on a hypocortisolism is the genetically caused adrenogenital syndrome: instead of cortisol and aldosterone, androgen is synthesized, causing a virilisation in girls and premature puberty in boys. By contrast, adrenal hyperfunction – hallmarked by a hypercortisolism – which is also known as Cushing's syndrome, induces adiposity, edemas, osteoporosis, hypertension, heightened blood glucose levels, depression and cognitive disturbances (morning cortisol after dexamethasone suppression test >7.2 nmol/l; KMI Diagnostics, 2006).

The impact of psychosocial stress on cortisol in humans is well explored and has been described in many cases for the normal population as well as for different clinical populations. With the onset of a psychosocial stressor, the HPA axis is activated and therefore cortisol increases. The averaged peak of cortisol after psychosocial stress is reached around 15 to 20 minutes after the onset of the stress (Dickerson & Kemeny, 2004; Kirschbaum & Hellhammer, 2007). Although these findings are fairly stable, there are

considerable individual differences in the diurnal cycle of cortisol (Smyth et al., 1997) as well as when responding to stress (Kudielka et al., 2009). These differences are presumably due to a great extent to the above-mentioned influencing factors. Along with such factors, in particular early stress experiences in life appear to affect the HPA axis, and therefore cortisol levels, on a lifelong basis (e.g. Heim et al., 2000; Heim et al., 2008). Furthermore, any form of chronic stress has an evident impact on the HPA axis, although the direction of the influence is still ambiguous (Melamed et al., 2006; Kudielka et al., 2006).