Interaction between the Hypothalamic-Pituitary-Adrenal Axis and the Circadian Clock System in Humans

Dissertation zur Erlangung der naturwissenschaftlichen Doktorwürde
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II. Table of Contents

III. Index of Abbreviations

ACTB	*Actin beta*
ACTH	Adrenocorticotropic hormone
AIC	Akaike`s information criterion
ANOVA	Analysis of variance
ANS	Autonomic nervous system
ARNT	*Aryl hydrocarbon receptor nuclear translocator*
AVP	Arginine vasopressin
BMAL1	*Brain and muscle ARNT-Like 1*
BMAL2	*Brain and muscle ARNT-Like 2*
BMI	Body mass index
Ca^{2+}	Calcium
cAMP	Cyclic-adenosine monophosphate
CCG	Clock-controlled gene
cDNA	Complementary DNA
ChIP-Seq	Chromatin immunoprecipitation sequencing
CKI	Casein kinase I
CLOCK	*Circadian locomotor output cycles kaput*
CNS	Central nervous system
CO_2	Carbon dioxide
CORT	Cortisol
CRE	cAMP response element
CREB	cAMP response element binding protein
CRH	Corticotropin-releasing hormone
CRY1	*Cryptochrome 1*
CRY2	*Cryptochrome 2*
CUP1	*Copper resistance-associated metallothionein*
DBD	DNA-binding domain
DBP	*D-box binding protein*
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Dinatriummethylendiaminteraacetat

EGTA	Ethylenglycol-bis(aminoethylether)
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal bovine serum
For	Forward primer
FSH	Follicular-stimulating hormone
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*
GG-ε	Greenhouse-Geisser epsilon
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GILZ	*Glucocorticoid-induced leucine zipper gene*
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HCl	Hydrochloric acid
HeLa	Human cervical cancer cells
HepG2	Human hepatocellular liver carcinoma
Hes1	*Hairy and enhancer of split-1*
Hes7	*Hairy and enhancer of split-7*
HPA	Hypothalamus-pituitary-adrenal axis
Hz	Hertz
KCl	Potassium chloride
LB	Luria-Bertani
LBD	Ligand-binding domain
LH	Luteinizing hormone
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
M-MuLV	Moloney murine leukemia virus
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NF-κB	Nuclear factor kappa B
NPAS2	*Neuronal PAS domain protein 2*

NR3C1	*Nuclear receptor subfamily 3, group 3, member 1*
NTD	N-terminal regulatory domain
p53	Tumor suppressor transcription factor
PAS	PER-ARNT-SIM
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PER1	*Period 1*
PER2	*Period 2*
PER3	*Period 3*
PMSF	Phenylmethylsulonylfloride
PPIA	*Peptidylprolyl isomerase A*
PRL	*Prolactin*
PVN	Paraventricular nucleus
REM	Rapid eye movement
Rev	Reverse primer
ROR	Retinoic acid receptor-related orphan receptor
RORE	Retinoic acid receptor-related orphan receptor response element
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulfate
Sgk1	*Serum/glucocorticoid regulated kinase 1*
SIM	Fly developmental regulator single-minded
SIRT1	Sirtuin histone deacetylase
SOC	Super optimal broth
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TE	Tris-EDTA
Tm	Melting temperature
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
TSST	Trier social stress test

IV. Index of Figures

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VI. Index of Publications

Several parts of this thesis were published as “Original Research Article” in an international peer-reviewed journal.

Yurtsever T, Schilling TM, Kölsch M, Turner JD, Meyer J, Schächinger H, Schote AB (2016). The acute and temporary modulation of *PERIOD* genes by hydrocortisone in healthy subjects. *Chronobiology International*, 33:1222–1234

VII. General abstract

Rotation of the Earth creates day and night cycles of 24 h. The endogenous circadian clocks sense these light/dark rhythms and the master pacemaker of the mammals situated in the suprachiasmatic nucleus of the hypothalamus entrains the physical activities according to this information. The circadian machinery is built from the transcriptional/translational feedback loops generating the oscillations in all nucleated cells of the body. In addition, unexpected environmental changes, called stressors, also challenge living systems. A response to these stimuli is provided immediately via the autonomic nervous system and slowly via the hypothalamus–pituitary–adrenal (HPA) axis. When the HPA axis is activated, circulating glucocorticoids are elevated and regulate organ activities in order to maintain survival of the organism. Both the clock and the stress systems are essential for continuity and interact with each other to keep internal homeostasis.

The physiological interactions between the HPA axis and the circadian clock system are mainly addressed in animal studies, which focus on the effects of stress and circadian disturbances on cardiovascular, psychiatric and metabolic disorders. Although these studies give opportunity to test in whole body, applying the unpleasant techniques, controlling and manipulating the parameters at the high level, and generalization of the results to humans are still a debate. On the other hand, studies established with cell lines cannot really reflect the conditions occurring in a living organism. Thus, human studies are absolutely necessary to investigate mechanisms involved in stress and circadian responses.

The studies presented in this thesis were intended to determine the effects of cortisol as an end-product of the HPA axis on *PERIOD* (*PER1*, *PER2* and *PER3*) transcripts as circadian clock genes in healthy humans. The expression levels of *PERIOD* genes were measured under baseline conditions and after stress in whole blood. The results demonstrated here have given better understanding of transcriptional programming regulated by pulsatile cortisol at standard conditions and short-term effects of cortisol increase on circadian clocks after acute stress. These findings also draw attention to interindividual variations in stress response as well as non-circadian functions of *PERIOD* genes in the periphery, which need to be examined in details in the future.

CHAPTER 1: INTRODUCTION

1.1. Stress and the hypothalamic-pituitary-adrenal axis

1.1.1. Stress and stressors

Stress is a favorite word used in our daily conversations and refers to an internal or external challenge that threatens the physiological homeostasis of the body. Definition of stress as a concept, based on homeostasis, returns back to the ancient Greek and since then it was developed by philosophers and scientists (Johnson et al., 1992). In our modern life, it is defined as follows: "*One way of looking at stress is as a condition where expectations, whether genetically programmed, established by prior learning, or deduced from circumstances, do not match the current or anticipated perceptions of the internal or external environment, and this discrepancy between what is observed or sensed and what is expected or programmed elicits patterned, compensatory responses* (Goldstein & Kopin, 2007)."

A *stressor* is a stimulus that perturbs homeostasis. Stressors can be psychological, physical and social as well as specific to challenge metabolic and cardiovascular steady state. Psychological stressors are emotional processes and may cause frustration, fear or anxiety (Johnson et al., 1992), while intense radiation, heat, noise, cold and poisons are among physical stressors (Pacak & Palkovits, 2001). Poverty, divorce and unemployment are examples of social stressors in humans. Exercise, hypoglycemia, upright tilt, hemorrhage and heat exposure are stressors challenging metabolic and cardiovascular steady state (Pacak & Palkovits, 2001).

In mammals, the autonomic stress system (ANS) immediately responds through sympathetic and parasympathetic activations after stress, whereas the hypothalamus–pituitary–adrenal (HPA) axis provides a sluggish response, in which circulating glucocorticoids are elevated (Ulrich-Lai & Herman, 2009). The brain starts stress response and regulates the activations of the ANS and the HPA axis. In the ANS response, preganglionic sympathetic neurons in the spinal cord are activated. Signals from these neurons is either transferred to prevertebral ganglia, which project to peripheral effector organs, or paravertebral ganglia, which project to the adrenal medulla. This sympathetic activation results in the classical "fight or flight" response, which elevates circulating concentrations of adrenaline and noradrenaline, blood pressure and heart rate, vasoconstriction and energy mobilization. Parasympathetic activations

can also occur during stress and have reverse consequences of the sympathetic system (Ulrich-Lai & Herman, 2009). The HPA axis and its response to stress will be explained in the following section.

1.1.2. The HPA axis

The HPA axis is made up of three components: hypothalamus, pituitary gland and adrenal glands. The interactions among these organs are stress-responsive and help organisms to maintain homeostasis as well as to regulate the physiological functioning.

1.1.2.1. Hypothalamus

The hypothalamus is a brain region consisting of a number of small nuclei (Figure 1) and exists in the nervous systems of all vertebrates. It controls certain metabolic processes and the activities of the autonomic nervous system. The major functions of the hypothalamus include regulation of body temperature, cardiovascular functions, water balance, feeding and gastrointestinal activity, uterine contractility, sleep-wake cycle and milk ejection from the breast (Hall & Guyton, 2011, pp 715-716). Beside these activities, the hypothalamus also affects behavioral functions (Hall & Guyton, 2011, p 717).

The neurons of the hypothalamus synthesize and secrete certain hormones, which stimulate or inhibit the release of the pituitary hormones. The hypothalamic hormones controlling the release of the anterior pituitary hormones are gonadotropin-releasing hormone (GnRH), prolactin-inhibiting hormone (Dopamine), growth hormone-releasing hormone (GHRH), growth hormone inhibitory hormone (Somatostatin), corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) (Hall & Guyton, 2011, p 883).

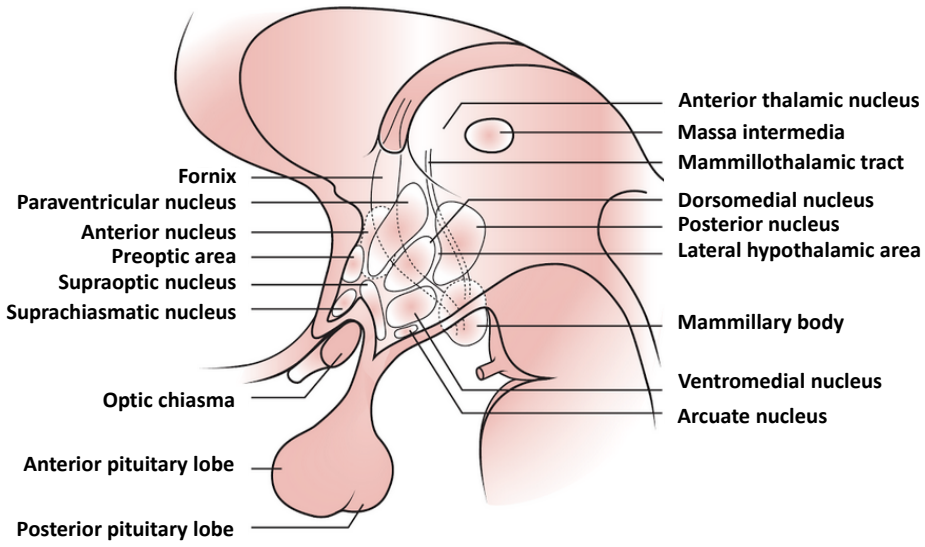

Figure 1: Small nuclei of the hypothalamus (retrieved from Brazis et al., 2011, p 420)

1.1.2.2. Pituitary gland

The pituitary is a pea-size gland, which controls the functions of other endocrine glands. It is made up of two parts, adenohypophysis and neurohypophysis. The adenohypophysis contains the anterior and intermediate lobes, whereas neurohypophysis is also called the posterior lobe (Zhu et al., 2007). The hormones secreted by the cells of the anterior pituitary regulate the metabolic functions and can be summarized as two different gonadotrophic hormones, luteinizing hormone (LH) and follicular-stimulating hormone (FSH), growth hormone (GH), prolactin, thyroid-stimulating hormone (TSH) and adrenocorticotropic hormone (ACTH) (Zhu et al., 2007). The posterior pituitary gland is a neuronal projection of the supraoptic and paraventricular nuclei (PVN) of the hypothalamus. The endings of these nerve tracts originating from the hypothalamus store and release two posterior pituitary hormones, oxytocin and vasopressin (or antidiuretic hormone) (Hall & Guyton, 2011, pp 904-905).

1.1.2.3. Adrenal gland

The adrenal glands are small and yellowish organs located above and slightly medial to the kidneys. The right adrenal gland has a pyramidal shape while the left one has a semi-lunar structure (Stocksley, 2001, p 204). Each adrenal gland consists of two different parts, the adrenal cortex (outer) and the adrenal medulla (inner).

The adrenal medulla is controlled by the sympathetic nervous system and secretes catecholamine hormones, epinephrine (adrenalin) and norepinephrine (noradrenalin), in response to sympathetic stimulations. Epinephrine and norepinephrine play an important role in the “fight or flight” reaction and affect different organs although they have almost the same consequences. Increasing the blood flow and heart rate, inhibition of the gastrointestinal system, increasing the blood sugar and pupil dilation are some effects caused by these hormones (Hall & Guyton, 2011, p 736).

The adrenal cortex synthesizes and secretes different groups of hormones, which are known as corticosteroids: glucocorticoids (cortisol in humans), mineralocorticoids (aldosterone) and androgens. Although all corticosteroids are synthesized from cholesterol, slight structural differences cause differential functions in the body (Nussey & Whitehead, 2001, p119). Mineralocorticoids control salt and water balances, whereas glucocorticoids have effects on carbohydrate, protein and fat metabolisms as well as on immune system (Seifter et al., 2005, p 540). Adrenal androgens are weak steroids and have a little effect when reproductive glands are regularly functional (Nussey & Whitehead, 2001, p119).

1.1.3. Stress response

Stress stations of the brain (prefrontal cortex, hippocampus and amygdala) transfer the stress signal to the PVN of the hypothalamus to initiate the HPA axis activation. The PVN secretes CRH and arginine vasopressin (AVP), which synergistically control the release of ACTH from the anterior pituitary (Kalogeras et al., 1996). ACTH consecutively stimulates the secretion of glucocorticoids from the adrenal cortex (Figure 2).

The HPA axis has both a baseline and a stress-related activity. Under baseline conditions, CRH and AVP have circadian secretion profiles, which are made up from ultradian rhythms with a frequency of two to three episodes per hour (Engler et al., 1989). The amplitudes of the CRH and AVP pulses increase in the morning hours. This results in an increase of ACTH secretion and subsequently glucocorticoid production. Therefore, cortisol reaches its zenith in the early morning. During the day, a discrete but small peak after lunch and a nadir in the late evening are observed. When the HPA axis is chronically stressed, a decrease of morning zenith, an increase in evening nadir and a large cortisol response induced by lunch occurs (Chrousos & Gold, 1998). When the HPA axis is exposed to acute stress, the amplitude of

CRH and AVP pulsations elevates and as a consequence, the ultradian secretory episodes of ACTH and cortisol are increased (Tsigos & Chrousos, 2002).

Glucocorticoids, as the end-products of the HPA axis, are essential for the basal activity of the HPA axis as well as for the termination of the stress-induced HPA response. Glucocorticoids form a feedback loop to inhibit their own secretion by acting on the hypothalamus and the pituitary (Figure 2, Walker et al., 2010).

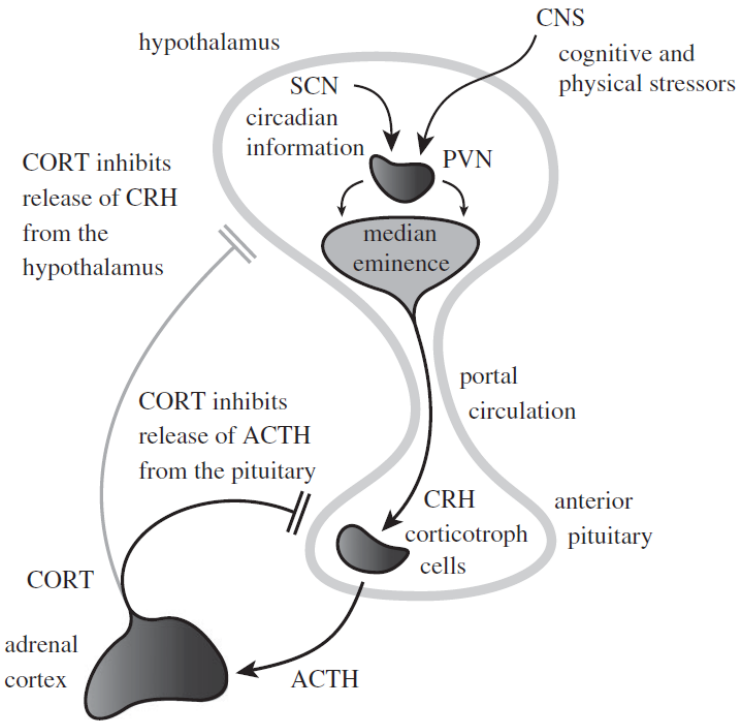

Figure 2: HPA axis and its regulation. The PVN gets inputs from the suprachiasmatic nucleus (SCN) and central nervous system (CNS), and releases CRH to trigger ACTH secretion from the anterior pituitary. ACTH stimulates adrenal cortex to secrete cortisol (CORT). With the feedback inhibition cortisol prevents the release of ACTH from the pituitary and the release of CRH from the PVN (retrieved from Walker et al., 2010).

1.1.4. Effects of Glucocorticoids

The primary glucocorticoid is cortisol in humans, which regulates a wide range of functions in the body. The well-known effect of cortisol is to stimulate the gluconeogenesis in the liver. In order to do this carbohydrate formation, cortisol activates the expression of DNA transcription to increase the enzymes involved in the gluconeogenesis and induces mobilization of amino acids from other tissues to the liver. Second effect of cortisol is the

stimulation of fat breakdown in adipose tissue, which causes an elevation of the fatty acid concentration in the plasma. Another effect of cortisol is the mobilization of amino acids, causing a reduction of protein stocks in extrahepatic cells and an induction of liver and plasma proteins (Hall & Guyton, 2011, p 928-929). Beside these metabolic effects, cortisol also suppresses the immune system (Jefferies, 1991), decreases bone formation (Chyun et al., 1984) and has an impact on cognition (Lupien et al., 2007). In addition, glucocorticoids alter the electrical potential of the neurons (Gower, 1993) and the stability of messenger RNAs (mRNAs), in turn affecting the translation of the glucocorticoid-target proteins (Cooper, op. 2005, p107).

1.1.5. Glucocorticoid receptors

Glucocorticoids exert their functions via glucocorticoid receptors (GRs) at their target tissues. The GR proteins are transcription factors and encoded by the *NR3C1* gene, which stands for nuclear receptor subfamily 3, group 3, member 1. Human *NR3C1* is located at chromosome 5q31.3 (Figure 3A) and has 16 transcript variants. Two transcripts are encoded through alternative use of two terminal exons 9 alpha (9α) and 9 beta (9β) (Kino & Chrousos, 2004). The canonical isoform, GRα, has 9 exons and 7,286 base pairs (bps) transcript length (Ensembl, release 87 - Dec 2016, Uniprot identifier: P04150-1, v1) (Figure 3A). *GRα* is widely expressed through the body and mediates nearly all functions of glucocorticoids, whereas GRβ cannot bind glucocorticoids and inhibits GRα actions (Bamberger et al., 1995). At least eight initiation sites encode *GRα* mRNA into multiple *GRα* isoforms. These isoforms are termed as A, B, C1, C2, C3, D1, D2 and D3 and have differential transcriptional activities on target genes (Lu & Cidlowski, 2005). GRα contains 777 amino acids and distinct functional domains: N-terminal regulatory domain (NTD), DNA-binding domain (DBD), hinge region and ligand-binding domain (LBD) (Figure 3B, Kino & Chrousos, 2011).

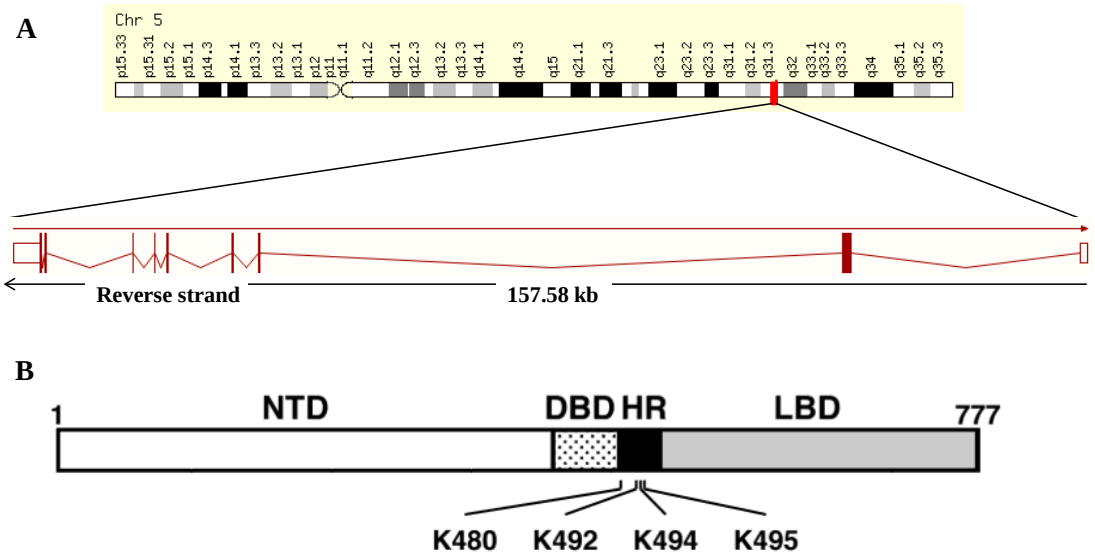

Figure 3: Gene and protein structure of GRα. A. Chromosome location and genomic structure of *NR3C1*. Chr: Chromosome, kb: kilo base (Modified from Genecards and Ensembl, release 87 - Dec 2016) B. Functional domains of GRα. NTD: N-terminal regulatory domain, DBD: DNA-binding domain, HR: Hinge region, LBD: Ligand-binding domain, K: lysine (Retrieved from Kino & Chrousos, 2011).

GRs are situated in the cytoplasm and form complexes with heat-shock proteins 20, 50, 70, 90 and other possible proteins when the glucocorticoid ligand is absent. As soon as its ligand binds to the C-terminal LBD, a conformational change occurs, GR dissociates from the heat-shock proteins, dimerizes and relocates into the nucleus (Kino & Chrousos, 2011). Translocation into the nucleus through the nuclear pore requires ATP and is mediated by the nuclear localization signal-1 located at the DBD and the nuclear localization signal-2 located at the LBD (Savory et al., 1999).

In the nucleus, ligand-activated GR binds to the specific DNA sequences, known as glucocorticoid response elements (GREs), in the promoters of glucocorticoid-responsive genes and either activates (Pratt, 1990) or represses (Scheinman et al., 1995) the transcription of target genes. On the other hand, GR also regulates the transcription of non-GRE containing genes, which are under the control of other transcription factors. GR interacts with these factors, such as activator protein 1 and nuclear factor κB (NF-κB), and indirectly modulates the expression of these genes (Kino & Chrousos, 2004).

GR has two transactivation domains, which are located at the NTD and LBD. Through these domains, GR acts on many proteins and protein complexes, known as co-activators (Kino &

Chrousos, 2004). Co-activators have endogenous histone acetyltransferase activity to remodel the chromatin structure and promote binding of transcription complex to DNA (McKenna et al., 1999). On the other hand, co-repressors oppose the function of co-activators and keep the steroid receptors in the inactive form. Co-repressors might have histone deacetyltransferase activity or recruit other factors, which have deacetyltransferase activity, to condense chromatin structure and block binding of transcription complex to DNA (McKenna et al., 1999). Depending on their concentrations and target tissue type, there are some co-activators repressing the transcription of target genes (Kino et al., 1999); thus, the difference between co-activators and co-repressors is not exact.

1.1.6. Ultradian rhythms of glucocorticoids

Glucocorticoids are secreted from the adrenal glands in a circadian manner, which is actually created from an underlying ultradian rhythm (Veldhuis et al., 1989; Windle et al., 1998). Ultradian glucocorticoid secretion takes place with pulses of 60 to 90 minutes in humans (Veldhuis et al., 1989; Trifonova et al., 2013) and about 60 minutes in rats (Windle et al., 1998), where hormone release is followed by termination of the secretion and fast removal of hormone from the circulation (Windle et al., 1998). This pulsatile secretion of the hormone is essential for the responsiveness of the HPA axis to stress (Lightman, 2008) and mRNA expression of glucocorticoid-regulated genes (Stavreva et al., 2009).

The suprachiasmatic nucleus (SCN) of the hypothalamus regulates the circadian activity of the HPA axis and cortisol release; however, mechanisms regulating the ultradian activity are not well-known. First, a hypothalamic pulse generator was thought to be involved in the ultradian activity, in which CRH was released episodically (Ixart et al., 1991; Mershon et al., 1992). However, studies in sheep revealed that surgical disconnection of the hypothalamus from the pituitary did not destroy the pulsatile glucocorticoid secretion, suggesting that a pulsatile CRH signal does not drive the ultradian rhythm of cortisol (Engler et al., 1990). Later, via theoretical modelling it has been shown that feed-forward and feedback interactions between the pituitary and adrenal cortex sufficiently generate the glucocorticoid ultradian pulses (Walker et al., 2010). Currently, the origin of ultradian pulsatility is still under investigation.

1.2. The circadian clock system

Circadian rhythms are physiological, mental and behavioral changes with a period of approximately 24 h. Sleep-wake cycle, cardiovascular activities like heart rate and blood pressure (Morris et al., 2012), hormone secretion (Tsang et al., 2014), body temperature (Refinetti, 2010) and the immune system (Scheiermann et al., 2013) are some of the processes displaying daily fluctuations and generated by endogenous biological clocks located in the brain and peripheral tissues.

In mammals, the master clock is the SCN, which is situated in the hypothalamus (Figure 1, Moore & Silver, 1998). However, clocks are also known to be present in other brain regions like the olfactory bulb (Granados-Fuentes et al., 2004), cerebral cortex (Abe et al., 2001) and limbic forebrain (Lamont et al., 2005), as well as peripheral tissues like liver, lung and skeletal muscle (Yamazaki et al., 2000). Moreover, peripheral blood mononuclear cells (PBMCs) (Boivin et al., 2003; Burioka et al., 2005), oral mucosa and skin (Bjarnason et al., 2001), visceral and subcutaneous adipose tissue explants (Gomez-Abellan et al., 2012) and hair follicle cells (Akashi et al., 2010) were used to investigate circadian clock system in humans.

1.2.1. Molecular components of the mammalian circadian oscillators

Transcriptional/translational feedback loops in the circadian clocks generate the circadian rhythms. Two transcription factors, CLOCK (its paralog NPAS2) and BMAL1 (its paralog BMAL2), activate the transcription of target genes such as *PERIOD* (*PER)1*, *PER2*, *PER3* and *CRYPTOCHROME* (*CRY*)*1* and *CRY2* in the main loop (Figure 4). When PER and CRY proteins are produced, they heterodimerize, enter the nucleus and repress the transcriptional activity of CLOCK-BMAL1 to inhibit their own transcription. When PER and CRY complexes are degraded, CLOCK-BMAL1 can activate a new cycle of *PER/CRY* transcripts (Ko & Takahashi, 2006). In the auxiliary loop, CLOCK-BMAL1 heterodimers regulate their own transcription (Figure 4). Two nuclear receptors, REV-ERBα and REV-ERBβ, bind retinoic acid receptor-related orphan receptor response elements (ROREs) in the *Bmal1* promoter and repress transcription of *Bmal1*, whereas the members of RORs (α, β and γ) activate transcription of *Bmal1* (Ko & Takahashi, 2006). REV-ERBs and RORs compete to

bind ROREs and regulate the circadian oscillation of *Bmal1* (Guillaumond et al., 2005). Thus, this loop assists the stabilization of the main loop (Ko & Takahashi, 2006).

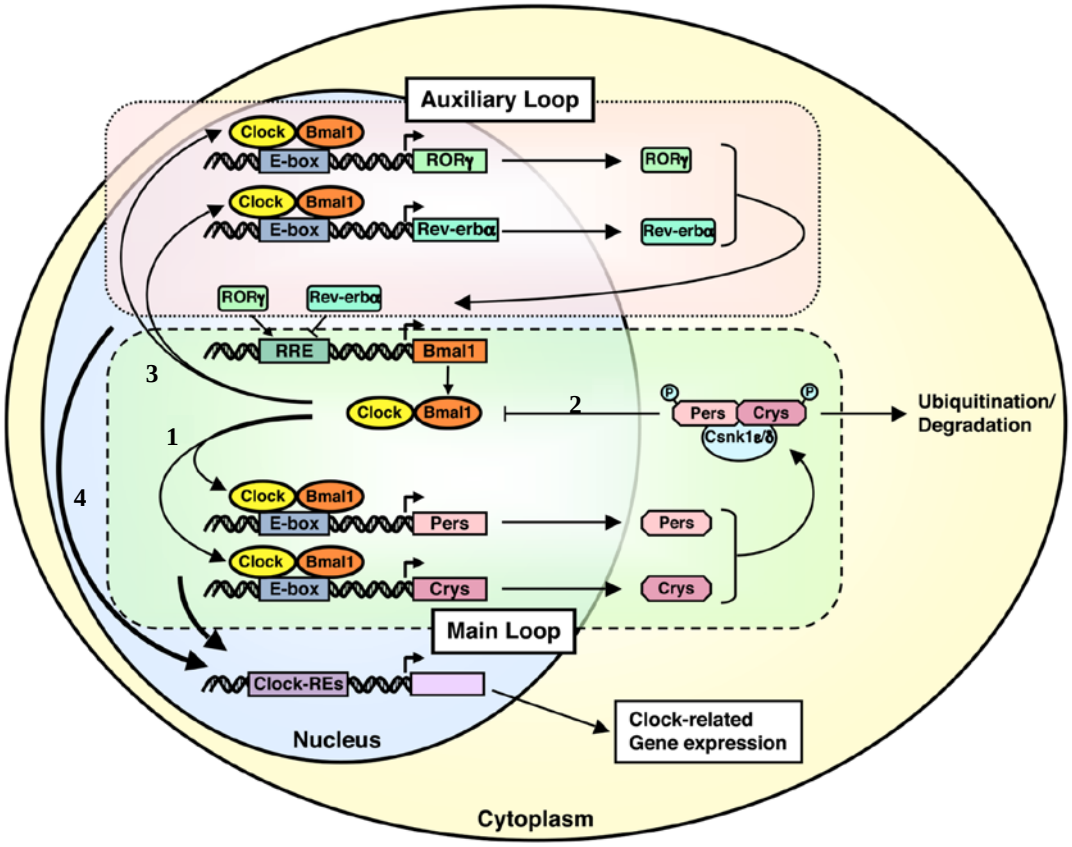

Figure 4: Transcriptional/translational feedback loops of the circadian clocks. 1. In the main loop, CLOCK-BMAL1 heterodimer activate the transcriptions of murine *Pers* and *Crys* by binding E-box elements in the promoter region. 2. When Pers and Crys are produced, they form a complex with casein kinase 1ε and δ. After phosphorylation, they translocate back to the nucleus to inhibit their own transcription by repressing the transcriptional activity of the CLOCK-BMAL1 heterodimer. 3. In the auxiliary loop, RORα activates whereas Rev-erbα represses the transcription of *Bmal1* by binding the retinoic acid receptor-related orphan receptor response element (RRE) in the promoter of *Bmal1*. 4. Several clock-related genes are regulated by these transcription factors. Clock: circadian locomotor output cycle kaput, Bmal1: brain-muscle-arnt like protein 1, Pers: periods, Crys: cryptochromes, Csnk1ε/δ: caseine kinase 1ε/δ, P: phosphate residue, RORγ: retinoic acid receptor-related orphan nuclear receptor γ (Retrieved and modified from Nader et al., 2010)

Beside these feedback loops, non-transcriptional circadian oscillations have been described in human red blood cells, which have no nucleus or DNA to perform transcription. Cytoplasmic redox reactions such as peroxiredoxin oxidation-reduction, NADH/NADPH fluctuations and hemoglobin dimer-tetramer transpositions display circadian oscillations (O'Neill & Reddy,

2011). Interestingly, these oscillations also exist in the nucleated cells and are interconnected with the transcriptional/translational feedback loops (O'Neill & Reddy, 2011). Thus, nuclear circadian processes as well as cytoplasmic processes contribute to generate the circadian rhythms in eukaryotic cells.

1.2.1.1. *PERIOD* genes and proteins of circadian timing system

PERIOD family genes have three homologs: *PER1, PER2* and *PER3*. These genes are widely and rhythmically expressed in mammals. Human *PER1* is located at chromosome 17p13.1 and has 20 splice variants, which have not been fully described. The canonical isoform has 23 exons and 4,707 bps transcript length (Ensembl, release 87 - Dec 2016, Uniprot identifier: O15534-1, v2) (Figure 5A). *PER2* is located at chromosome 2q37.3 and has four splice variants. The canonical isoform has 23 exons and 6,385 bps transcript length (Ensembl, release 87 - Dec 2016, Uniprot identifier: O15055-1, v2) (Figure 5B). *PER3* is located at chromosome 1p36.23 and has nine splice variants. The canonical isoform has 21 exons and 6,203 bps transcript length (Ensembl, release 87 - Dec 2016, Uniprot identifier: P56645-1, v4) (Figure 5C). Furthermore, analysis of the promoter regions revealed functional GREs in human *PER1* (Reddy et al., 2009), *PER2* (Reddy et al., 2009) and *PER3* (Archer et al., 2010), which are the key elements in the interactions between the HPA axis and the circadian clock system.

PERIOD genes seem to be redundant; however, they have differential effects on time-keeping of circadian oscillations. Murine *Per1* and *Per2* were rapidly induced by light in the SCN, but not *Per3* (Albrecht et al., 1997; Shearman et al., 1997; Zylka et al., 1998). In a recent study, *Per1* and *Per2* have been reported to cooperate rigidly in order to keep the circadian rhythm approximately 24 h (Tamiya et al., 2016). On the other hand, *Per3* has been shown to be not crucial for the main clock function (Shearman et al., 2000; Bae et al., 2001). Although only *Per1* and *Per2* are important to generate circadian rhythms in the SCN and eye (Shearman et al., 1997), it has been reported that *Per3* is involved in period determination of peripheral clocks (Pendergast et al., 2012a). Moreover, preferences in sleep timing (Archer et al., 2008) and delayed sleep phase syndrome (Archer et al., 2010) have been shown to be associated with *PER3* in humans, suggesting an essential role for *PER3* in output functions of the clock system. When three homologs of *Period* genes were knocked out, two extra-SCN oscillators, food entrainable oscillator and methamphetamine-sensitive circadian oscillator, got shorter

and had a 21-h period of rhythmicity (Pendergast et al., 2012b). Even though these rhythms are not expressed under normal conditions, this shows the importance of *Period* genes in circadian rhythms. When we take these results into account, it seems that *PERIOD* genes are not interchangeable and interact with different components to response to different input cues.

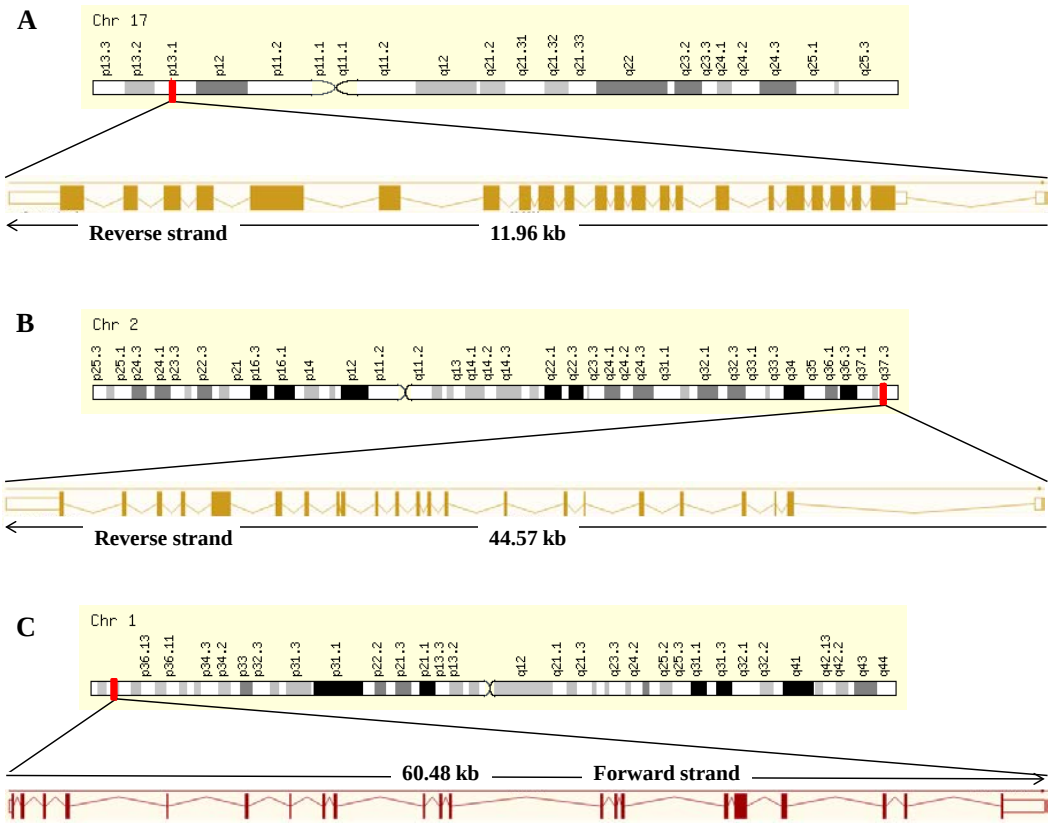

Figure 5: Chromosome locations and genomic structures of *PER1* (A), *PER2* (B) and *PER3* (C). Chr: Chromosome, kb: kilo base (Modified from Genecards and Ensembl, release 87 - Dec 2016)

PERIOD proteins contain two PAS domains: PAS-A and PAS-B. PAS stands for **P**er-**A**rnt-**S**im, where Per is fly clock protein PERIOD, Arnt is the vertebrate aryl hydrocarbon nuclear translocator and Sim is the fly developmental regulator single-minded (Henry & Crosson, 2011). These domains are essential for homo- and heterodimer formation of PERIODs, and interactions with other transcription factors and kinases. The studies on the crystal structures of PAS domains of these mouse PERIOD homologs revealed structural differences, which cause distinct molecular interactions (Hennig et al., 2009; Kucera et al., 2012) and distinct circadian and/or non-circadian functions, confirming the non-redundancy of these proteins.

1.2.2. Post-translational mechanisms

The autoregulatory transcriptional/translational feedback loops complete one cycle in approximately 24 hours, which is controlled by post-translational modifications like acetylation, phosphorylation and ubiquitination. These mechanisms will briefly be explained in this section.

The central clock protein CLOCK has histone acetyltransferase properties (Doi et al., 2006); however it can also acetylate non-histone proteins, such as BMAL1. This BMAL1 acetylation recruits CRY1 (Hirayama et al., 2007), in turn inactivating the CLOCK-BMAL1 complex. Moreover, a sirtuin histone deacetylase, SIRT1, physically interacts with CLOCK and deacetylases BMAL1 (Nakahata et al., 2008) and PER2 (Asher et al., 2008), controlling the generation of the circadian rhythms. The main transcriptional/translational loop is tightly regulated by these enzymatic feedback loops.

The serine/threonine protein kinase casein kinase I ε (CKIε) and casein kinase I δ (CKIδ) bind to PERs and phosphorylate them, which has several functional effects. The first effect of phosphorylation of the PERs is the recruitment of ubiquitin ligase, polyubiquitination and degradation by the proteasome (Akashi et al., 2002; Eide et al., 2005). Moreover, CKIε can also phosphorylate CRYs, which results in similar degradation (Yagita et al., 2002). The other effect of phosphorylation is the induction of nuclear translocation (Akashi et al., 2002). The decision on cellular retention or nuclear entry of the circadian proteins is made by phosphorylation in a way that when PERs and CRYs exist at the same time, they form a heterodimer to relocate to the nucleus; otherwise, phosphorylation mediates ubiquitination and degradation by proteolysis (Yagita et al., 2002). Mutations in these kinases have been found to result in abnormal circadian phenotypes. For example, a mutation in mouse CKIε accelerates degradation and accordingly shortens the circadian period (Lowrey et al., 2000). Mutations of PER2 phosphorylation site and of CKIδ have been reported to cause familial advanced sleep phase syndrome in humans (Toh et al., 2001; Xu et al., 2005).

A small ubiquitin-related modifier protein modification has also been shown to be essential in clock gene expression and clock rhythmicity (Cardone et al., 2005). These show the importance of post-translational regulation in the generation of the ~24-h molecular clock. It seems that keeping the circadian clock ticking ~24 h is a result of complex molecular interactions.

1.2.3. Clock-controlled genes

Clock-controlled genes (CCGs) are the genes which are not involved in the clockwork, but are regulated by core oscillator components. The rhythmic transcription of these clock output regulators is activated via E-box, RORE and D-box motifs (Ueda et al., 2005). The *D-box binding protein* (*DBP*) and the *arginine vasopressin* gene are well-studied CCGs and have been reported to be directly regulated by CLOCK-BMAL1 heterodimers (Jin et al., 1999; Ripperger et al., 2000). Microarray analysis revealed that up to 10% of the mammalian transcriptome might be controlled by the circadian clock and this regulation of transcription is tissue-specific. CCGs in the SCN are involved in protein synthesis, processing and secretion as well as in energy production to drive the circadian rhythm of the mammals. On the other hand, CCGs in the liver are involved in nutrient and intermediate metabolisms as well as in immune mechanisms (Panda et al., 2002). It seems that CCGs are important connections between the core clock machinery and cellular functions.

1.2.4. Cellular makeup of the circadian clock system

The master clock, the SCN contains two tiny aggregates of closely compacted neurons in the hypothalamus, above the optic chiasm (Moore et al., 2002). SCN neurons generate long-lasting self-sustained oscillations via synaptic and paracrine mechanisms. Cyclic-adenosine monophosphate (cAMP), Ca^{2+} signaling (O'Neill & Reddy, 2012) and vasoactive intestinal peptide signaling (Harmar, 2003) are paracrine mechanisms, which keep the SCN cells in synchrony. However, the SCN must be entrained by light in order to adapt the circadian rhythms to geophysical time.

The SCN receives light signals from classical rod and cone photoreceptor cells as well as photosensitive ganglion cells expressing the light receptor melanopsin in the inner retina layer (Ruby et al., 2002). These photic signals are transmitted to the SCN through the retino-hypothalamic tract and transduced to activate the ionotropic glutamate receptors, which mediate Ca^{2+} influx in postsynaptic neurons (Ding et al., 1994). Several protein kinases are activated and the cAMP response element binding protein (CREB) is phosphorylated by the Ca^{2+}-triggered pathways (Obrietan et al., 1998). CREB, as a transcription factor, binds to cAMP response elements (CREs) in the promoters of two circadian clock genes, *PER1* and *PER2*, and activates the transcription of these genes in a CLOCK-BMAL1 independent

manner (Travnickova-Bendova et al., 2002). The induction of PER1 and PER2 causes an inhibition of their own transcription, which resets the phase of the transcriptional rhythm of these genes.

The light-entrained SCN transmits this information to the slave clocks via several pathways such as signaling proteins secreted from the SCN, feeding time and body temperature. Signaling proteins, transforming growth factor-α (Kramer et al., 2001) and Prokineticin 2 (Cheng et al., 2002), have been identified as diffusible factors, which are rhythmically produced and secreted by SCN neurons and inhibit the locomotor activity in rodents. Feeding has been shown to change the phase of circadian gene expression in the peripheral tissues like liver, kidney, heart and pancreas, although the phase of the clock genes in the SCN is not affected (Damiola et al., 2000; Stokkan et al., 2001). Natural body temperature and ambient temperature can synchronize the peripheral clocks in mammals (Brown et al., 2002; Buhr et al., 2010; Morf et al., 2012). In addition, blood-born factors affect the circadian expression of various genes in the peripheral tissues (Balsalobre et al., 1998). Glucocorticoid hormones are also candidates for signals entraining peripheral clocks, since they modulate the expression of *Per* genes (Balsalobre et al., 2000; Yamamoto et al., 2005; Reddy et al., 2009).

1.2.5. Ultradian oscillations

Ultradian rhythms can be described as molecular, physical and behavioral processes which have a period shorter than 24 h and repeat several times per day. Rapid eye movement (REM)-non-REM sleep cycle (Aserinsky & Kleitman, 1953), cellular metabolic rhythms (Brodsky, 2014), cell fate determination (Isomura & Kageyama, 2014), hormones such as glucocorticoids (Lightman & Conway-Campbell, 2010) and gene expression (Hughes et al., 2009) are well-known examples of ultradian rhythms. These rhythms are common across species and governed by intrinsic or extrinsic signals. For example, a dopaminergic ultradian oscillator (Blum et al., 2014) and the retrochiasmatic area of the hypothalamus (Gerkema et al., 1990) have been found to drive the ultradian rhythm of behavioral arousal in the mammalian brain. On the other hand, deprivation of rest and food revealed an independent clock regulation of ultradian feeding rhythms in voles (Gerkema & van der Leest, 1991). However, the biological mechanisms driving these ultradian rhythms are not well-known. In the next section, the ultradian rhythms of gene expression will be detailed and the recent findings about cyclic gene regulations will be consolidated.

1.2.5.1. Ultradian rhythms of gene expression

Ultradian oscillations have been discovered in the regulation of several genes, which are governed by intrinsic signals. Tumor suppressor transcription factor p53 (Lev Bar-Or et al., 2000), NF-κB signaling (Ashall et al., 2009), the segmentation clock in mammals (Hirata et al., 2002; Hirata et al., 2004) and genes involved in metabolic cycles of the budding yeast *Saccharomyces cerevisiae* (Tu et al., 2005) are some examples of endogenously driven oscillators. In these systems, negative feedback loops are formed and molecules regulate their own expression to generate an oscillatory behavior.

The pulsatile transcriptional response mediated by ultradian hormone release is one of the oscillations generated extrinsically. GR-regulated gene pulsing has especially been well-studied. The ultradian hormone fluctuations are detected by GRs, which are associated with chaperon machinery. Then, short-leaving GR-hormone complexes are formed and a dynamic exchange occurs with the regulatory elements, which generates oscillations of the transcriptional responses (Stavreva et al., 2009). A differential response of the glucocorticoid target genes to pulsatile versus continuous cortisol delivery has been identified in HeLa cells. Pulsatile cortisol treatment caused a reduction in cell viability and proliferation in comparison to continuous exposure (McMaster et al., 2011). Moreover, the transcriptional pulsing of a circadian gene, *Per1*, was experimentally confirmed in cultured cells (Stavreva et al., 2009; Conway-Campbell et al., 2011), rat liver (Stavreva et al., 2009), and rat hippocampus (Conway-Campbell et al., 2010) after ultradian glucocorticoid exposure.

In conclusion, oscillatory regulations are common in cellular dynamics and transcriptional fluctuations are a result of dynamic interactions of different regulatory mechanisms; however, many of the molecules involved in these mechanisms remain to be discovered.

1.3. Interaction between the HPA axis and the circadian clock system

The circadian clock system and the HPA axis interplay with each other at multiple levels to coordinate the physiological activities. Several studies report the regulation of the HPA axis by the SCN and peripheral clocks, while the HPA axis also influences the clock system.

1.3.1. Circadian regulation of the HPA axis

The SCN directly projects to the PVN of the hypothalamus and controls the circadian secretion of glucocorticoids in at least two different ways. One pathway is HPA-dependent, where two neurotransmitters are involved. The first one is an unknown SCN transmitter triggering the activity of the HPA axis (Buijs & Kalsbeek, 2001), while the second one is an inhibitory SCN transmitter, vasopressin, suppressing the activity of the HPA axis (Kalsbeek et al., 1992). The daily oscillation of glucocorticoids in mammals is driven by this rhythm, which also modulates stress-induced corticosterone secretion (Buijs et al., 1997). In the second pathway, the central clock controls the release of glucocorticoids in a HPA axis-independent manner via the autonomic nervous system. This autonomic control modulates the adrenal responses to ACTH (Buijs et al., 1999; Ishida et al., 2005; Ulrich-Lai et al., 2006). Therefore, the SCN maintains an optimum production of glucocorticoids in a dual mechanism.

Several studies report the regulation of the HPA axis by the peripheral circadian clocks. In a mouse study, the loss of *Per1* resulted in an elevation of glucocorticoids during the day with an impaired daily rhythm (Dallmann et al., 2006). Similar results were found for *Per2*. *Per2*-deficient mice lost the glucocorticoid rhythm, although corticosterone response is unimpaired (Yang et al., 2009). The loss of circadian rhythm of ACTH and corticosterone has been found in *Per2/Cry1* double mutant mice (Oster et al., 2006). In another study, it has been shown that peripheral CLOCK protein negatively regulates the activity of GR through acetylation of multiple lysine residues located in the hinge region of GR (Nader et al., 2009; Charmandari et al., 2011). This was confirmed by a further study, which showed that the CLOCK/BMAL1 complex reduced the maximal efficiency and maximal transactivation of GR (Han et al., 2014). In the same study, it was also reported that fibroblast cells deficient in BMAL1 and

PERs became hypersensitive to glucocorticoids. Moreover, it has been demonstrated in mouse embryonic fibroblast that *Cry1* and *Cry2* physically interact with GR; and *Cry1*- and/or *Cry2*-deficient mice suffer from hypercortisolism, suggesting that these genes are involved in the suppression of the HPA axis activity (Lamia et al., 2011). There are also experiments showing the effect of stress on clock gene mutant animals, confirming the circadian clock regulation of the HPA axis. For example, genetic loss of *Per1* results in a functional anomaly of hormonal responses of the PVN after an acute stress (Zhang et al., 2011). In an additional study, *Bmal1*-deficient mice suffered from hypocortisolism and showed blunted behavioral responses to stress (Leliavski et al., 2014).

1.3.2. The HPA axis regulation of the circadian clock

Glucocorticoids are the end-product of the HPA axis and secreted in response to stress as mentioned in the section 1.1.3. Glucocorticoids bind to GRs and regulate the expression of several genes, including the clock genes. Synchronization and phase resetting of the circadian clocks by glucocorticoids has been proven not only in animals but also in human studies.

The administration of glucocorticoids or glucocorticoid hormone analogs modulates the expression of clock genes in cell culture. In a study of cultured rat-1 fibroblasts, one hour treatment with dexamethasone activated *Per1* expression, whereas 44 hours treatment induced the expression of *Per1*, *Per2*, *Per3*, *Cry1*, *Rev-erbα* and *DBP* (Balsalobre et al., 2000). Treatment of cultured hepatic cells with prednisolone increased the expression of *PER1* in dose-dependent manner as well as *BMAL1* mRNA levels, while the expression of *PER2*, *CRY1*, *DBP* and *REV-ERBα* was considerably decreased (Koyanagi et al., 2006). In primary mesenchymal stem cells, the transcriptional oscillations of the genes involved in the positive and negative feedback loops were stimulated by dexamethasone (So et al., 2009). In another study, dexamethasone induced the transcription of *Per1* and *Per2* in the mouse embryonic fibroblasts (Cheon et al., 2013). Furthermore, pulsatile treatment of corticosterone revealed transcriptional pulsing of *Per1* nascent mRNA in mouse mammary adenocarcinoma cells (Stavreva et al., 2009).

Beside cell culture studies, there are various *in vivo* reports confirming the glucocorticoid regulation of the circadian clock. *In vivo* dexamethasone treatment resulted in a phase-shift of circadian clock genes in rat liver, kidney and heart (Balsalobre et al., 2000). Continues

administration of prednisolone decreased the amplitude of circadian clock oscillations in mouse liver and skeletal muscle (Koyanagi et al., 2006). In another study, circadian clocks in the kidney and lung have strongly been entrained by GCs (Sujino et al., 2012). Moreover, pulsatile corticosterone treatment induced the transcriptional pulsing of *Per1* in rat liver (Stavreva et al., 2009) and hippocampus (Conway-Campbell et al., 2010).

Influence of stress on circadian rhythm has been demonstrated by several animal experiments. In a study of mice, levels of *Per1* mRNA were induced after an acute physical stress in the peripheral tissues (Yamamoto et al., 2005). Repeated psychosocial stress has been found to have more negative effects on behavior, physiology and immunology during the active/dark phase of male mice, suggesting the time-of-day dependency of stress effects on peripheral clocks (Bartlang et al., 2012). Chronic subordinate stress caused phase shifting of the clock gene rhythms in the adrenal and anterior pituitary (Razzoli et al., 2014). Confirming these results, acute restraint or injection stress has phase-advanced the adrenal clock gene rhythm (Engeland et al., 2016). In another mouse study, peripheral clocks were entrained by sub-acute physical and psychological stressors (Tahara et al., 2015).

Comparison to the animal studies, there is a limited number of human studies confirming the GC regulation of clock genes. In these studies, the responsiveness of *PER1* transcript was specifically determined. Dexamethasone induced the expression of *PER1* in human bronchial epithelium and PBMCs *in vitro* (Burioka et al., 2005; Burioka et al., 2007). Hydrocortisone administration resulted in an elevation of *PER1* expression in the EBV-transformed peripheral lymphocytes (Charmandari et al., 2011). In human epithelial carcinoma cell line, treatment with dexamethasone stimulated the expression of circadian clock genes, including *PER1* (Reddy et al., 2009). In another study, the expression patterns of clock genes were modulated by dexamethasone exposure in white adipose tissue (Gomez-Abellan et al., 2012).

Beside these *in vitro* reports, only a few human studies have been performed *in vivo*. Burioka et al. demonstrated that after infusion of 20 mg prednisolone, *PER1* expression was increased in PBMCs (2007). Cuesta et al. reported a resetting of clock genes after Cortef (hydrocortisone tablet) administration (2015). Moreover, our group showed that hydrocortisone infusion caused an acute and temporary induction of *PER1* and *PER3* mRNAs in whole blood (Yurtsever et al., 2016).

1.3.3. Association of HPA axis-clock system interactions with development of health disorders

Since the HPA axis and the circadian clock system mutually interact with each other, abnormality or impairment in the function of either system may cause development of pathological conditions. Therefore, it is worth to discuss the effect of these interactions on human health.

Continuous stimulation of the HPA axis by several stressors or applying high concentrations of glucocorticoids results in various disorders. Acute stress has been associated with panic attacks and psychotic episodes, gastrointestinal symptoms, hypersensitive or hyposensitive attacks, angiokinetic phenomena as well as allergic reactions, whereas chronic stress triggers anxiety, sleep disorders or excessive daytime sleepiness, depression, osteoporosis, insulin resistance, hypertension, obesity, atherosclerotic cardiovascular diseases, type 2 diabetes mellitus, immunosuppression and neurovascular degenerative disease (Chrousos, 2009).

Shift work, jet lag and socioeconomic status can be considered as the stressors of our modern society, which disrupt circadian rhythms and have consequences on human health. Shift work has been related to metabolic disturbances (Karlsson et al., 2003), diabetes and weight gain (Pan et al., 2011), vascular disease (Vyas et al., 2012), hypertension (Kubo et al., 2013), and coronary heart disease (Vetter et al., 2016). Jet lag affects cortisol rhythms although only a few time zones are crossed (Doane et al., 2010). Circadian misalignment even for eight days caused an elevation in blood sugar and insulin levels, and in arterial pressure of human subjects, associated with increased risk for metabolic and cardiovascular diseases (Scheer et al., 2009).

The interactions between the HPA axis and the circadian clock have also consequences for the regulation of the immune system. In a human study, it has been demonstrated that natural killer cells, neutrophils, monocytes, T and B lymphocytes are controlled by the circadian clock (Born et al., 1997). *Per2* deficient mice had a decrease in the serum levels of cytokines gamma interferon and interleukin-1β after a lipopolysaccharide (LPS) administration and showed more resistance to endotoxic shock induced by LPS (Liu et al., 2006), suggesting the circadian clock regulation of the immune system. In a study of rats, neuroinflammatory reaction to an LPS administration was enhanced during the rest phase when compared to the animals treated during the active phase, showing the influence of time of day on immune system (Fonken et al., 2016). On the other hand, monocytes, granulocytes, macrophages, B

and T cells were affected (Dumbell et al., 2016) and the production of cytokines was inhibited by glucocorticoids (Ashwell et al., 2000). Moreover, in the epithelial club cells, the circadian clock controlled the pulmonary bacterial responses, which was entrained by glucocorticoids (Gibbs et al., 2014). This report nicely presents the effects of the interaction between the HPA axis and the circadian clock system on immune system.

There are various studies demonstrating that continuous exposure to stress or circadian disruption is associated with mood disorders. Continuous exposure of social stress led to the development of depression-like behaviors in male mice (Kudryavtseva et al., 1991) and rats (Rygula et al., 2006). *Clock* mutant mice showed behaviors similar to human patients in the manic state, suggesting the clock system regulation of behavior and mood (Roybal et al., 2007). Moreover, single-nucleotide polymorphisms in clock genes were associated with winter depression in humans (Partonen et al., 2007).

The HPA axis activation and/or circadian disturbances are related to the sleep disorders. Chronic activation of the stress system is associated with chronic insomnia (Vgontzas et al., 1998; Vgontzas et al., 2001) and obstructive sleep apnea (Buckley & Schatzberg, 2005). On the other hand, circadian disruptions can lead sleep disorders through two underlying mechanisms (Zhu & Zee, 2012). First one is the dysfunction of the circadian clock system, which causes irregular sleep-wake disorder, advance sleep phase disorder, delayed sleep phase disorder and free-running disorder. Second one is the misalignment of the internal clock with the environmental light/dark cycle, which results in insomnia, daytime sleepiness and difficulty in falling asleep (Zhu & Zee, 2012).

As a conclusion, various health disorders or illnesses are caused by the combined functions of the stress and the circadian systems. Thus, it is inevitable to reduce both stressors and circadian-based stressors to promote human well-being and improve quality of life.

CHAPTER 2: OBJECTIVES OF THE THESIS

The main objective of this thesis was to determine the interaction between cortisol and the expression of *PERIOD* genes in human subjects in short intervals for a few hours. In order to investigate this interaction, three studies were designed to analyze the mRNA levels of *PERIOD* genes. The first study, baseline study, aimed to determine the gene expression under non-treated, baseline conditions. The second study was a psychological stress study, where the gene expression was determined after Trier Social Stress Test (TSST). The last study, pharmacological stress study, was designed to measure gene expression after infusing donors with escalating doses of glucocorticoid analogue, hydrocortisone.

Based on the aforementioned studies from the literature, we first hypothesized that the expression of *PERIOD* genes was correlated to the cortisol under baseline conditions. In relation to this, *PERIOD* gene transcripts were expected to be pulsatile and show ultradian rhythms in whole blood. Secondly, an increase in the expression of *PER1* was predicted because of the possible increase in cortisol levels after psychological stress. We finally hypothesized that *PER1* mRNA levels would be induced in a dose-dependent manner after infusing the subjects with different doses of hydrocortisone. *PER2* and *PER3* genes might also be responsive to the stress after psychological and pharmacological treatments.

The secondary objective of this thesis was to determine the interaction between cortisol and the expression of *PERIOD* genes *ex vivo* and in cell lines. The mRNA levels of *PERIOD* genes in whole blood were expected to increase after hydrocortisone administration in an *ex vivo* setting and this increase was hypothesized to be directly dependent on GR activity. Moreover, we aimed to investigate downstream genes of glucocorticoid-induced PER1 as a transcription factor in human hepatocellular liver carcinoma cell line (HepG2). Cells were treated with hydrocortisone and transfected with an expression vector carrying the *PER1* cDNA in order to induce *PER1* expression. Then, these conditions were planned to be used for the chromatin immunoprecipitation, subsequently for next generation sequencing, to determine the possible target genes of glucocorticoid-induced PER1.

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

3.1.1. Subjects

3.1.1.1. Baseline study

A total of 17 male participants (age range 19 - 38, mean 27.5 ± 6.7 years) were included in this study. Health status of the participants was determined by a health questionnaire. Exclusion criteria were chronic or acute illnesses, medication within the previous two weeks and a BMI <18 or >30. Ethical approval of the study was obtained from the Luxembourg National Research Ethics Committee (CNER) and the National Data Protection Committee (CNPD) in accordance with the latest revision of the Declaration of Helsinki and all participants provided written informed consent.

3.1.1.2. Psychological stress study

In total, 6 male and 8 female subjects (age range 19 - 31, mean 25 ± 3) participated in this study. Women who did not use ethinyl-estradiol containing oral contraceptives were included and tested within 10 days immediately after ovulation since the activity of HPA axis is influenced by the menstrual cycle phase (Kirschbaum et al., 1999). A commercial luteinizing hormone detection urine test was applied to detect ovulation. None of the subjects smoked more than five cigarettes per day and had any history of chronic physical or psychological diseases. They reported themselves to be healthy and free of medication at the time of experiment. Ethical approval of the study was obtained from the Ethical Committee of the State's Medical Association (Landesärztekammer Rheinland-Pfalz) in accordance with the latest revision of the Declaration of Helsinki and written informed consent was obtained by all participants.

3.1.1.3. Pharmacological stress study

40 participants (20 males, 20 females) were included in this study and received a monetary reward for participation. The age range of the subjects was 20 - 30 years with mean age of 23.1 years. The BMI range was 19.5 - 26.5 kg/m^2 with mean BMI of 21.9 kg/m^2. Age and BMI were similar among dosing groups. The health status of the participants was determined by a physician using a standard medical examination and interview procedure. Any acute or chronic somatic or psychiatric illnesses; any history of psychiatric, cardiovascular, sleep- or stress-related disorders; glaucoma, pregnancy, smoking, increased caffeine consumption; any illicit drug intake within the last six months; any family history of epilepsy or aneurysms were specified as exclusion criteria for participation. Ethical approval of the study was obtained from the Ethical Committee of the State's Medical Association (Landesärztekammer Rheinland-Pfalz) in accordance with the latest revision of the Declaration of Helsinki and written informed consent was obtained by all participants.

3.1.2. Cell line

Table 1: Cell line used in the studies

Cell line	***Type***
HepG2	Human hepatocellular liver carcinoma

3.1.3. Bacterial strains

Table 2: Bacterial strains used in the studies

Bacterium	***Stain***	***Company***
Escherichia coli K12	XL1-Blue	Stratagene, La Jolla, CA
	DH5α	BRL Life Technologies, Gaithersburg, MD

3.1.4. Vectors

Table 3: Cloning and expression vectors used in the studies

Vector	*Manufacturer*
p TZ57R (TA)	Thermo Scientific, Schwerte (Germany)
pcDNA3.1(+)	Invitrogen, Waltham, MA

3.1.5. Oligonucleotides

Table 4: Primers for real-time PCR and optimized PCR conditions

Genes	*Primer sequence (5' - 3')*	*Product size (bp)*	*Tm (°C)*	*MgCl2 (mM)*	*Primers (µM)*
PER1	for: gtggctgtctccttcctgac rev: gttaggcggaatggctggta	293	60	1	0.1
PER2	for: ggagagcgttacctctgagc rev: gtgaaactgtggaacacgcc	195	60	1	0.2
PER3	for: tcggaaccttgctgtctcac rev: aacgatcttcagggtgcagg	211	60	1	0.1
GILZ	for: gcacaatttctccatctccttctt rev: tcagatgattcttcaccagatcca	146	60	2	0.2
GR	for: gttgattttccaaaagg rev: caatgctttcttccaa	170	60	1	0.2
PRL	for: aatgttcatttctggccagt rev: ttccctttcctggtcatct	118	60	2	0.2
GAPDH	for: gaaggtgaaggtcggagtc rev: gaagatggtgggatttc	146	60	2	0.5
ACTB	for: ggccacggctgcttc rev: gttggcgtacaggtctttgc	208	60	1	0.5
PER1 cDNA PCR1	for: acactcctgcgaccaggtac rev: atcctaggctggtgatgggg	4181	59	1,50	0.2

PER1 cDNA PCR2	for:ctaagaattcccagacatgagtggccccct agaa rev:atccgtttaaactggagtctagctggtgca gtttcc	3873	59	1,5	0.2

Table 5: Primers for sequencing

	Primer	***Sequence (5' - 3')***	***Tm (°C)***	***GC content***
	Fwd2_pcDNASEQ	agagcagcaagagcacaa	56.79	50.00
	Fwd3_pcDNASEQ	ggatgtgggagtcttctatg	54.64	50.00
	Fwd4_pcDNASEQ	aagaagattctgcagttggc	56.32	45.00
	Fwd5_pcDNASEQ	agggccagcagcttttta	56.72	50.00
	Fwd6_pcDNASEQ	agtcagtgtagcttcagct	55.66	47.37
	Fwd7_pcDNASEQ	tttggtgaccccaatggt	56.18	50.00
	Fwd8_pcDNASEQ	aaatactttggcagcatcga	55.73	40.00
	Fwd9_pcDNASEQ	aggaaactgcaccagcta	55.68	50.00
PER1 cDNA	Fwd10_pcDNASEQ	gccgatggcatttgtgtt	56.69	50.00
specific primers	Rev2_pcDNASEQ	gattcctctccaaaggtgg	54.85	52.63
	Rev3_pcDNASEQ	cgctggtcctcagaaaac	55.72	55.56
	Rev4_pcDNASEQ	ggaaagtgcgtcctgattg	56.63	52.63
	Rev5_pcDNASEQ	gtgaggggtgtgagacata	55.73	52.63
	Rev6_pcDNASEQ	ttcttgtctcccacatgga	55.51	47.37
	Rev7_pcDNASEQ	tgcttcaccagatgcaca	56.77	50.00
	Rev8_pcDNASEQ	gtcctcaggatgcaggaa	55.87	55.56
	Rev9_pcDNASEQ	actcagacgtgatgtgct	55.85	50.00
	Rev10_pcDNASEQ	tgttggcatcggtgtcat	56.86	50.00
pcDNA specific	Fwd1 _T7	taatacgactcactataggg	50.32	40.00
primers	Rev1_BGH	tagaaggcacagtcgagg	55.62	55.56
TA vector specific	M13_for	tgtaaaacgacggccagt	64.00	50.00
primers	M13_rev	caggaaacagctatgac	56.00	47.00

3.1.6. Reagents

Table 6: Reagents used in the studies

Reagents	*Manufacturer*
Agar agar	Roth, Karlsruhe
Agarose LE	Biozym, Oldendorf
Ampicillin	Roth, Karlsruhe
Bromphenol blue	Serva, Heidelberg
Buffer 2	New England Biolabs, Frankfurt am Main
Buffer *Eco*RI	Thermo Scientific, Waltham, MA (USA)
Charcoal filtered fetal bovine serum	Lonza, Rockland, ME (USA)
Chloroform:isoamyl alcohol	AppliChem, Darmstadt
Dimethylsulfoxide (DMSO)	Merck, Darmstadt
Dinatriummethylendiaminteraacetat (EDTA)	Roth, Karlsruhe
dNTPs (ATP, TTP, CTP, GTP)	Fermentas, St- Leon-Roth
Eagle's Minimum Essential Medium (EMEM)	Lonza, Rockland, ME (USA)
Ethanol (100%)	Merck, Darmstadt
Ethidiumbromid	Roth, Karlsruhe
Ethylenglycol-bis(aminoethylether) (EGTA)	Sigma-Aldrich, München
Fetal bovine serum (FBS)	Biochrom, Berlin
Ficoll 400	Roth, Karlsruhe
Formaldehyde	Roth, Karlsruhe
Gene Ruler 1 kb DNA Ladder	Fermentas, St- Leon-Roth
Gene Ruler 1 kb plus DNA Ladder	Fermentas, St- Leon-Roth
Gene Ruler 100bp DNA Ladder	Fermentas, St- Leon-Roth
Glycerine	Roth, Karlsruhe
Glycine	Merck, Darmstadt
Hydrocortisone	Sigma-Aldrich, München
Hydrocortisone for infusion	Rotexmedia, Trittau
Magnesium chloride ($MgCl_2$)	Invitrogen, Waltham, MA (USA)
Non-essential amino acids	Lonza, Rockland, ME (USA)
Penicillin/Streptomycin	Lonza, Rockland, ME (USA)
Phenol:chloroform:isoamyl alcohol	Roth, Karlsruhe
Phenylmethylsulonylfloride (PMSF)	Sigma-Aldrich, München

Phosphate-buffered saline (PBS)	Lonza, Rockland, ME (USA)
RU486	Sigma-Aldrich, München
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe
Sodium pyruvate	Lonza, Rockland, ME (USA)
Sodium-butyrate	Merck, Darmstadt
Sodium chloride (NaCl)	Roth, Karlsruhe
Sodium chloride for infusion	Braun, Melsungen
Sodium-deoxycholate	Sigma-Aldrich, München
SYBR green	Lonza, Rockland, ME (USA)
T4 DN ligase buffer	Thermo Scientific, Waltham, MA (USA)
Tris-borate- EDTA (TBE) buffer Rotiphorese	Roth, Karlsruhe
Tris-HCl	Roth, Karlsruhe
TritonX-100	Roth, Karlsruhe
Trypan blue	Lonza, Rockland, ME (USA)
Trypton/Pepton	Roth, Karlsruhe
Xylencyanol	Serva, Heidelberg
Yeast extract	Roth, Karlsruhe

3.1.7. Machines

Table 7: Machines used in the studies

Machines	***Manufacturer***
Centrifuge Avanti J-25 I	Beckman Coulter, Brea, CA (USA)
CEQ8000 Genetic Analysis System	Beckman Coulter, Brea, CA (USA)
D-50 Digital-Camera	Nikon, Düsseldorf
Diagenode Bioruptor® UCD-200 sonicator	Diagenode, Liège (Belgium)
DNA engine Opticon® 2 system	Biorad, Hercules, CA (USA)
Electrophoresis Power Supply EPS200	Pharmacia Biotech, Freiburg
Electrophoresis Power Supply EPS3500XL	Pharmacia Biotech, Freiburg
Gel chamber	Pharmacia Biotech, Freiburg
Incubator	Heraeus Instruments, Hanau
Incubator for cell culture	Sanyo, München
Microfuge centrifuge	Beckman Coulter, Brea, CA (USA)

Microscope	Wilovert, Wetzlar
Microwave	Sharp Electronics, Hamburg
Multigene™ Gradient PCR Thermocycler	Labnet, Edison, NJ (USA)
Scale Europe 4000 AR	Gibertini, Paderno Dugnano MI (Italy)
Shaker incubator	Gallenkamp, Loughborogh (UK)
Sterile bank for cell culture	Jouan, Fernwald
Thermomixer 5436	Eppendorf, Hamburg
UV/VIS Spektrophotometer	Picodrop, Cambridge (UK)
UV/VIS Spektrophotometer	Nanodrop 2000, ThermoScientific, Waltham, MA (USA)
UV-Illuminator N-90M	INTAS UV-System, Wiesloch
Vortex mixer 7-2020	neoLab, Heidelberg
Water bath for cell culture	Lauda-Brinkmann, Delran, NJ (USA)
Water bath SW21	Julabo, Schilbach

3.1.8. Media and Buffers

Table 8: HepG2 culture medium (500 ml)

Content	*Stock*	*Amount (ml)*
EMEM		437.5
10 % FBS		50
0.5 % Penicillin/streptomycin	20000 u/ml	2.5
1 % Non-essential amino acids	100x	5
1 % Sodium pyruvate	100 mM	5

Table 9: HepG2 culture medium for stimulation (500 ml)

Content	*Stock*	*Amount (ml)*
EMEM		437.5
10 % Charcoal filtered FBS		50
0.5 % Penicillin/streptomycin	20000 u/ml	2.5
1 % Non-essential amino acids	100x	5
1 % Sodium pyruvate	100 mM	5

Table 10: LB medium (500 ml)

Content	*Amount*
NaCl	5 g
Trypton	5 g
Yeast extract	2.5 g
Water	Up to 500 ml

Table 11: LB-agar medium (500 ml)

Content	*Amount*
NaCl	5 g
Trypton	5 g
Yeast extract	2.5 g
Agar agar	10 g
Water	Up to 500 ml

Table 12: SOC medium (1 L)

Content	*Amount*
NaCl	0.5 g
Trypton	20 g
Yeast extract	5 g
Water	Up to 1 L
1 M $MgCl_2$	10 ml
1 M $MgSO_4$	10 ml
2 M Glucose	10 ml

Table 13: PBS/Na-butyrate buffer (10 ml)

Content	*Final concentration*	*Stock concentration*	*Amount (ml)*
Na-butyrate	20 mM	2 M	0.1
1x PBS			9.9

Table 14: Lysis buffer (2.5 ml)

Content	*Final concentration*	*Stock concentration*	*Amount (ml)*
Tris–HCl, pH 8.0	50 mM	1 M	0.1250
EDTA	10 mM	0.5 M	0.0500
SDS	1 % (wt/vol)	10 %	0.2500
protease inhibitor mix	1x	20 x	0.1250
PMSF	1mM	200 mM	0.0125
Na-butyrate	20mM	2 M	0.0250
Water			1.9125

Table 15: RIPA ChIP buffer (12 ml)

Content	*Final concentration*	*Stock concentration*	*Amount (ml)*
Tris–HCl, pH 7.5	10 mM	1 M	0.120
EDTA	1 mM	0.5 M	0.024
EGTA	0.5 mM	0.4 M	0.015
SDS	0.1 % (wt/vol)	10%	0.120
TritonX-100	1 % (vol/vol)	25%	0.480
Na-deoxycholate	0.1 % (wt/vol)	10%	0.120
NaCl	140 mM	5 M	0.333
protease inhibitor mix	1x	20x	0.600
PMSF	1 mM	200 mM	0.060
Na-butyrate	20 mM	2 M	0.120
water			10.130

Table 16: RIPA buffer (16 ml)

Content	*Final concentration*	*Stock concentration*	*Amount (ml)*
Tris–HCl, pH 7.5	10 mM	1 M	0.160
EDTA	1 mM	0.5 M	0.032
EGTA	0.5 mM	0.4 M	0.020
SDS	0.1 % (wt/vol)	10%	0.160
TritonX-100	1 % (vol/vol)	25%	0.640
Na-deoxycholate	0.1 % (wt/vol)	10%	0.160
NaCl	140 mM	5 M	0.440
water			14.380

Table 17: TE buffer (3 ml)

Content	*Final concentration*	*Stock concentration*	*Amount (ml)*
Tris–HCl, pH 8.0	10 mM	1 M	0.03
EDTA	10 mM	0.5 M	0.06
water			2.91

Table 18: Complete elution buffer (3 ml)

Content	*Final concentration*	*Stock concentration*	*Amount (ml)*
Tris–HCl, pH 7.5	20 mM	1 M	0.0600
EDTA	5 mM	0.5 M	0.0300
NaCl	50 mM	5 M	0.0300
Na-butyrate	20 mM	2 M	0.0300
SDS	1 % (wt/vol)	10 %	0.3000
proteinase K	50 µg/mL	20 mg/mL	0.0075
water			2.5425

Table 19: Elution buffer (7 ml)

Content	*Final concentration*	*Stock concentration*	*Amount (ml)*
Tris–HCl, pH 7.5	20 mM	1 M	0.14
EDTA	5 mM	0.5 M	0.07
NaCl	50 mM	5 M	0.07
water			6.72

Table 20: Loading dye for gel electrophoresis

Content	*Amount*
Ficoll 400	15 g
Bromphenol blue	250 mg
Xylencyanol	250 mg
Water	Up to 100 ml

3.1.9. Commercial kits

Table 21: Kits used in the studies

Kit	*Manufacturer*
Freeze 'N Squeeze DNA gel extraction spin columns	Bio-Rad, Hercules, CA (USA)
gabControl® luteinizing hormone detection urine test	gabmed, Nettel-Loberich
Gel extraction kit	PeqGOLD, Darmstadt
GenomeLab dye terminator cycle sequencing kit	Beckman Coulter, Brea, CA (USA)
InsTAclone PCR cloning kit	Thermo Scientific, Waltham, MA (USA)
Liaison® cortisol *in vitro* diagnostic competitive immunoluminometric sandwich assay	Diasorin, Saluggia (Italy)
Lipofectamine® 3000 transfection reagent	Invitrogen, Waltham (USA)
PAXgene blood RNA tubes	PreAnalytiX, Hombrechtikon (Switzerland)
PAXgene blood RNA kit	PreAnalytiX, Hombrechtikon (Switzerland)
Plasmid miniprep kit II	PeqGOLD, Darmstadt
RevertAid first strand cDNA synthesis kit	Thermo Scientific, Waltham, MA (USA)
RiboPure-blood kit	Ambion, Foster City, TX (USA)
Rneasy mini kit	Qiagen, Hilden
Salivary cortisol ELISA kit	Salimetrics, Newmarket (UK)
SuperScript® III first strand cDNA synthesis kit	Invitrogen, Waltham (USA)

3.1.10. Enzymes and proteins

Table 22: Enzyme and proteins used in the studies

Enzymes and proteins	*Manufacturer*
Anti-PER1 antibody (sc-7724)	Santa Cruz Biotechnology, Santa Cruz, CA (USA)
*Eco*RI	Thermo Scientific, Waltham, MA (USA)
*Eco*RV	New England Biolabs, Frankfurt am Main
IgG control antibody (sc-2027)	Santa Cruz Biotechnology, Santa Cruz, CA (USA)
Platinum *Taq* DNA polymerase	Invitrogen, Waltham, MA (USA)
Proteinase K	Qiagen, Hilden
Rnase A	Qiagen, Hilden
T4 DNA ligase	Fermentas, St- Leon-Roth
Trypsin with Versene	Lonza, Rockland, CA (USA)

3.1.11. Consumable materials

Table 23: Other materials used in the studies

Consumable materials	*Manufacturer*
6 well tissue plate	Sarstedt, Nümbrecht
Bioruptor® microtube	Diagenode, Liège (Belgium)
EDTA Vacutainer tubes	BD Diagnostics, Preanalytical Systems, Heidelberg
Neubauer counting chamber	Marienfeld, Lauda Königshofen
Heparinized Vacutainer tubes	BD Diagnostics, Preanalytical Systems, Heidelberg
Intravenous catheter	Vasofix Braunüle, Braun, Melsungen
Magnetic rack	Invitrogen, Waltham, MA (USA)
Pierce™ ChIP-grade protein A/G magnetic beads	Thermo Scientific, Waltham, MA (USA)
Saliva collection devices (Salimetrics oral swabs)	Salimetrics, Newmarket (UK)
Salivettes® sampling devices	Sarstedt, Nümbrecht

Syringe pump	Infusomat fm, Braun, Melsungen
Tissue culture flask T75 (Stand., Vent. Cap)	Sarstedt, Nümbrecht

3.1.12. Databases and software

Table 24: Databases and software used in the studies

Databases and Software	***Websites/Manufacturer***
GeneCards	www.genecards.or
Ensembl Genome Browser	www.ensembl.org
MJ Opticon Monitor Analysis Software version 3.1	Biorad, Hercules, CA (USA)
National Center for Biotechnology Information	www.ncbi.nlm.nih.gov
R software version 1.1.2	www.r-project.org
SPSS 21	SPSS, IL (USA)

3.2. Methods

3.2.1. Saliva and blood sampling

3.2.1.1. Baseline study

An indwelling intravenous cannula was inserted in the arm of the participants and EDTA blood was drawn every 15 minutes from 08:00 h until 16:00 h for the analysis of *PER1*, *PER2* and *PER3* messenger RNA (mRNA) and plasma cortisol. For mRNA stabilization, 300 µl blood were pipetted into 1.3 ml of RNAlater® incubated at room temperature for two hours and stored at -20 ˚C until analysis. With saliva collection devices saliva was collected concurrently with blood sampling for the analysis of free cortisol. Samples were stored at -20 ˚C until analyzed.

3.2.1.2. Psychological stress study

Salivary samples were collected using Salivettes® sampling devices at 2 min before TSST and 2, 10, 20, 30, 45, 60, 120, 150, 180, 210, 240, 270 min after TSST. Samples were stored at -20 ˚C until analyzed. An indwelling intravenous cannula was inserted in the arm of the participants and blood samples were drawn into EDTA Vacutainer tubes concurrently with saliva sampling for mRNA analysis. For mRNA stabilization, 300 µl blood were pipetted into 1.3 ml of RNAlater®, incubated at room temperature for two hours and stored at -20 ˚C until analysis.

3.2.1.3. Pharmacological stress study

A flexible intravenous catheter was inserted in the right arms of the participants at the beginning of the experiment. The beginning of the experiment varied from 12:00 h to 14:25 h with a mean of 12:54 h ± 00:50 h and two subjects were treated per day. Participants were brought to a waiting room to have a 45-minute resting period in order to avoid a potential carry-over stress effects from the insertion of the needle. Then, blood was drawn every 15 minutes up to 165 minutes for the analysis of *PER* mRNA and for the analysis of plasma cortisol. Salivary samples were collected using Salivettes® sampling devices concurrently

with blood sampling. For mRNA stabilization, 300 µl blood were pipetted into 1.3 ml of RNAlater®, incubated at room temperature for two hours and stored at -20 ˚C until analyzed.

3.2.2. Study protocol

3.2.2.1. Baseline study

Donors who live and work very close to the Clinical Investigation and Epidemiological Center, Luxembourg, were included in the study to reduce the time spent between awakening and arrival. All participants were informed about the study in details several days prior to the experiment and were told that they had the right to stop the experiment at any time. The experimental procedure (represented in Figure 6A) started from the time of arrival. Participants were allowed to do normal ambulatory activity during the experiment and received meals at 9:00 h and 12:00 h.

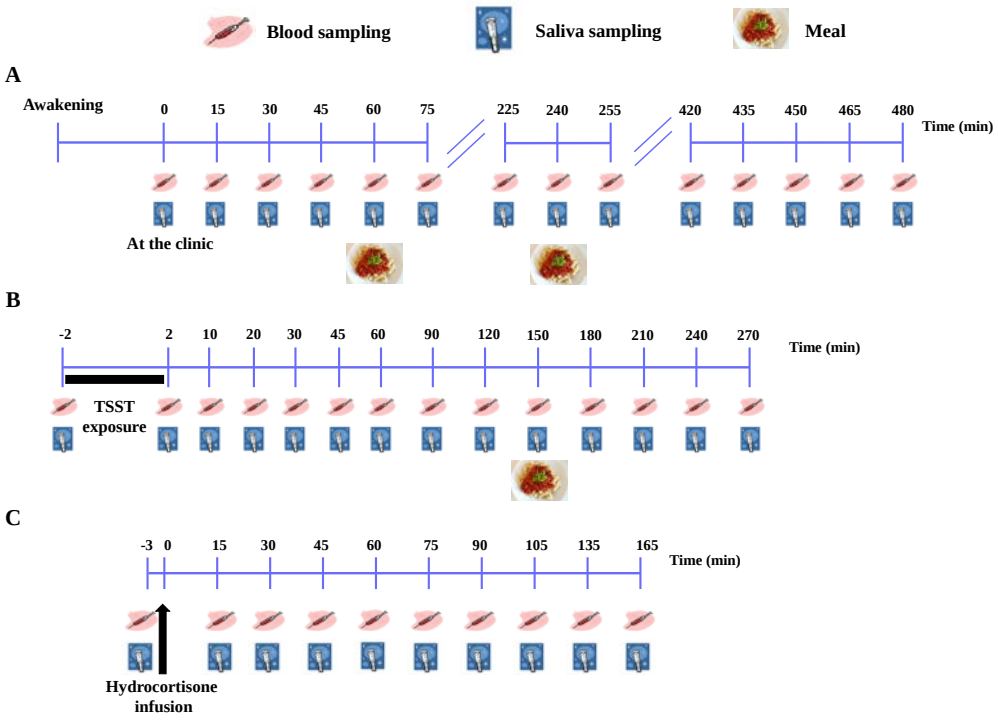

Figure 6: Schematic representation of the experimental design for baseline study (A), psychological stress study (B) and pharmacological stress study (C).

3.2.2.2. Psychological stress study

The standardized laboratory stress test, TSST, consists of a three minutes preparation period, a five minutes public speaking and a five minutes mental arithmetic task in front of an audience. Subjects arrived at 13:00 h and subsequently an indwelling cannula was inserted in the left arm of the participants before the TSST starts. The TSST was started at 14:00 h (T = 0 min) when subjects were taken into the TSST room and introduced to the task. A light meal was provided since the sampling period was long. The meal was announced at 120 min and provided at 150 min post-TSST. Experimental procedure was represented in Figure 6B.

3.2.2.3. Pharmacological stress study

A flexible intravenous catheter connected to a syringe pump was inserted in the left arm of the participants at the beginning of the experiment. After the resting period, the pharmacological manipulations were applied. Participants were randomly assigned to five groups, with an equal number of males and females, and received a saline placebo solution (0.9 % NaCl) or 3 mg, 6 mg, 12 mg, 24 mg of hydrocortisone (Figure 6C). For further details on the procedure of the study see Schilling et al., 2013.

3.2.3. Biochemical analysis of cortisol

In the pharmacological stress study, free cortisol concentrations in the salivary samples were determined by a time resolved fluorescence immunoassay as described previously (Dressendorfer et al., 1992), whereas they were measured by commercial ELISA in the baseline study. The time resolved fluorescence immunoassay sensitivity was 0.173 nmol/L. The intra-assay coefficient of variation was in the span of 4.0 % and 6.7 %, and the corresponding inter-assay coefficient of variation was in the span of 7.1 % and 9.0 %. The commercial ELISA had a minimal detection limit of 0.03 ng/ml, and intra- and inter-assay variability of 3.5 % and 5.08 %. Cortisol concentrations in the plasma were determined by an *in vitro* diagnostic competitive immunoluminometric sandwich assay. The assay sensitivity was 15 ng/ml. Intra-assay variability was 2.96 % and inter-assay variability was 5.58 % (according to the company).

3.2.4. Gene expression analysis

Total RNA was isolated from blood using the RiboPure-Blood Kit, according to the manufacturers' protocol. First strand synthesis of total cDNA (following an established protocol at the Luxembourg Institute of Health) was carried out at 55 °C using 200 U/µl SuperScript III reverse transcriptase, 250 ng/µl dN6 primers in a 60 µl reaction containing 375 mM KCl, 250 mM Tris-HCl, 15 mM MgCl2, 10 mM dithiothreitol and 500 µM dNTPs. Amplification of cDNA by PCR was performed in 25 µl reactions containing 200 µM dNTPs, MgCl2, gene specific primers (see Table 4), 5 U Platinum *Taq* DNA polymerase and 1x concentrated SYBR green. Thermal cycling was performed in an Opticon® 2 system. Cycling conditions were: one cycle at 94 °C for 4 min, followed by 40 cycles each including denaturation at 94 °C for 30 sec; annealing at gene specific temperatures (see Table 4) for 30 sec and elongation at 72 °C for 30 sec. The primer sequences and optimized PCR conditions including concentrations of magnesium chloride and primers are shown in Table 4. All primers were synthesized by Biomers (Ulm, Germany). All genes were assayed in triplicates for each sample on three separate PCR plates. The threshold cycle (Ct) values and PCR efficiency were computed with the MJ Opticon Monitor Analysis Software, version 3.1. The criterion for the PCR efficiency was between 90 % and 110 % (Broeders et al., 2014). For each gene a melting curve analysis was performed. Each investigated PCR product showed only single peak and no primer-dimer peaks or artifacts.

Schote and her colleagues measured the expression of three housekeeping genes β-actin (*ACTB*), peptidylprolyl isomerase A (*PPIA*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in the peripheral blood mononuclear cells in order to determine the most stable reference gene (Schote et al., 2007). The assumption for the stability was that the Ct ratio of at least two genes would be identical for all donors and for *GAPDH* the ratios between Ct values were the most similar. Therefore, Ct values of the target mRNAs were normalized to *GAPDH* as the most stable internal reference gene. The relative expression of each target mRNA was calculated using the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ relative quantification method where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal reference gene}}$ and $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{internal reference gene}})$ sample A - $(Ct_{\text{target gene}} - Ct_{\text{internal reference gene}})$ sample B (Schmittgen & Livak, 2008b). $2^{-\Delta\Delta Ct}$ method was used in the baseline study and the last time point was the calibrator, while $2^{-\Delta Ct}$ method was used in the stress studies. $2^{-\Delta Ct}$ method gives individual data points, independent from a calibrator. Since different participants were used for different stress studies, we could relate the expressions of the genes among different groups by using the $2^{-\Delta Ct}$ method.

3.2.5. *Ex vivo* Hydrocortisone and RU486 treatment

Blood samples were drawn by venipuncture into heparinized Vacutainer tubes from five healthy subjects (2 males, 3 females). Blood was incubated in falcon tubes (2.5 ml in each) either with 10 µM hydrocortisone, 5 µM RU486, which is a GR antagonist, a combination of both or ethanol as vehicle for one hour in a water bath at 37 °C. RU486 was added to the whole blood 15 min before hydrocortisone administration. In order to isolate total RNA, blood was transferred into the PAXgene Blood RNA tubes to stabilize the intracellular RNA in the treated samples. Blood samples were incubated for 18 hours in these tubes to ensure complete lysis of the blood cells. Subsequently, RNA was isolated with the PAXgene Blood RNA Kit according to the manufacturers' protocol. First strand synthesis of total cDNA (following an established protocol at the University of Trier) was carried out at 42 °C using 200 U/µl RevertAid M-MuLV reverse transcriptase, 0.5 µg/µl Oligo$(dT)_{18}$ primers in a 40 µl reaction containing 250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT and 10 mM dNTPs. Amplification of cDNA by real-time PCR was performed in 25 µl reactions as described above.

3.2.6. Cell Culture

The human hepatocellular liver carcinoma cell line (HepG2) was cultured in tissue culture flasks T-75 with complete growth medium. This medium includes Eagle's Minimum Essential Medium (EMEM) with L-glutamine containing 10 % fetal bovine serum (FBS), 0.1 mg/ml penicillin/streptomycin, 1 % non-essential amino acids and 1 % sodium pyruvate. When they reached 90 % - 95 % confluency, they were split 1:4 every 3 days. In order to split, cells were rinsed with 1 x PBS twice and treated with 3 ml trypsin-Versene for 5 - 7 min at 37°C. When cell layer is dispersed, trypsin was deactivated by adding 13 ml complete growth medium. 4 ml of this medium was transferred in a new flask containing 21 ml complete growth medium and cells were incubated at 37 °C in humidified atmosphere with 5 % CO_2.

3.2.6.1. Hydrocortisone stimulation

HepG2 cells were cultured in 6 well tissue culture plates with special complete growth medium containing 10 % charcoal filtered FBS, 0.1 mg/ml penicillin/streptomycin, 1 % non-essential amino acids and 1 % sodium pyruvate and incubated at 37 °C in humidified atmosphere with 5 % CO_2. Cell number was determined with a Neubauer chamber and $2x10^5$ cells were seeded in 6 well plates and incubated 24 h before stimulation. 1 mg hydrocortisone was dissolved in 1 ml ethanol (100 %) and dilutions were prepared with complete growth medium containing 10 % charcoal filtered FBS. After 24 h, cells reached 50 % confluency and were rinsed with 1 x PBS twice. 3 ml special complete growth medium containing 1 µM or 10 µM hydrocortisone was added into the appropriate wells of the plate and incubated for another 24 h until they reached approximately 80 % confluency.

3.2.6.2. Overexpression of *PER1*

3.2.6.2.1. Sequencing of pcDNA3.1-PER1 and sub-cloning into TA vector

The expression vector, pcDNA3.1 (+), containing human *PER1* cDNA was provided by Sigal Gery (Cedars-Sinai Medical Center, Los Angeles, CA). Plasmid was delivered on a filter paper. The paper, where plasmid was dropped, was cut into 6 small pieces with sterilized tweezer and scissor. These pieces of paper were placed into a 2 ml microtube, 130 µl of sterile water was added on the top and tube was incubated at 37 °C for 1 h in a thermomixer. In order to transform the pcDNA-*PER1* plasmid into DH5α and XL-1 blue bacterial cells, 2 µl water from this tube was added into the competent bacterial cells and incubated on ice for 30 min. Cells were heat-shocked at 42 °C for 90 sec and incubated on ice for 2 min. 800 ml SOC medium was added in each tube and solutions were transferred into conical tubes appropriate for bacterial growth. After 1 h incubation at 37 °C, 100 µl of the transformed bacteria were plated on LB agar plates containing ampicillin. Colony PCR was performed to check the presence of *PER1* cDNA in 25 µl reactions containing 200 µM dNTPs, $MgCl_2$, *PER1* primers (see Table 4), 5 U Platinum *Taq* DNA polymerase and 1x concentrated SYBR green. Thermal cycling was performed in an Opticon® 2 system. Cycling conditions were: one cycle at 94 °C for 4 min, followed by 40 cycles each including denaturation at 94 °C for 30 sec; annealing at 60 °C for 30 sec and elongation at 72 °C for 30 sec. PCR products were visualized in a 1.5 % agarose gel with ethidium bromide. After confirming the presence of *PER1*, bacterial colonies

were culture in LB medium containing ampicillin overnight and large amounts of plasmid was isolated by using plasmid miniprep kit II according to manufacturer`s instructions.

Before transfection, pcDNA3.1-*PER1* was sequenced by using GenomeLab dye terminator cycle sequencing kit according to the manufacturer`s instructions. Sequencing revealed that the guanine of the starting codon (ATG) was missing in the sequence of *PER1* cDNA. Therefore, total RNA was isolated from human blood cells and first strand cDNA synthesis was performed as explained in the section 3.2.6. cDNA was amplified by nested PCR. First PCR was performed in 50 µl reactions containing 200 µM dNTPs, $MgCl_2$, *PER1* cDNA PCR1 primers (Table 4), 5 µl cDNA and 5 U Platinum *Taq* DNA polymerase. Thermal cycling was performed in a Multigene^{TM} gradient PCR thermocycler. Cycling conditions were: one cycle at 94 °C for 2 min, followed by 40 cycles each including denaturation at 94 °C for 15 sec; annealing at 59 °C for 30 sec and elongation at 68 °C for 4 min, final elongation at 68 °C for 10 min. The product of this reaction was used as a template in the second PCR. Second PCR was performed in 50 µl reactions containing 200 µM dNTPs, $MgCl_2$, *PER1* cDNA PCR2 primers (Table 4), 5 µl product of first PCR and 5 U Platinum *Taq* DNA polymerase. PCR products were loaded on a 0.8 % agarose gel. The band at around 4000 bp was cut and DNA was extracted by freeze `n squeeze DNA gel extraction columns according to manufacturer`s instructions. In order to increase the DNA concentration after gel extraction, the flow-through from the column was mixed with 2.5 x ethanol (100 %) and centrifuged at 4 °C for 30 min at 14,000 rpm in a microfuge centrifuge. Supernatant was removed and pellet was washed with 70 % ethanol and centrifuged at 4 °C for 15 min at 14,000 rpm. After removing the supernatant, pellet was resolved in 30 µl TE buffer. 23 µl of this extracted DNA was mixed with 3 µl p TZ57R (TA) vector, 10 x T4 DNA ligase buffer and 1 µl T4 DNA ligase, and incubated at 4 °C overnight. Ligated cDNA-TA vector was transformed into XL-1 blue cells by InsTAclone PCR cloning kit according to manufacturer`s instructions. LB agar plates containing the transformed XL-1 blue cells were incubated 24 h at 37 °C. Colony PCR was performed with *PER1* primers (Table 4) in order to check the insertion of *PER1* cDNA into TA vector. Gel electrophoresis was used to visualize the PCR product. After confirming the insert in the TA vector, the plasmid was sequenced by using sequencing specific primers for TA vector and *PER1* cDNA (Table 5). Sequencing revealed that 347 bp were missing in exons covering 16, 17 and 18. Therefore, this *PER1* transcript could not be used for cloning into pcDNA. We needed another strategy to insert the missing guanine in the sequence of pcDNA-*PER1* vector.

3.2.6.2.2. Replacement strategy

In order to insert the guanine of the starting codon (ATG) in the sequence of *PER1* cDNA in pcDNA, a sequence replacement was performed. A specific region (1466 bp) covering the missing guanine from TA vector-*PER1* was cut by two restriction enzymes (*Eco*RI and *Eco*RV). Consequently, pcDNA3.1-*PER1* was also cut by the same enzymes which gave an opportunity to make a replacement in the expression vector. pcDNA-*PER1* and TA-*PER1* were restricted by the same enzyme but in separate tubes. Restriction was performed in 50 µl reactions containing 36 µl pcDNA-*PER1* or 40 µl TA-*PER1*, 10 x *Eco*RI buffer and 5 µl *Eco*RI. Mixtures were incubated at 37 °C for 1 h. In order to get rid of *Eco*RI and buffer, ethanol precipitation was performed as previously explained. Pellets in the tube were resuspended in 31 µl water and mixed with 4 µl 10 x buffer 2 and 5 µl *Eco*RV to get in total 40 µl restriction reactions. Solutions were incubated at 37 °C for 1 h and enzyme was inactivated at 80 °C for 20 min. 1 % gel was prepared and restricted plasmids were loaded. 7822 bp long sequence from pcDNA-*PER1* and 1466 bp long sequence from TA-*PER1* were cut from the gel and DNA fragments were extracted by gel extraction kit. Ligation was performed in 30 µl reactions containing 21 µl extracted *PER1* cDNA, 5 µl extracted pcDNA, 3 µl 10 x T4 DNA ligase buffer and 1 µl T4 DNA ligase. Ligation was incubated at room temperature for 2 h. 5 µl ligation mixture was used for transformation into DH5α and XL-1 blue bacterial cells as explained above. After 24 h incubation at 37 °C, cells grown in the agar plates were checked for the presence of *PER1*. Plasmids were isolated and sequenced by specific primers for pcDNA and *PER1* cDNA (Table 5).

3.2.6.2.3. Transfection protocol

HepG2 cells were cultured in 6 well tissue culture plates with complete growth medium and incubated at 37 °C in humidified atmosphere with 5 % CO_2. 2 x 10^5 cells were seeded in 6 well plates and incubated 24 h before transfection. Three µg of pcDNA3.1 expression vector containing human *PER1* cDNA was diluted with culture medium and P3000 reagent following the manufactures instruction in order to get 125 µl DNA mixture. 7.5 µl Lipofectamine® 3000 was diluted with 117.5 µl culture medium and added to 125 µl DNA mixture. After incubating 5 min at room temperature, this 250 µl mixture was added dropwise to cells that reached 50 % confluency in the 6 well plates. Cells were incubated for another 24 h until they reach 80 % confluency. The overexpression of *PER1* was determined via qPCR.

3.2.7. Chromatin immunoprecipitation

HepG2 cells were cultured until they reached 90 % - 95 % confluency in the tissue culture flasks T-75 for non-treated cells and 80 % confluency in the 6 well plates for stimulated and transfected cells as explained in section 2.2.7. Multiple flasks and plates were handled simultaneously. Cells were rinsed with ice-cold PBS/Na-butyrate buffer (20 ml for the flask and 3 ml for the plate) and treated with trypsin-Versene (5 ml for the flask and 600 µl for the plate) for 10 min at 37°C. When the cell layer was dispersed, trypsin was suppressed by adding complete growth medium (10 ml for the flask and 2 ml for the plate). After getting single cells suspension, multiple flasks and plates from the same conditions were pooled and transferred in three 50 ml conical tubes (1 tube for non-treated cells, 1 tube for stimulated cells and 1 tube for transfected cells). Cells were centrifuged at 1500 rpm for 5 min at room temperature and supernatant was discarded. Cells were resuspended in 25 ml ice-cold PBS/Na-butyrate buffer and centrifuged at 1500 rpm for 5 min. After removing the supernatant, cells were resuspended in 21.6 ml complete growth medium. In order to fix cells for chromatin immunoprecipitation, 600 µl formaldehyde solution (1 % final concentration) was added and tubes were incubated for 10 min at room temperature by swirling occasionally. Cross-linking was stopped by adding 2.5 ml of 1.25 M glycine (0.125 M final concentration) and incubating for 5 min at room temperature by swirling occasionally. After centrifugation at 1500 rpm for 5 min, supernatant was removed and cell pellets were resupended in 1.5 ml PBS/Na-butyrate buffer. 8 x 10^5 cells were aliquoted per 1.5 ml microtube. After another round of centrifugation, supernatant was discarded and cell pellets were resuspended in lysis buffer. Then the lysates were transferred into 1.5 ml Bioruptor® microtubes and sonicated using a Diagenode Bioruptor® UCD-200 sonicator. The power setting was adjusted to “high” and shearing was performed 3 x 10 min for 30 sec at 30 sec intervals. In order to analyze sheared chromatin, 500 µl of the sheared lysate (two samples from each condition) was treated with 5 µl RNAse A for 1 h in a thermomixer at 37 °C with 350 rpm shaking. Then 2 µl proteinase K was added and lysate was incubated at 68 °C for 1 h at 350 rpm shaking. DNA fragments were isolated by phenol-chloroform extraction, followed by ethanol precipitation and resuspension in TE buffer. DNA was quantified with Nanodrop and visualized on a 1.5 % agarose gel. The sonication resulted in chromatin fragments of 100 - 10,000 bp in length with a mean of 300 bp. Sheared chromatin was incubated with 3 µg of goat polyclonal antibody against *PER1* and 3 µg of normal rabbit IgG antibody as a negative control overnight at 4 °C. Then immunoprecipitation was carried out using Pierce™ ChIP-grade protein A/G magnetic beads. Sheared chromatin was incubated with the beads for 30 min at 4 °C to avoid non-

specific binding. The antibody-bound beads were incubated with precleared chromatin for 2 h at 4 °C. Then, beads were washed 5 x with RIPA buffer, and 1 x TE buffer. In order to reverse the cross-linking, complete elution buffer was added to the samples and incubated for 2 h at 68 °C with shaking. Genomic DNA fragments were isolated by phenol-chloroform extraction, followed by ethanol precipitation and resuspension in water. DNA was quantified using the Picodrop for photometric assessment and real-time PCR with *PRL* primers (Table 4) for gene specific determination.

3.2.8. Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualize PCR products and sonicated DNA fragments. 1 g of agarose was measured and mixed with 100 ml 1 x TBE buffer in a microwave flask in order to get 1 % gel. Agarose was microwaved for 2 - 2.5 min until completely dissolved. Flask containing the agarose solution was left to cool down for 5 min. 1.5 µl ethidium bromide was added to the solution and the agarose was poured into a gel tray with a well comb. Poured gel was solidified at room temperature for 20 - 25 min. When solidified, gel was placed in an electrophoresis chamber filled with TBE buffer. Samples were mixed with 6 x loading dye and loaded into the wells of gel. A molecular weight ladder was also loaded into one well. Gel was run at 120 V for 1.5 hours and DNA fragments were visualized with a device, which has UV light.

3.2.9. Statistical analysis

3.2.9.1. Baseline study

For the final analyses, a time slot from 8:00 h to 16:00 h was selected since this represents the time commonly shared among the majority of donors. Gene expression data are expressed as mean ± SD.

3.2.9.1.1. Spectral analysis

Spectral analysis and correlation tests were performed by Thomas Maierhofer and Felix Günther, Institute for Statistics, Ludwig-Maxilians-University, Munich**.** Missing time points were imputed by linear interpolation for all genes. Data were individually standardized and analyzed based on a methodology previously described (Wichert et al., 2004). The spectral density of the individual time series of the *PERIOD* genes was estimated and a periodogram was computed as a graphical device to detect potential periodicity in our data. The peaks at the frequencies corresponding to any periodicities were observed. The statistical significance of each frequency was determined with a critical $p < 0.05$. The association between gene expression and cortisol concentrations in both plasma and saliva samples was determined via Pearson correlation and Kendall`s τ rank correlation in R software version 1.1.2 using the GeneCycle package. In addition, cross-correlation analysis was computed between the time series of *PERIOD* genes and cortisol concentrations with 15 min time lags.

3.2.9.1.2. Deconvolution analysis

For the deconvolution analysis, Pulse_XP software was used (Johnson et al., 2008). Pulse_XP is an integrated software package designed for the analysis of hormone pulsatility time series data. It is a non-commercial freeware that can be downloaded from http://mljohnson.pharm.virginia.edu/home.html. A model was first estimated and then the estimated model was refined to generate statistically significant profiles by using Autodecon algorithm (Johnson et al., 2008). Akaike`s information criterion < 70 was used as a measure of the relative goodness of fit of the statistical model (Akaike, 1974) and runs test was performed for randomness to demonstrate that there is no statistical difference between fitted curves and observed data (i.e $p > 0.05$).

3.2.9.2. Stress studies

The statistical analyses were calculated using IBM SPSS software (version 21) with a critical $\alpha = 0.05$. Data were analyzed using repeated measures ANOVAs. Data were checked for outliers, normality of distribution, homogeneity of variance and sphericity. A Greenhouse-Geisser correction of degrees of freedom was applied whenever appropriate. The applied epsilon (GG-ε) is reported along with the degrees of freedom. Results of $2^{-\Delta Ct}$ analysis were log10 transformed to confirm the assumptions of normality. Interactions were decomposed by

multiple pairwise comparisons (paired t-tests) where the pre-stress time point was compared to all of the post-stress time points. In the psychological stress study, cortisol concentrations were analyzed by non-parametric two-sample Wilcoxon rank-sum test (two-sided). In the pharmacological stress study, the success of the pharmacological cortisol manipulation was assessed using the two baseline plasma samples acquired before cortisol administration, and a blood sample acquired directly 15 min after cortisol administration. The average of the two baseline values was subtracted from the post-intervention value for each individual, resulting in a cortisol difference score. These scores were then compared between adjacent dose steps by non-parametric two-sample Wilcoxon rank-sum test (two-sided).

For pharmacological stress study Bonferroni-Holms corrections for multiple testing were applied and corrected values are reported in the results section. Effect sizes in Cohen`s *d* for t-tests or in partial eta square (η_p^2) for F-tests are reported.

Gene expression after RU486 administration was calculated separately for the control blood and the hydrocortisone treated blood using repeated measures ANOVA (SPSS version 21), to determine the effects of hydrocortisone and RU486 on the gene expression. Interactions were further decomposed by contrasting the blood treated with RU486 to blood treated without RU486, separately for hydrocortisone *vs*. control blood.

CHAPTER 4: RESULTS

4.1. Expression of *PERIOD* genes under baseline conditions

4.1.1. Expression profiles of *PERIOD* genes

In our study, we determined the relative mRNA levels of three genes under baseline conditions by real-time PCR. The relative expression of *PER1* in all individuals varied from 0.01 to 28.03 with a mean of 1.75 ± 3.09 during the sampling period (Figure 7). A range of 0.03 to 7.08 was found for *PER2* with a mean of 0.92 ± 0.81 (Figure 8). The relative expression of *PER3* fluctuated from 0.03 to 18.92 with a mean of 1.13 ± 1.31 (Figure 9). These results demonstrate high interindividual variability in expression of these genes in healthy donors.

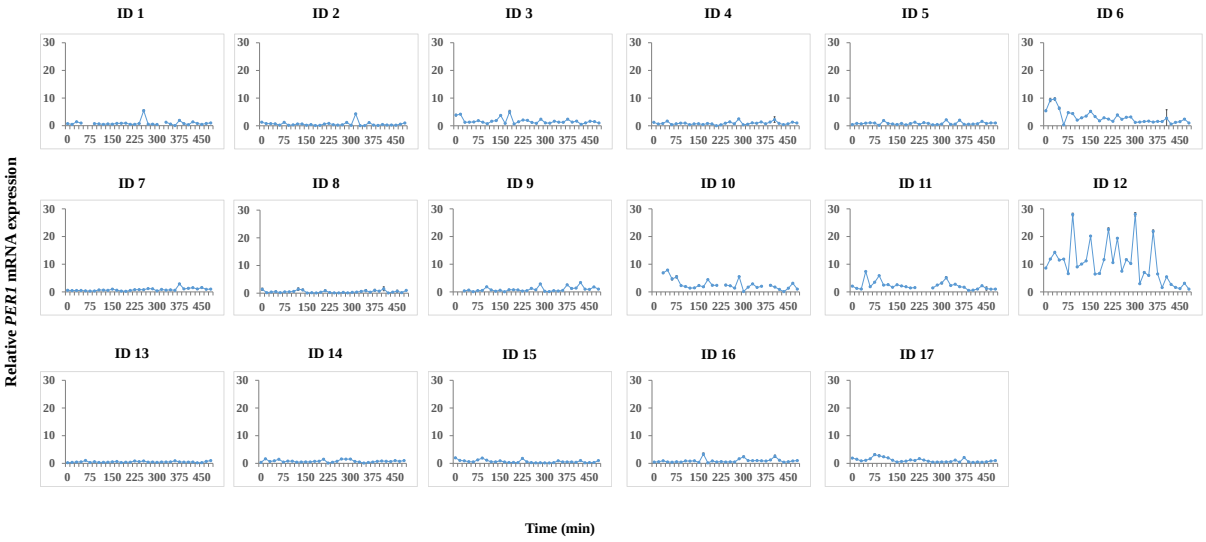

Figure 7: *PER1* mRNA expression relative to *GAPDH* was calculated using the $2^{-\Delta\Delta Ct}$ method over 8 h in 17 subjects. Error bars represent the standard deviation of PCR triplicate measurements of the same sample.

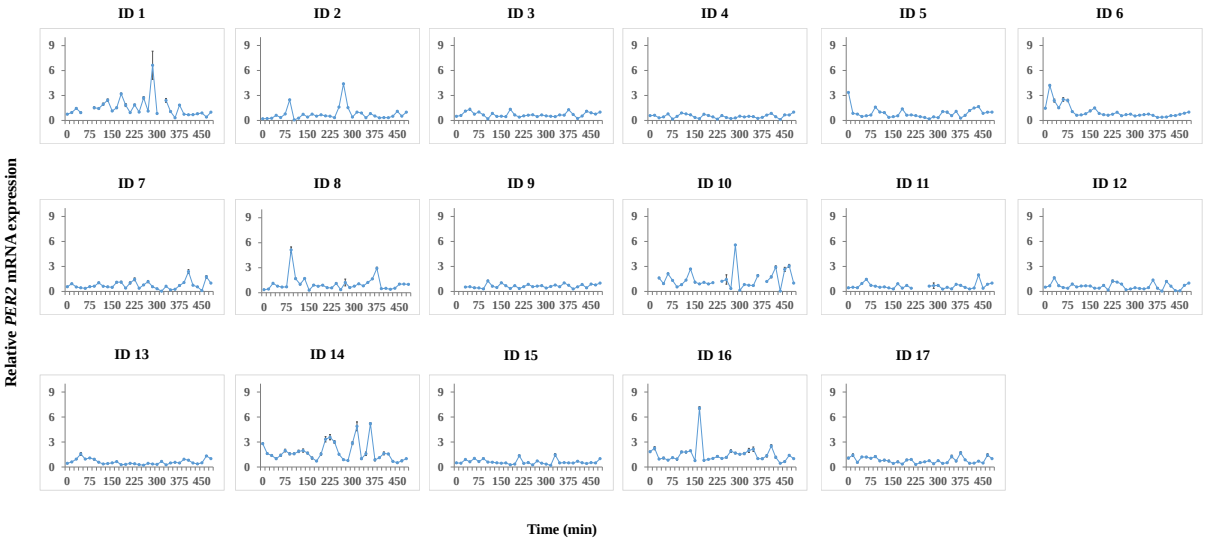

Figure 8: *PER2* mRNA expression relative to *GAPDH* was calculated using the $2^{-\Delta\Delta Ct}$ method over 8 h in 17 subjects. Error bars represent the standard deviation of PCR triplicate measurements of the same sample.

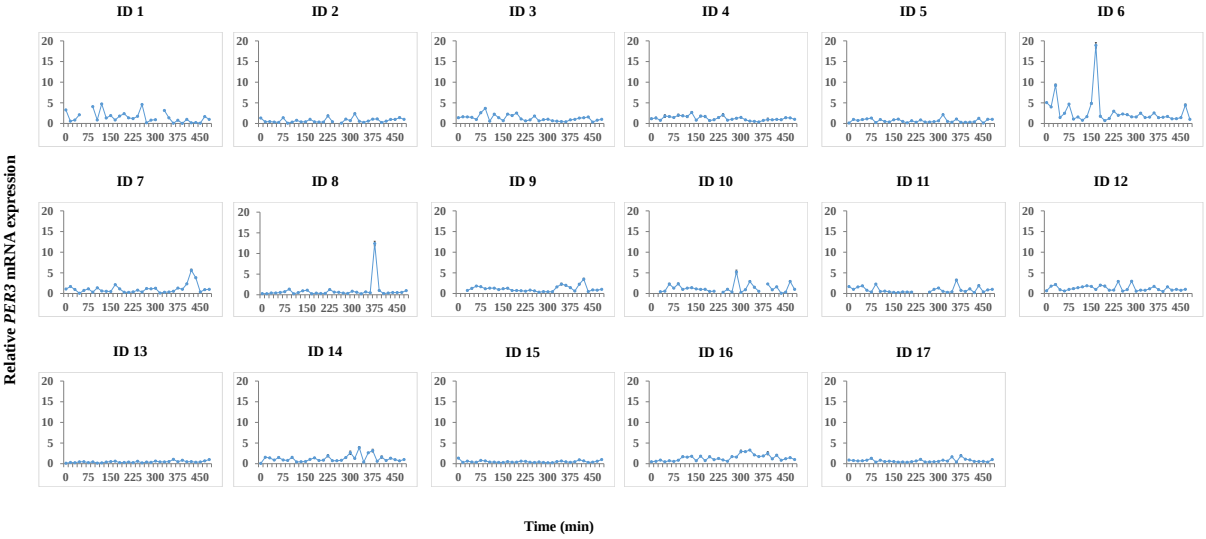

Figure 9: *PER3* mRNA expression relative to *GAPDH* was calculated using the $2^{-\Delta\Delta Ct}$ method over 8 h in 17 subjects. Error bars represent the standard deviation of PCR triplicate measurements of the same sample.

4.1.2. Spectral analysis of gene expression data

A periodogram was used to depict the periodic components of the gene expression time series from each subject (Figure 10). Spectral analyses of the dominant frequencies revealed a clear periodic structure in the ultradian range only in two donors for each gene (Figure 11). Donors ID 4 and 11 showed a pulsatile *PER1* expression, while donors ID 6 and 15 in *PER2*, and ID 9 and 13 in *PER3* showed ultradian rhythm. Since this periodic structure was determined in

different individuals with different period length in the time series of the gene expression, it suggests that the periodicity detected was random and not a common characteristic of these genes. Thus, *PERIOD* genes do not show pulsatile expression profiles in our setting.

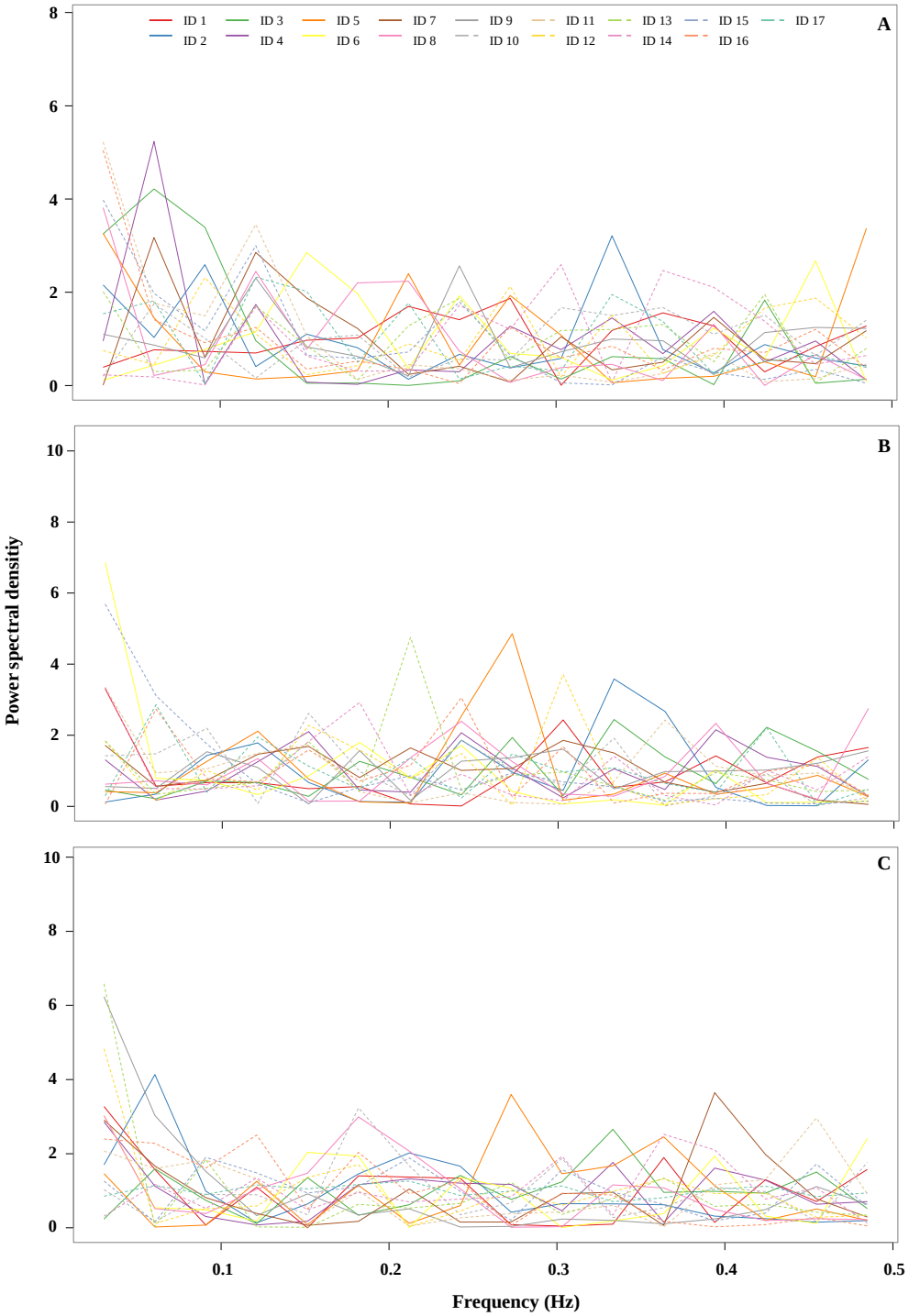

Figure 10: The spectral density of the individual time series of the *PERIOD* genes was estimated and periodograms were computed to detect potential periodicity in the time series of *PER1* (A), *PER2* (B) and *PER3* (C) expression. Periodograms show a sequence of peaks at certain frequencies. The statistical significance of each frequency was determined with a critical $p < 0.05$. Figure was provided by Thomas Maierhofer and Felix Günther, Institute for Statistics, Ludwig-Maxilians-University, Munich.

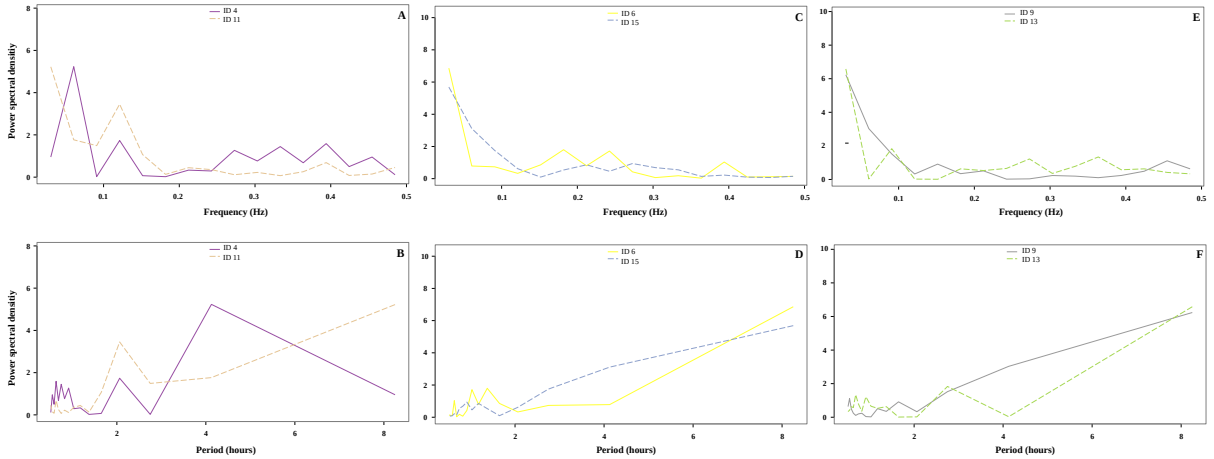

Figure 11: Individual periodograms of subjects showing a clear periodicity in the expression of *PER1* (A and B), *PER2* (C and D) and *PER3* (E and F). After estimating the spectral density of individual time series of the gene expression, periodograms were computed. Two frequencies passed significance test in each gene (A, C and E) and the length of periodic gene expression was shown in hours (B, D and F)). Figure was provided by Thomas Maierhofer and Felix Günther, Institute for Statistics, Ludwig-Maxilians-University, Munich.

4.1.3. Spectral analysis of cortisol concentration data

A periodogram was used to depict the periodic components of the cortisol concentration data from each subject (Figure 12). Spectral analyses of the dominant frequencies revealed a four-hour periodicity in the plasma cortisol data of 14 donors and in the saliva cortisol data of 13 donors (Figure 13). Plasma cortisol data from three donors (ID 2, 8 and 16) and saliva cortisol data from four donors (ID 2, 3, 6 and 16) did not show pulsatile profiles after spectral analyses. Thus, these analyses detected only four-hour pulses in our setting.

4.1.4. Deconvolution analysis of gene expression data

Trifonova and colleagues investigated cortisol pulsatility in the saliva of this cohort previously (2013). They generated statistically significant models of cortisol secretion and elimination in plasma and saliva by using a multi-parameter deconvolution technique. Therefore, we applied the same method in order to detect any putative periodic structures in the gene expression data.

Fully automated and statistically-based deconvolution procedure, Autodecon, generated statistically significant models of gene expression time series in each individual. In addition,

estimates of the peak number, half-life and interpeak intervals were included in the model. Table 25 summarizes the parameters of the individual models.

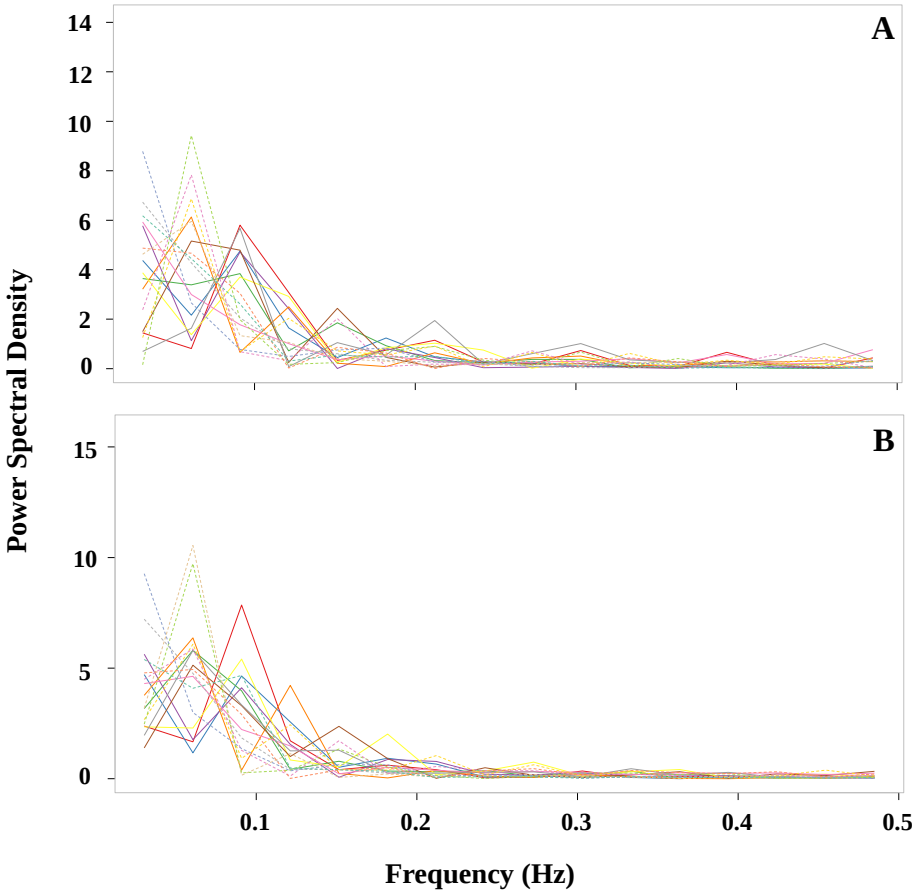

Figure 12: The spectral density of the individual time series of cortisol concentrations was estimated and periodograms were computed to detect potential periodicity in the time series of saliva cortisol (A) and plasma cortisol (B). Periodograms show a sequence of peaks at certain frequencies. The statistical significance of each frequency was determined with a critical $p < 0.05$. Figure was provided by Thomas Maierhofer and Felix Günther, Institute for Statistics, Ludwig-Maxilians-University, Munich.

Deconvolution analysis revealed pulsatile structures in the *PER1* expression of 13 donors. Mean peak number of its expression was estimated as 2.31 ± 1.55 and mean half-life of mRNA was predicted to be 89.4 ± 222.53 min, while nine donors showed pulses in the expression of *PER2* with a mean peak number of 2.56 ± 1.24 and a mean half-life of 14.23 ± 17.61 min. Mean peak number of *PER3* expression was estimated as 1.73 ± 0.9 and mean half-life of mRNA was predicted to be 12.71 ± 16.82 min in eleven donors.

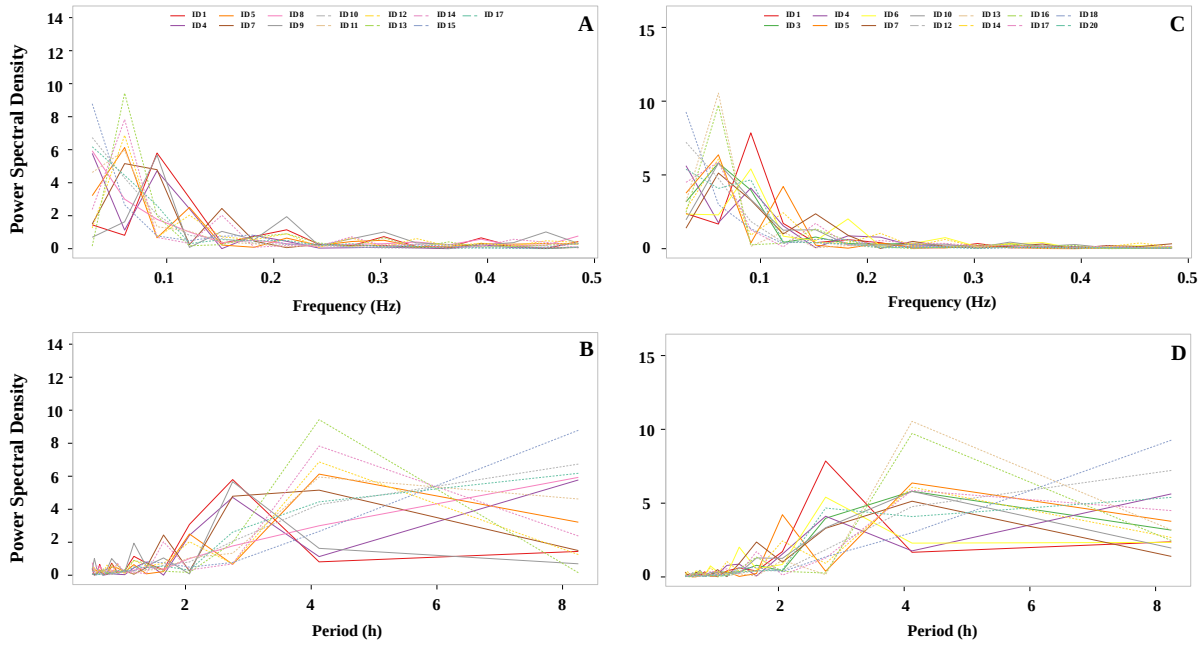

Figure 13: Individual periodograms of subjects showing a clear periodicity in the saliva cortisol (A and B), and plasma cortisol (C and D). After estimating the spectral density of individual cortisol concentration data, periodograms were computed. Frequencies passed significance test were shown in A and C. The length of pulses was shown in B and D. Figure was provided by Thomas Maierhofer and Felix Günther, Institute for Statistics, Ludwig-Maxilians-University, Munich.

Only two donors (ID 13 and 17) showed pulsatile expression in all three genes (see Figure 14). The Autodecon provided statistically significant models for these donors. Parameters of gene expression model of these donors are shown in Table 25.

The half-life of *PER1* mRNA was calculated to be 1.95 min and 1.78 min in ID 13 and 17, respectively. Four peaks were predicted with 101.99 ± 13.23 min interpulse interval for donor ID 13 and with 88.78 ± 35.45 min interpulse interval for donor ID 17. The half-life of *PER2* mRNA was calculated as 4.2 min for donor ID 13 and 33.25 min for donor ID 17. Three peaks were predicted with 166.44 ± 64.65 min interpulse interval for donor ID 13 and with 145.63 ± 65.37 min interpulse interval for donor ID 17. The half-life of *PER3* mRNA was estimated to be 10.55 min for donor ID 13 and 21.65 min for donor ID 17. One peak was predicted in the gene expression data of donor ID 13, while two peaks were predicted for donor ID 17 with 63.54 min interpulse interval.

Table 25: Quantitative parameters of gene expression data resolved by deconvolution analysis

	Donor ID	*Half-life(min)*	*Peak number*	*Interpeak interval (min ± SD)*
	2	1.14	1	-
	3	12.38	2	76.04
	4	752.5	1	-
	5	0.89	3	96.7
	6	3.49	1	-
	7	4.58	1	-
PER1	8	2.09	2	277.83
	9	18.77	2	197.17
	12	1.56	6	64.87 ± 9.57
	13	1.95	4	101.99 ± 13.23
	15	271.72	1	-
	16	186. 53	2	99.27
	17	1.78	4	88.78 ± 35.45
	1	5.14	2	200.64
	3	1.77	1	-
	4	1	5	109.12 ± 110.46
	10	21.26	3	95.57 ± 6.31
PER2	13	4.2	3	166.44 ± 64.65
	14	9.01	2	111.6
	15	51.13	3	203.22 ± 89.82
	16	1.27	1	-
	17	33.25	3	145.63 ± 65.37
	2	5.33	1	-
	3	23.87	1	-
	5	1.34	1	-
	7	6.36	3	135.19 ± 75.04
	8	3.44	3	116.05 ± 26.64
PER3	9	5.28	1	-
	10	3.14	3	140.9 ± 66.71
	13	10.55	1	-
	14	1.09	2	194.87
	16	57.76	1	-
	17	21.65	2	63.54

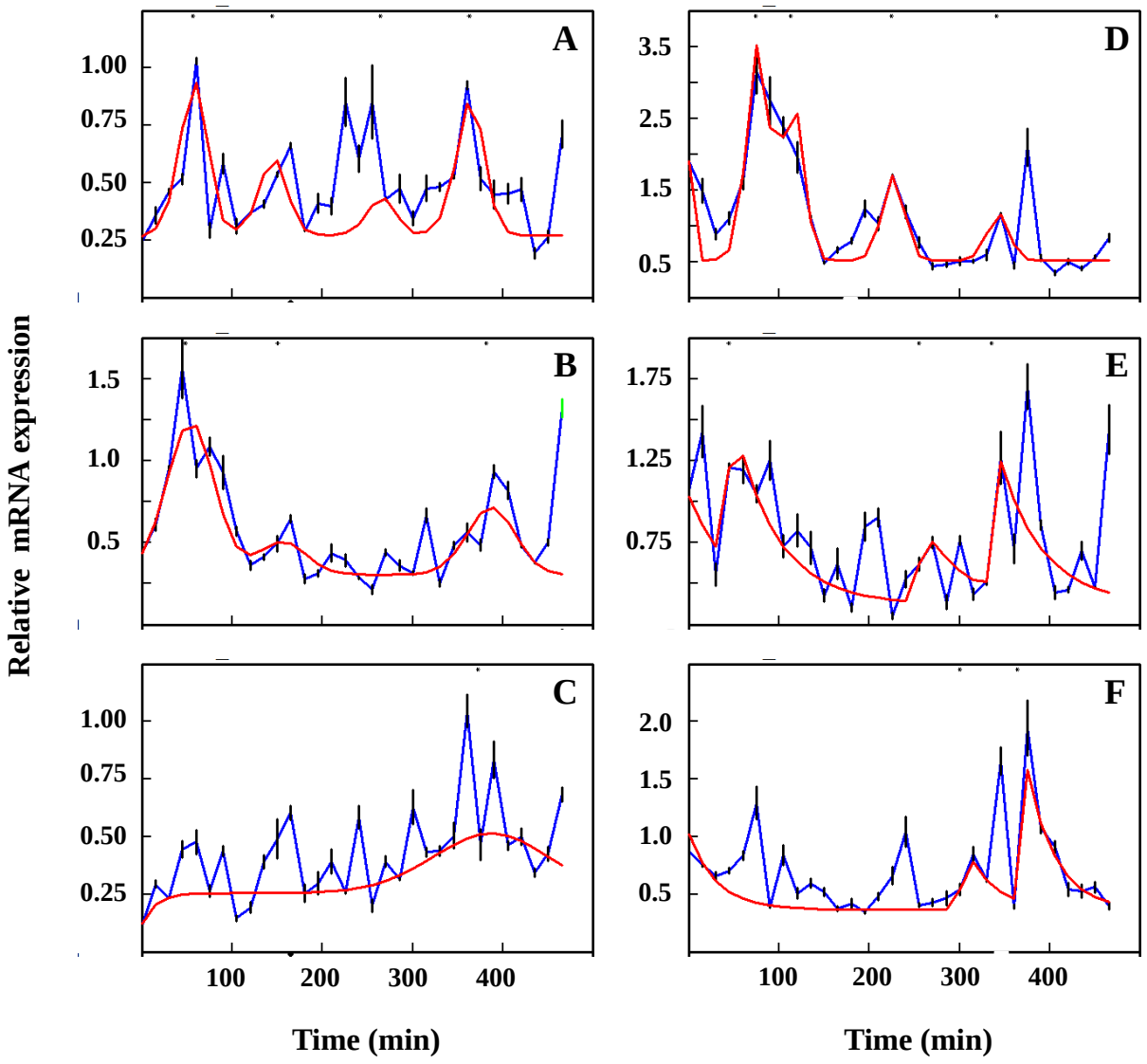

Figure 14: Expression of *PER1* (A and D), *PER2* (B and E) and *PER3* (C and F) was analyzed by deconvolution analysis for two donors (ID 13 shown in A, B and C, and ID 17 shown in D, E and F). Blue lines are actual gene expression time series data calculated by $2^{-\Delta\Delta Ct}$ method. Red lines are the curves fitted by Autodecon algorithm. Error bars represent the standard deviation. Green bar represents predicted standard deviation.

The Autodecon algorithm performed runs tests for randomness and computed the Akaike`s information criterion (AIC), which is one of the most well-known information criteria used in model selection. The statistical models of all donors reached a runs test significance of $p > 0.05$ and an AIC > 70, except donor ID 8 in *PER1* expression and donor ID 16 in *PER3* expression. Although randomness assumption is valid, these models with a higher value of AIC indicated that the fitted models were significantly different from the true model.

Therefore, these models are not the best models fitting to the data of *PERIOD* gene expression in our setting and need further analyses.

4.4.5. Relationship between *PERIOD* gene expression and cortisol concentrations

A Pearson's product-moment correlation test was computed to assess the relationship between *PERIOD* gene expression and cortisol levels. As shown in Figure 15, the distribution of cortisol levels and gene expression is right-skewed, indicating a non-normal distribution Therefore, an appropriate test for non-normal data, Kendall`s τ rank correlation, was used to evaluate the relationship between *PERIOD* gene expression and cortisol concentrations. Although some correlations were weak, the actual associations were significant (see Table 26). *PER1* was correlated with both plasma and saliva cortisol, while *PER3* was correlated with saliva cortisol and *PER2* was correlated with plasma cortisol. In addition, cross-correlation analysis was computed between the time series of *PERIOD* genes and cortisol concentrations with 15 min time lags. While there was a correlation between gene expression and cortisol levels in some subjects, this was not the case for the overall samples (see Figures in the Appendix section). Thus, we conclude that *PERIOD* genes, especially *PER1*, were weakly but significantly associated with cortisol concentrations under baseline conditions.

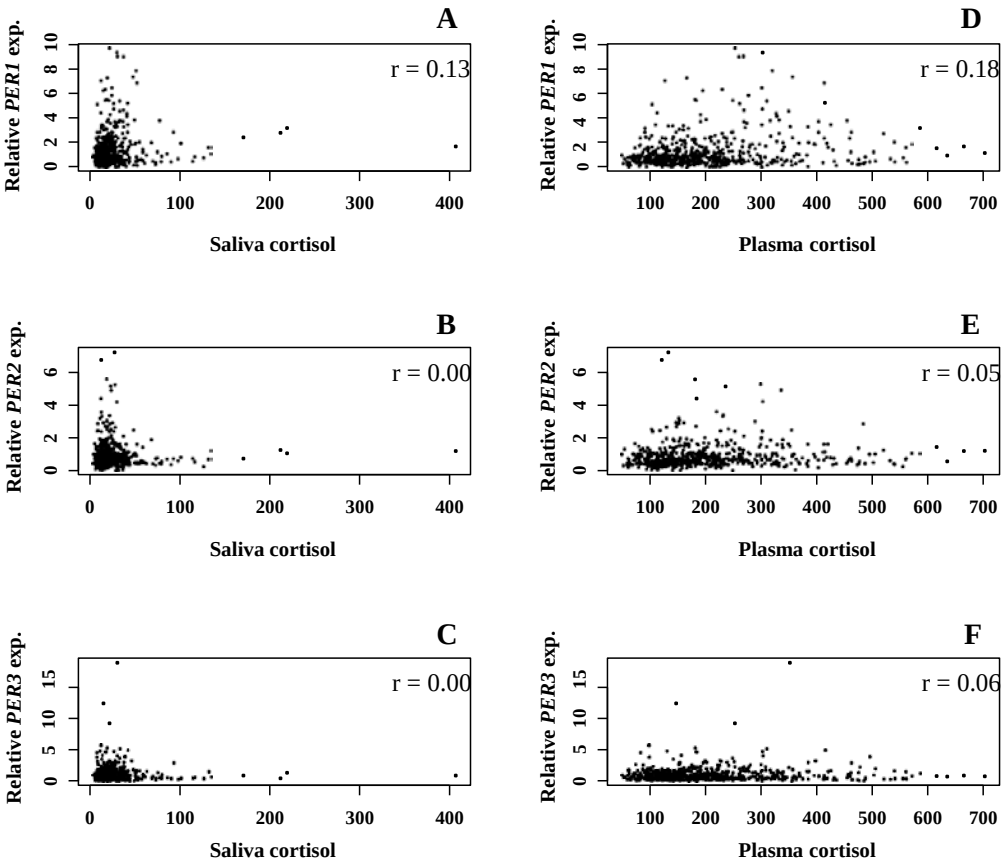

Figure 15: Correlations between saliva cortisol and *PER1* (A), *PER2* (B) and *PER3* (C) expression; and plasma cortisol and *PER1* (D), *PER2* (E) and *PER3* (F) expression. Figure was provided by Thomas Maierhofer and Felix Günther, Institute for Statistics, Ludwig-Maxilians-University, Munich.

Table 26: Kendall`s τ rank correlation

Gene	***Saliva cortisol***		***Plasma cortisol***	
	$\mathbf{r_\tau}$	***p***	$\mathbf{r_\tau}$	***p***
PER1	0.155	$< 0.001^{**}$	0.114	$< 0.001^{**}$
PER2	0.050	0.083	0.072	0.013^{*}
PER3	0.093	0.001^{*}	0.028	0.339

$^{*}p < 0.05$ $^{**}p < 0.001$

4.2. Expression of *PERIOD* genes after psychological stress

4.2.1. Cortisol concentrations

Participants showed differential cortisol responses to the psychological stress. After visual inspection, it was possible to divide them into groups of anticipators, responders and non-responders (Figure 16). There were seven responders including three anticipators and seven non-responders including one anticipator. Since these subgroups have very small numbers of participants, it is not possible to make proper statistical analysis. Therefore, all participants without subgroups were included for the analysis.

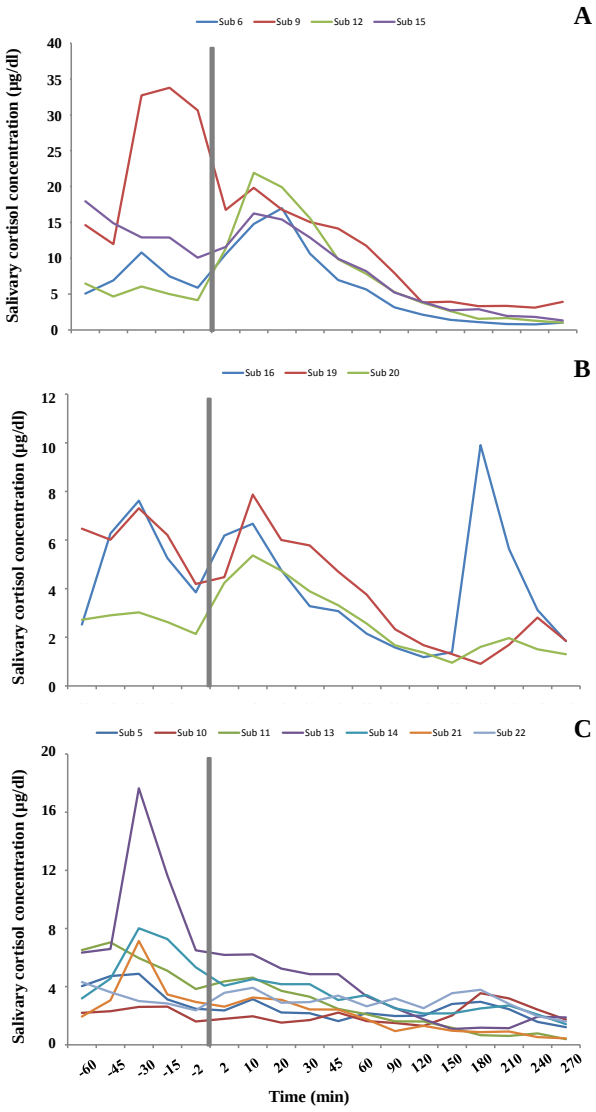

Figure 16: Salivary cortisol concentration profiles A: High cortisol responders B: Low cortisol responders C: Non-responders. The grey bars indicate the time point at which TSST was performed. Anticipators (Subjects 9, 16, 19 and 13) and late responder (Subject 16) were also included.

Mean saliva cortisol concentrations were shown in Table 27. When cortisol levels of pre-test were contrasted to all of the post-test time points separately, two-sample Wilcoxon rank-sum test revealed that cortisol concentrations of 10 min after TSST are significantly higher than the levels of 2 min pre-test (Z = -2.04, p = 0.041). With the time, cortisol concentrations leveled out to baseline levels.

Table 27: Salivary cortisol concentrations pre- and post-TSST

Time point min	***Mean cortisol µg/dl***	***Standard deviation***
-2	6.14	7.38
2	6.44	4.42
10	8.59	6.66
20	7.67	6.47
30	6.33	4.96
45	5.15	3.72
60	4.21	3.04
90	2.96	1.94
120	2.18	0.98
150	2.01	0.99
180	2.63	2.36
210	2.21	1.32
240	1.83	0.83
270	1.50	0.85

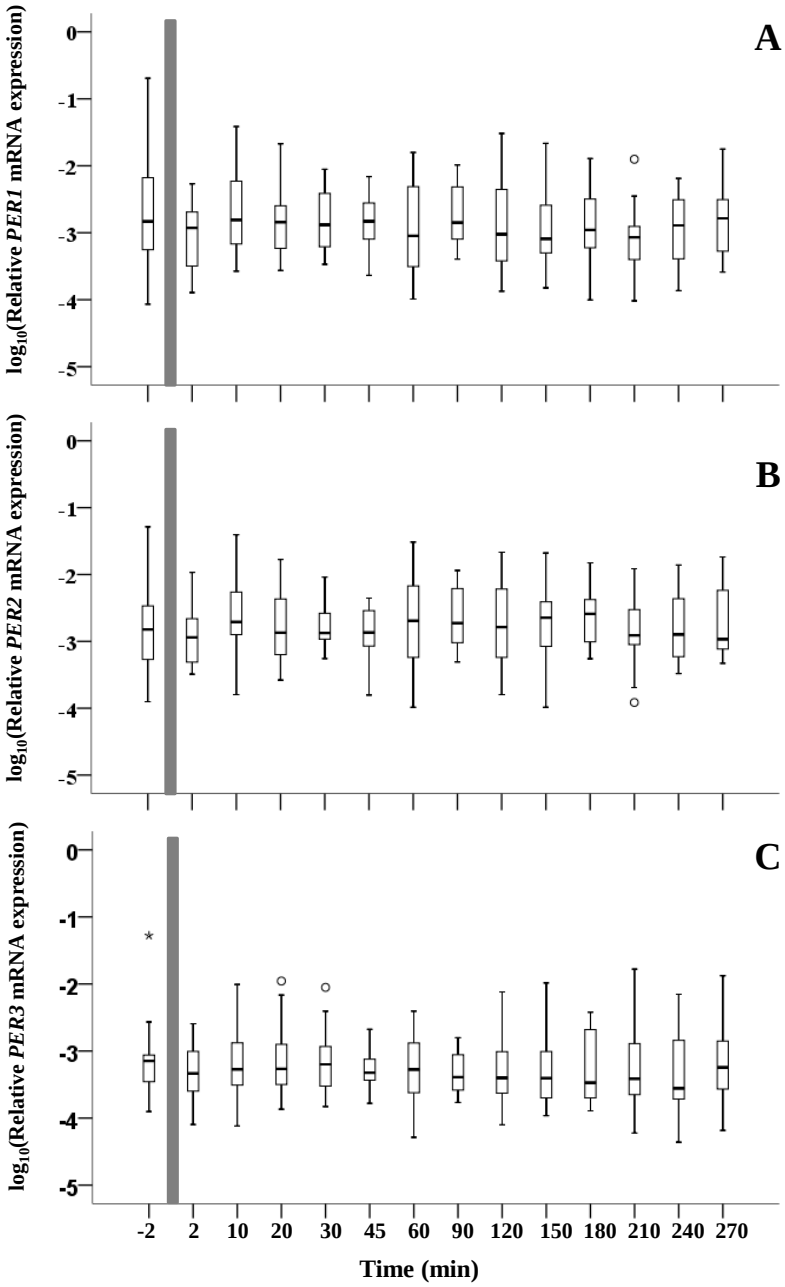

Figure 17: Influence of TSST on the mRNA levels of *PER1* (A), *PER2* (B) and *PER3* (C) expression. Each value represents the median ± standard deviation (n = 8). The grey bars indicate the time point at which TSST was performed. Boxes represent the interquartile range, with the median represented by the line inside the box. *Whiskers* indicate the highest and lowest values, excluding outliers (○ and *).

4.2.2. Assumption of normality

In each dependent variable (*PER1*, *PER2*, *PER3*) was tested for normality by visual inspection of distributions, z-scoring all data of individual participants and by testing skewness and kurtosis for significance. Visual inspection revealed no outlying data, which

was confirmed by z-scores of individual participants, which were all below ± z = 1.96. Importantly, all z-scores of skewness and kurtosis in each factor level combination were for both experimental settings below ± *z* = 2.58. Thus, normality of data was assumed.

4.2.3. Influence of TSST on gene expression

Data were analyzed separately for each gene (*PER1*, *PER2*, *PER3*) by calculating a repeated measures ANOVA. We expected to see an influence of *time of measurement* (- 2 min, + 2 min, + 10 min, + 20 min, + 30 min, + 45 min, + 60 min, + 90 min, + 120 min, + 150 min, + 180 min, + 210 min, + 240 min and + 270 min).

In the statistical model we observed no main effect of *time of measurement* on *PER1* expression (F[13, 169] = 1.15, p < 0.319, GG-ε = 0.46), *PER2* expression (F[13, 169] = 0.95, p = 0.501, GG-ε = 0.44) and *PER3* expression (F[13, 156] = 0.73, p = 0.735, GG-ε = 0.418). Therefore, we conclude that the psychosocial stress test could not induce the gene expression in whole blood (Figure 17).

4.3. Expression of *PERIOD* genes after pharmacological stress

This study was published in *Chronobiology International* (33(9):1222-1234, 2016).

4.3.1. Manipulation check

The analyses of the plasma cortisol data reported in this study indicated an almost linear relationship between escalating doses 15 min after hydrocortisone administration. Raw plasma cortisol concentrations reached a mean value of 8.66 ± 5.49 μg/dl in the control group. In comparison, a mean value of 14.84 ± 6.07 μg/dl in the group receiving 3 mg; 22.05 ± 4.51 μg/dl in the group receiving 6 mg; 32.86 ± 6.6 μg/dl in the group receiving 12 mg and 45.63 ± 10.71 μg/dl in the group receiving 24 mg hormone was measured 15 min after administration. *Two-sample* Wilcoxon rank sum tests revealed significant differences between plasma cortisol measures 15 min after infusion for all cortisol dosing groups (0 vs 3 mg: $Z = 2.74$, $p = 0.006$; 3 vs 6 mg: $Z = -3.31$, $p = 0.0009$; 6 vs 12 mg: $Z = -3.1$, $p = 0.002$; 12 vs 24 mg: $Z = -2.47$, $p = 0.014$).

4.3.2. Assumption of normality

In each dependent variable (*PER1*, *PER2*, *PER3*), each factor level combination (infusion group * time of measurement) in the *in vivo* study and (hydrocortisone * RU486) in the *ex vivo* study was tested for normality by visual inspection of distributions, z-scoring all data of individual participants and by testing skewness and kurtosis for significance. Visual inspection revealed no outlying data, which was confirmed by z-scores of individual participants, which were all below ± z = 2.58 *in vivo* and below ± z = 1.96 *ex vivo*. Importantly, all z-scores of skewness and kurtosis in each factor level combination were for both experimental settings below ± $z = 2.58$. Thus, normality of data was assumed.

4.3.3. Influence of hydrocortisone on gene expression *in vivo*

Data were analyzed separately for each gene (*PER1*, *PER2*, *PER3*, *GR*) by calculating a 5 (*infusion group*, 0 mg, 3 mg, 6 mg, 12 mg, 24 mg) * 10 (*time of measurement*, - 3 min, + 15 min, + 30 min, + 45 min, +60 min, + 75 min, + 90 min, + 105 min, + 135 min, + 165 min) repeated measures ANOVA. We expected to see an influence of hydrocortisone on the gene expression in some but not all dosing groups, and thus, expected an interaction *infusion group * time of measurement.* This interaction was followed up by contrasting the pre-infusion time point to all of the post-infusion time points separately in each dosing group (0, 3, 6, 12, 24 mg), summing up to nine non-orthogonal comparisons in each group.

4.3.3.1. *PER1*

Exposure of cultured cells and cell lines to dexamethasone caused transient increase in *PER1* expression (Balsalobre et al., 2000; Reddy et al., 2009); therefore, we wanted to examine the influence of different hormone doses on *PER1* mRNA levels *in vivo*. In the statistical model we observed a main effect of *time of measurement* (F[9, 315] = 9.24, $p < 0.001$, $\eta_p^2 = 0.21$, GG-ε = 0.53) but no main effect of *infusion group* (F[4, 35] = 2.43, $p = 0.066$, $\eta_p^2 = 0.22$). Importantly, the interaction *infusion group * time of measurement* (F[9, 315] = 2.60, $p = 0001$, $\eta_p^2 = 0.23$, GG-ε = 0.53) reached significance. We next contrasted the pre-infusion time point to all of the post-infusion time points separately in each dosing group (0, 3, 6, 12, 24 mg).

In the control group and the low-dose receiving group (3 mg), *PER1* did not show significant changes in expression (Figure 18A and Figure 18B, left panel). Paired-t test revealed significant differences in *PER1* expression 45 minutes (t(7) = -9.43, $p = 0.0003$, $d = -0.52$), 60 minutes (t(7) = -4.19, $p = 0.025$, $d = -0.82$), 75 minutes (t(7) = -6.12, $p = 0.003$, $d = -0.56$) and 90 minutes (t(7) = -7.29, $p = 0.001$, $d = -0.62$) after infusion in the participants receiving 6 mg hydrocortisone (Figure 18C, left panel). In the 12 mg hormone receiving group (Figure 18D, left panel), the significant increase in mRNA amount was observed 45 minutes (t(7) = -4.06, $p = 0.043$, $d = -1.68$) after infusion. At the highest dose (24 mg), *PER1* mRNA was significantly accumulated 75 minutes (t(7) = -4.82, $p = 0.017$, $d = -1.71$) after infusion and turned back to the baseline level within 15 minutes (Figure 18E, left panel).

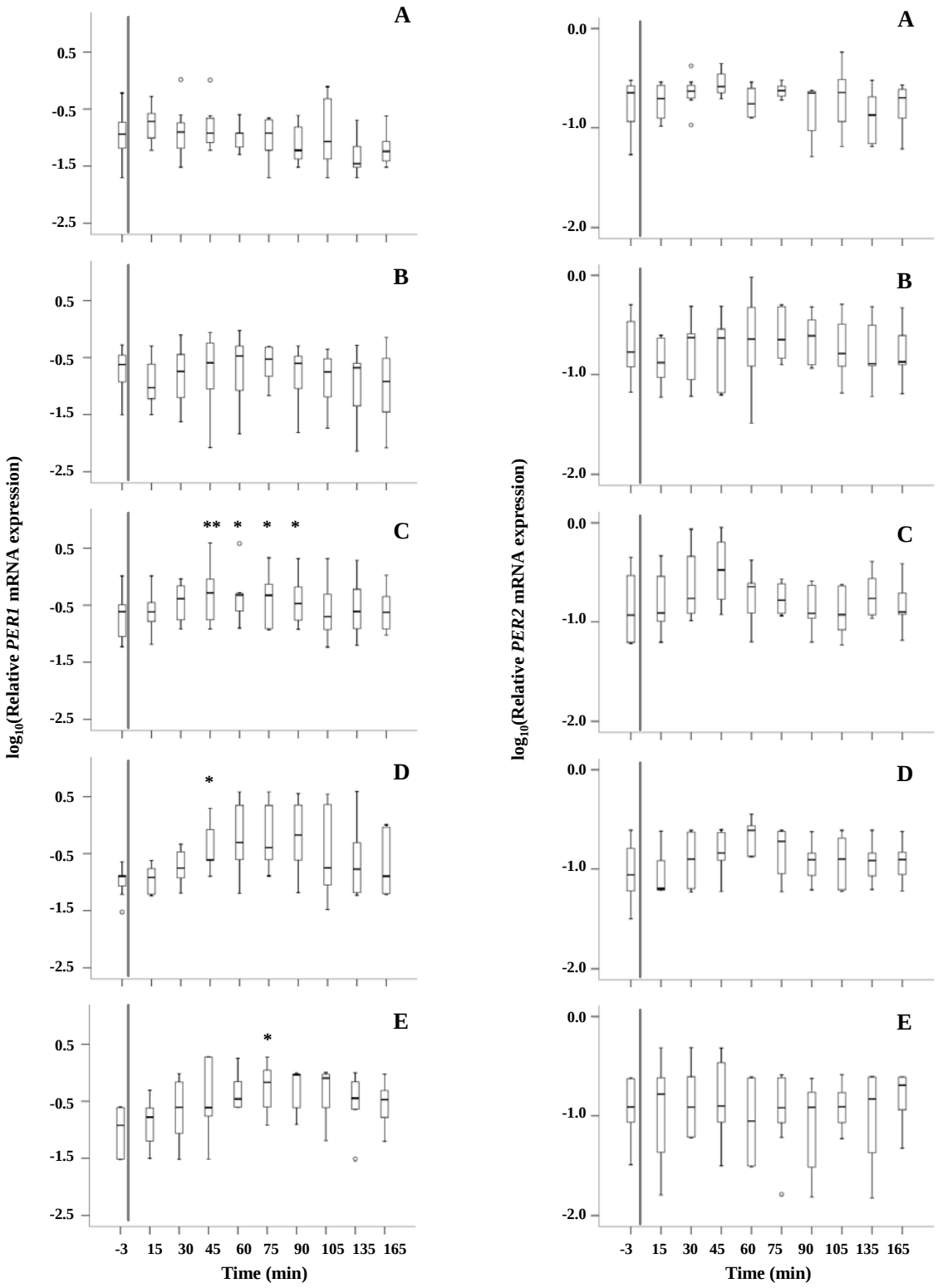

Figure 18: Influence of hydrocortisone on the mRNA levels of *PER1* (left panel) and *PER2* (right panel) expression by hydrocortisone infusion. Control group (A) and groups receiving 3 mg (B), 6 mg (C), 12 mg (D) and 24 mg (E) of hydrocortisone. Each value represents the median ± standard deviation (n = 8). *: $p < 0.05$ and **: $p < 0.01$. Post-infusion time points were compared with the pre-infusion time point in each group. The grey bars indicate the time 0, at which hormone infusion was administrated. Boxes represent the interquartile range,

with the median represented by the line inside the box. *Whiskers* indicate the highest and lowest values, excluding outliers (○).

4.3.3.2. *PER2*

A direct effect of glucocorticoids on *PER2* expression has been reported in human and mouse cell lines (Cheon et al., 2013; Reddy et al., 2009). Therefore, we wanted to show possible effects of hormone infusions on *PER2* mRNA levels *in vivo*. While a main effect of *time of measurement* (F[9, 315] = 3.28, p = 0.004, η_p^2 = 0.09, GG-ε = 0.70) was observed in the statistical model on *PER2* gene expression, neither a main effect of *infusion group* (F[4, 35] = 1.91, p = 0.13, η_p^2 = 0.18) nor an interaction *infusion group* * *time of measurement* (F[9, 315] = 1.37, p = 0.12, η_p^2 = 0.14, GG-ε = 0.70) was found. Thus, there was no influence of hydrocortisone on *PER2* expression in our sample (Figure 18, right panel) and accordingly no *post hoc* comparisons were calculated.

4.3.3.3. *PER3*

The regulation of *PER3* via glucocorticoids highly depends on the stressor and environmental conditions. Although murine *Per3* is not responsive to acute stress *in vivo* (Yamamoto et al., 2005), its expression was induced by dexamethasone *in vitro* (Balsalobre et al., 2000). Therefore, we also examined the influence of hormone infusion on the *PER3* expression. In the statistical model we observed a main effect of *infusion group* (F[4, 35] = 5.04, p = 0.003, η_p^2 = 0.37) and of *time of measurement* (F[9, 315] = 4.15, p < 0.001, η_p^2 = 0.11, GG-ε = 0.75) as well as an interaction *infusion group* * *time of measurement* (F[9, 315] = 3.80, p < 0.001, η_p^2 = 0.30, GG-ε = 0.75).

The expression of this gene fluctuated around the baseline in the control group and the group receiving 3 mg hormone without significant change (Figure 19A and Figure 19B, left panel). *Post hoc* paired t-tests showed a significant increase in *PER3* expression 30 minutes (t(7) = -10.27, p = 0.0002, d = -2.08), 45 minutes (t(7) = -7.56, p = 0.001, d = -1.37) and 60 minutes (t(7) = -4.44, p = 0.021, d = -0.86) after hormone administration in the participants receiving 6 mg hydrocortisone (Figure 19C, left panel).

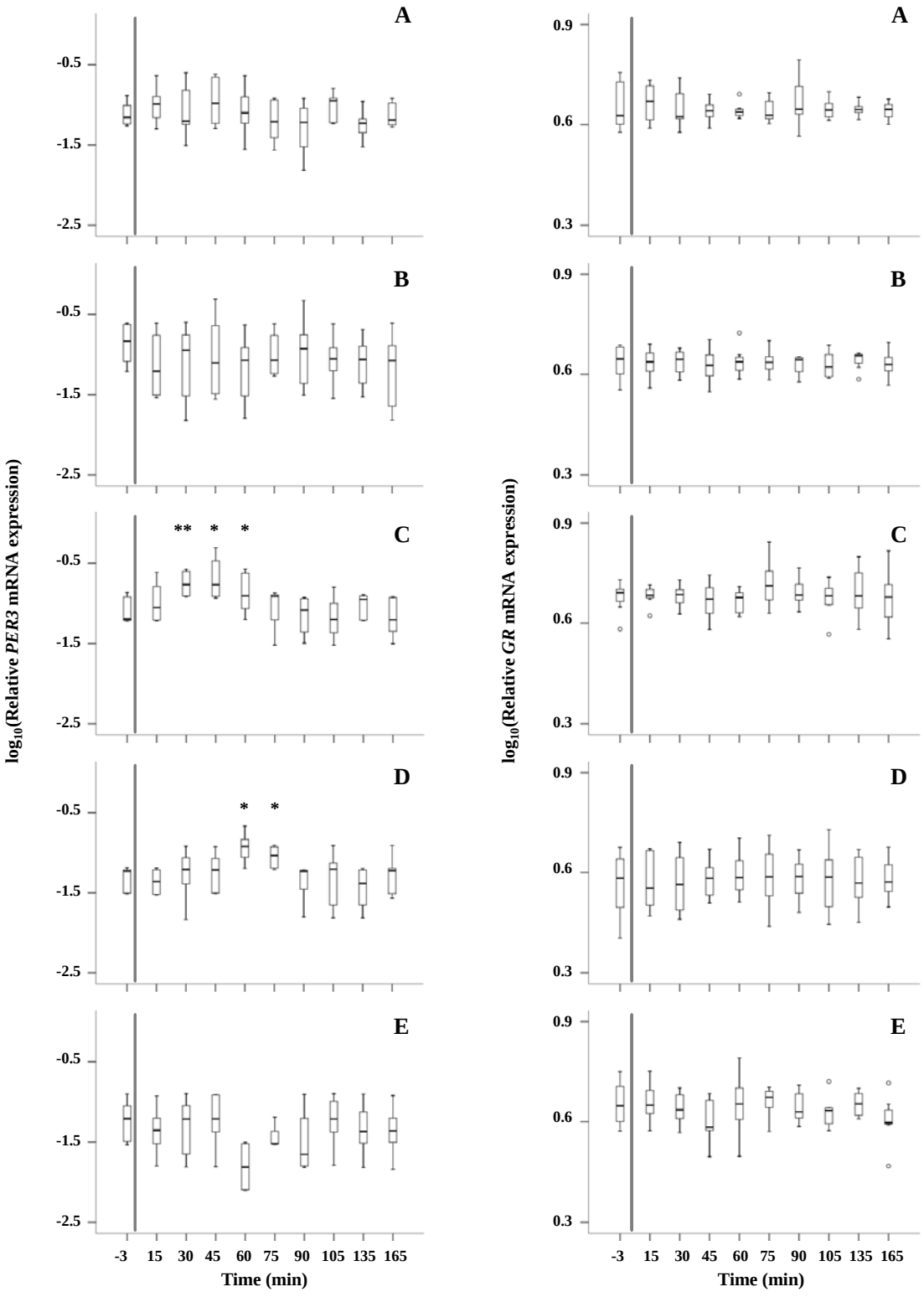

Figure 19: Influence of hydrocortisone on the mRNA levels of *PER3* (left panel) and *NR3C1* (right panel) expression by hydrocortisone infusion. Control group (A) and groups receiving 3 mg (B), 6 mg (C), 12 mg (D) and 24 mg (E) of hydrocortisone. Each value represents the median ± standard deviation (n = 8). *: $p < 0.05$ and **: $p < 0.01$. Post-infusion time points were compared with the pre-infusion time point in each group. The grey bars indicate the time 0, at which hormone infusion was administrated. Boxes represent the interquartile range,

with the median represented by the line inside the box. *Whiskers* indicate the highest and lowest values, excluding outliers (○).

In the group receiving 12 mg hormone, a significant induction was observed 60 minutes ($t(7) = -4.78$, $p = 0.018$, $d = -2.33$) and 75 minutes ($t(7) = -4.22$, $p = 0.031$, $d = -1.89$) after administration (Figure 19D, left panel). On the other hand, at the highest dose *PER3* expression did not display significant change (Figure 19E).

4.3.3.4. *GR*

When GR binds to a ligand, it influences the expression of its own gene (Kalinyak et al., 1987; Presul et al., 2007). In order to determine this regulation of hydrocortisone on *GR* expression, we measured the transcription of *GR* mRNA in our samples. While a main effect of *infusion group* ($F[4, 36] = 6.20$, $p = 0.001$, $\eta p^2 = 0.42$) was observed in the statistical model on *GR* expression, neither a main effect of *time of measurement* ($F[9, 306] = 1.48$, $p = 0.194$, $\eta p^2 = 0.042$, GG-$\varepsilon = 0.59$) nor an *interaction infusion group * time of measurement* ($F[36, 306] < 1$, $p = 0.85$, $\eta p^2 = 0.074$, GG-$\varepsilon = 0.59$) was found. Thus, there was no influence of hydrocortisone on *GR* expression in our sample and accordingly no *post hoc* comparisons were calculated (Figure 19, right panel).

4.3.4. Influence of hydrocortisone and RU486 on gene expression *ex vivo*

For each gene (*PER1*, *PER2*, *PER3*) and the positive control gene *Glucocorticoid-induced leucine zipper gene* (*GILZ*), a separate 2 (*hydrocortisone*) * 2 (*RU486*) repeated measures ANOVA was calculated. We assumed that the influence of hydrocortisone on the gene expression would be modulated by RU486 and, thus, an interaction of *hydrocortisone * RU486* was expected. Such interactions were further decomposed by contrasting the blood treated with RU486 to blood treated without RU486, separately for hydrocortisone vs. control blood.

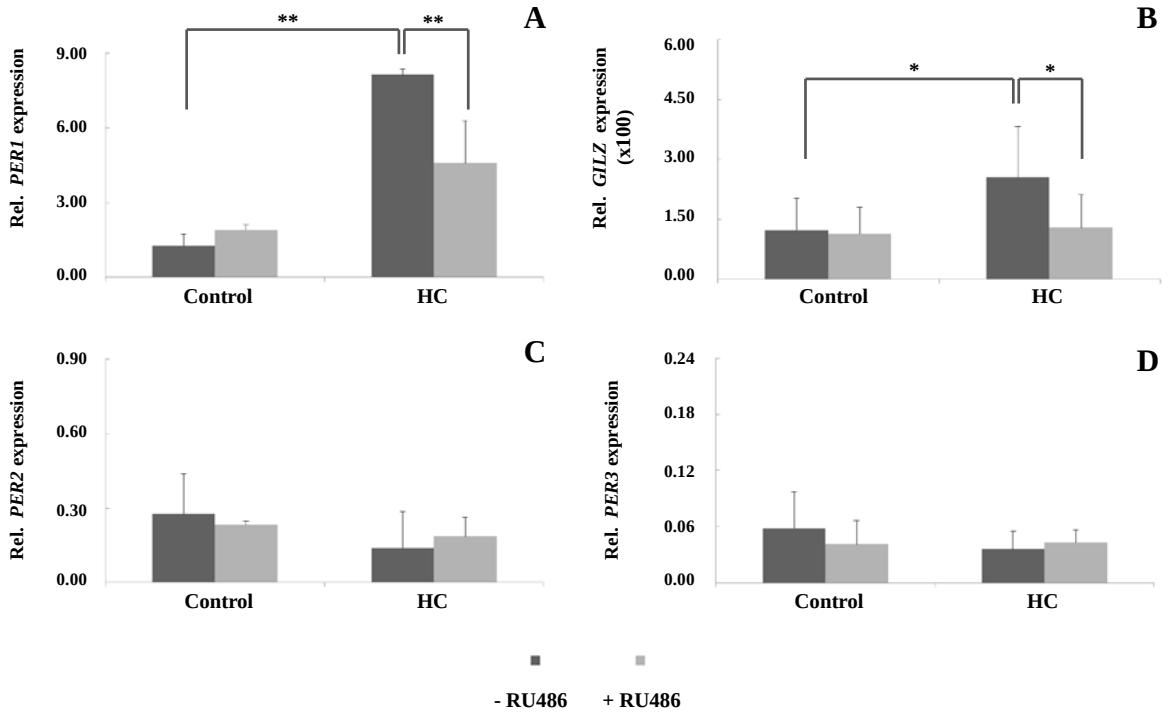

Figure 20: Influence of hydrocortisone and RU486 on the mRNA levels of *PER1* (A), *GILZ* (B), *PER2* (C) and *PER3* (D). For one hour, whole blood was treated with either 10 µM hydrocortisone (HC), 5 µM RU486, combination of both or ethanol (0.002%). Relative mRNA levels were calculated with $2^{-\Delta Ct}$ method. Each value represents the mean ± standard deviation (n = 5). *: $p < 0.05$ and **: $p < 0.01$.

4.3.4.1. *PER1*

In our statistical model for *PER1*, a main effect of *hydrocortisone* (F[1, 4] = 392.84, $p < 0.001$, $\eta_p^2 = 0.99$), of *RU486* (F[1, 4] = 15.09, $p = 0.018$, $\eta_p^2 = 0.79$) and an interaction *hydrocortisone* * *RU486* (F[1, 4] = 29.91, $p = 0.005$, $\eta_p^2 = 0.88$) was found. As expected, the hydrocortisone treatment resulted in a 6.5-fold increase in *PER1* transcript levels compared to the control. Paired t-tests showed that in the control blood RU486 did not influence *PER1* expression (t(4) = -2.21, $p = 0.092$, d = -0.97, Figure 20A), whereas in the hydrocortisone-treated blood the transcriptional activation was significantly diminished after administration of RU486 (t(4) = 4.81, $p = 0.009$, d = 2.58). However, there was no complete blocking of the induction of *PER1* mRNA in comparison to the control blood (t(4) = -5.29, $p = 0.006$, d = -1.88).

4.3.4.2. *PER2*

In our statistical model for *PER2*, we found no main effect neither of *hydrocortisone* (F[1, 4] = 3.20, p = 0.15, η_p^2 = 0.44), nor of *RU486* (F[1, 4] < 1, p = 0.94, η_p^2 < 0.01) nor an interaction *hydrocortisone * RU486* (F[1, 4] = 3.42, p = 0.14, η_p^2 = 0.46). Thus, the expression of *PER2* was not influenced by hydrocortisone or RU486 in the *ex vivo* study (Figure 20C).

4.3.4.3. *PER3*

As it was shown for *PER2*, the expression of *PER3* was not influenced by hydrocortisone or RU486 in the *ex vivo* study. Both main effects (*hydrocortisone* (F[1, 4] = 1.32, p = 0.31, η_p^2 = 0.25); *RU486* (F[1, 4] < 1, p = 0.46, η_p^2 = 0.14)) as well as the interaction *hydrocortisone * RU486* (F[1, 4] = 1.32, p = .32, η_p^2 = 0.25) failed to reach significance (Figure 20D).

4.3.4.4. *GILZ*

In our statistical model for *GILZ*, we observed a main effect of *hydrocortisone* (F[1, 4] = 7.68, p = 0.05, η_p^2 = 0.66) as well as an interaction *hydrocortisone * RU486* (F[1, 4] = 14.16, p = 0.02, η_p^2 = 0.78). The main effect of *RU486* was significant at trend level (F[1, 4] = 6.58, p = 0.06, η_p^2 = 0.62). *Post hoc* comparisons showed that in the hydrocortisone treated blood the expression of *GILZ* was significantly reduced in the presence of RU486 (t(4) = 3.24, p = 0.032, d = 1.07), whereas RU486 did not influence gene expression in the control (t(4) = 0.49, p = 0.65, d = 0.12) (Figure 20B).

4.4. Determination of the target genes of glucocorticoid-mediated PER1 as a transcription factor

It is not much known about the downstream genes of glucocorticoid-induced *PER1*; thus, we aimed to identify these genes by using chromatin immunoprecipitation-sequencing (ChIP-Seq). In this study, we increased the expression of *PER1* in HepG2 cells via stimulation with hydrocortisone and overexpression with an expression vector containing *PER1* cDNA. Then, chromatin immunoprecipitation was performed to determine the downstream genes of glucocorticoid-induced *PER1*.

4.4.1. Hydrocortisone stimulation

In order to find out the right concentration and stimulation time, HepG2 cells were treated with two different hydrocortisone concentrations (1 and 10 µM) for 1 h or 24 h. It was shown that 1 µM hydrocortisone concentration caused a 1.8-fold increase in the mRNA levels of *PER1* after 24 h incubation, whereas a 5.6-fold change was induced by 10 µM hydrocortisone (Figure 21).

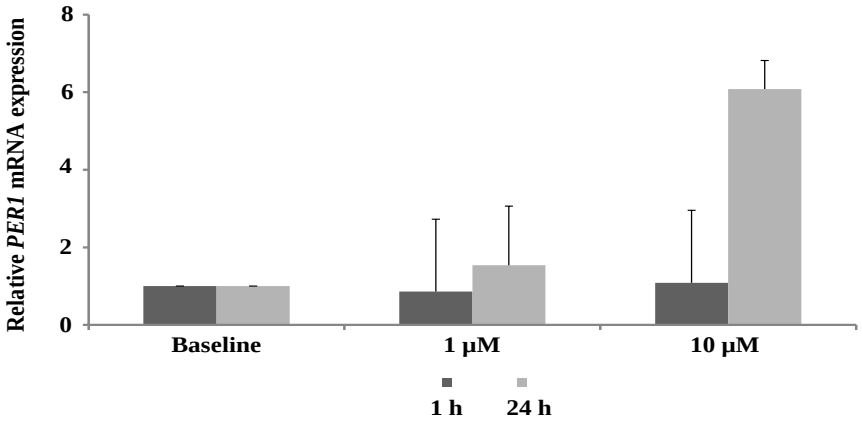

Figure 21: Influence of hydrocortisone on the mRNA levels of *PER1*. Relative mRNA levels were calculated with $2^{-\Delta\Delta Ct}$ method. Each value represents the mean ± standard deviation from 9 independent PCR results.

4.4.2. Overexpression of *PER1*

To determine the specific genes which are regulated only by GC-induced *PER1*, *PER1* was overexpressed in HepG2 cells using an expression vector containing the *PER1* cDNA. The expression vector containing the human *PER1* cDNA (pcDNA3.1-*PER1*) was kindly provided by Dr. Sigal Gery (Cedars Sinai Medical Center, CA 90048, USA). Before starting the overexpression analysis, pcDNA3.1-*PER1* was sequenced to control whether the plasmid contains the target gene, we are interested in. Sequencing revealed that the guanine of the starting codon (ATG) was missing in the sequence of *PER1* cDNA (Figure 22).

…tcactatagggagacccaagctggctagcgtttaaactctgcttactggcttatcgaaattaatacgactcacta
tagggagacccaagctggctagcgtttaaacttaagcttggtaccgagctcggatccactagtccagtgtggtgg
aattcgagctcggtaccc**AT**-AGTGGCCCCCTAGAAGGGGCTGATGGGG…

Figure 22: A part of pcDNA3.1-*PER1* after sequencing. Small letters: plasmid sequence. Capital letters: *PER1* cDNA sequence. AT-: start codon without G base.

PER1 cDNA was amplified from human blood cells by nested PCR and cloned into a TA vector. After confirming the sequence, a specific region (1466 bp) from TA vector-*PER1* was cut by two restriction enzymes (*Eco*RI and *Eco*RV). Consequently, pcDNA3.1-*PER1* was also cut by the same enzymes (Figure 23). This gave an opportunity to make a replacement in the expression vector. *PER1* sequence missing the guanine was replaced with the sequence containing the ATG codon from TA vector.

3 µg of pcDNA3.1-*PER1*, whose sequence was confirmed, was transfected into HepG2 cells using Lipofectamine® 3000 and cells were incubated for 24 h. The over-expression of *PER1* was determined by qPCR. The expression of *PER1* was increased approximately 43,000 to 100,000 fold compared to the non-transfected cells seeded in a 6-well plate.

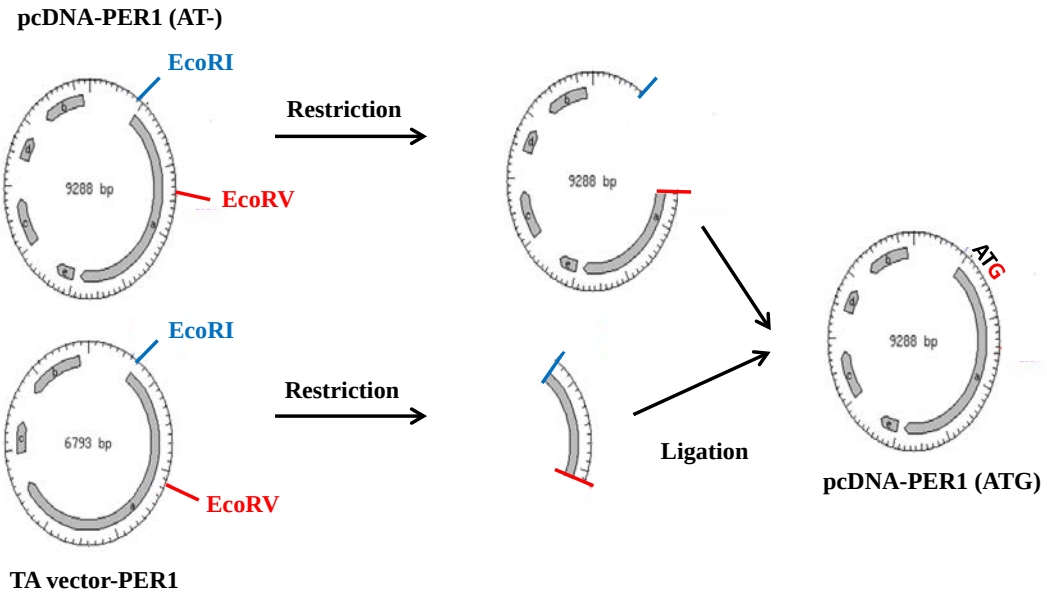

Figure 23: Replacement strategy. pcDNA-*PER1* and TA vector-*PER1* were restricted by *Eco*RI and *Eco*RV enzymes. ATG containing sequence from TA vector-*PER1* was ligated to pcDNA-*PER1*. Replacement was confirmed by sequencing.

4.4.3. Chromatin immunoprecipitation

In this study, the ChIP protocol provided by the Luxemburg Institute of Health was applied to investigate PER1 target genes. HepG2 cells, either non-treated, transfected or stimulated, were cross-linked using formaldehyde. Fixed cells were sonicated to shear the protein-DNA complexes into small fragments. Shearing is a challenging process which needs optimization depending on the cell type and duration of the fixation. The sonication resulted in chromatin fragments of 100 - 10,000 bp in length with a mean of 300 bp (Figure 24).

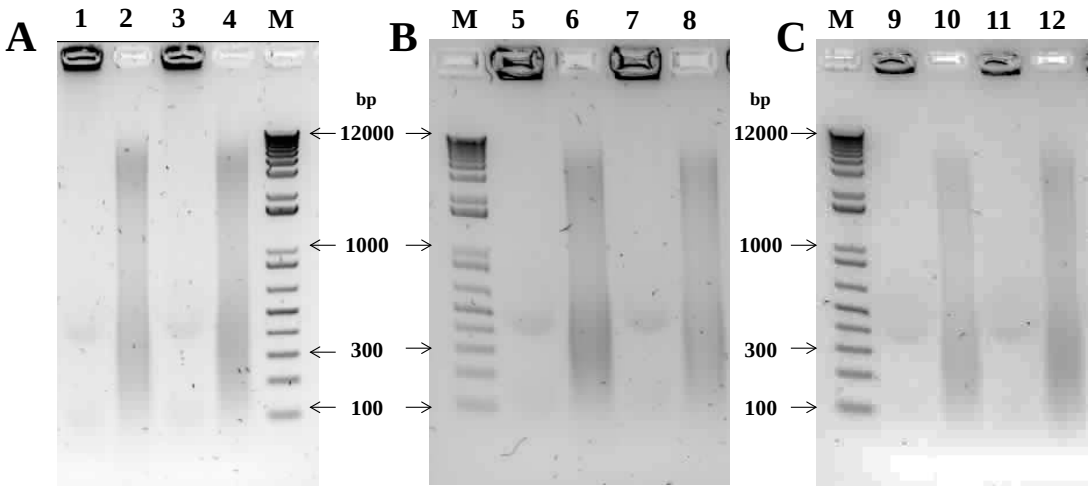

Figure 24: Shearing the DNA-protein complexes into small fragments by sonication. A: Cells under baseline conditions B: Transfected cells C: Stimulated cells. Samples are duplicates for each condition. Odd numbers show non-sheared samples. Even numbers show sheared samples. bp: Base pairs M: 1 kb plus DNA ladder

In order to precipitate PER1 associated with target DNA binding sites from the cell debris, a goat anti-PER1 antibody was used. After immunoprecipitation, de-crosslinking was performed and DNA fragments were purified. The quality of the precipitated DNA was determined via real-time PCR. It was previously reported that PER1 as a part of circadian system binds to the promoter of *prolactin* (*PRL*) gene in a rat pituitary tumor cell line (Bose & Boockfor, 2010). Thus, *PRL* gene was amplified in the precipitated DNA samples (Figure 25). % of input method (signal derived from the ChIP sample is divided by signal derived from the input) revealed 6.08 % of input for anti-PER1 antibody, 3.1 % of input for IgG control and 6.16 % of input for no antibody control. It has been shown that the specific antibody did not give a high level of enrichment in comparison to the background.

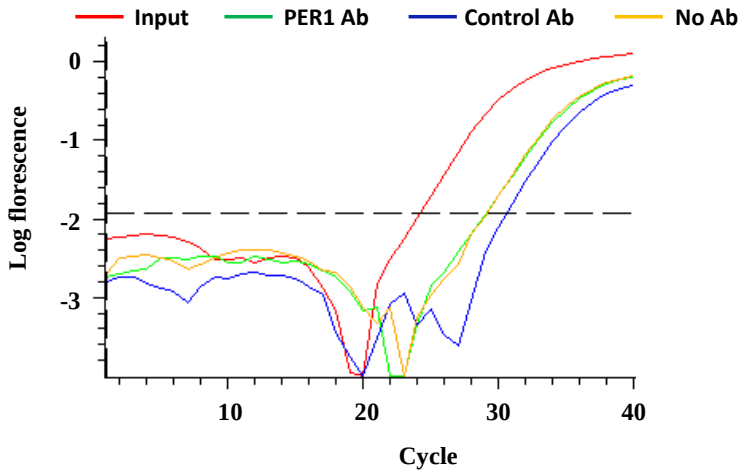

Figure 25: Graphical representation of *PRL* amplification in the immnunoprecipitated samples using real-time PCR. Ab: Antibody

Moreover, DNA samples were also controlled for the quality via Picodrop spectrophotometer. The anti-PER1 antibody sample has 14.2 ng/µl DNA with a ratio of 1.116 (A260/A280), IgG antibody sample has 10.1 ng/µl DNA with a ratio of 0.823 and no antibody sample has 10.9 ng/µl DNA with a ratio of 0.786, showing the low quantity and quality of the immunoprecipitated DNA (Figure 26). Samples showed an absorption maximum at 280 nm, suggesting protein contamination. As a result, ChIP in HepG2 cells performed using anti-PER1 antibody was not specific and revealed valueless DNA samples.

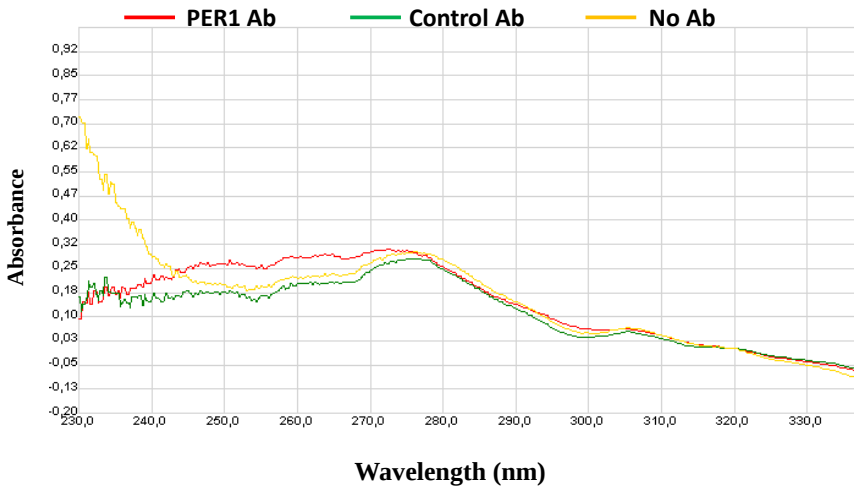

Figure 26: Absorption spectra of the immunoprecipitated samples showing an absorption max at 280 nm.

As a conclusion, ChIP performed in this study revealed the key steps of the protocol, which needs more optimization. For example, cross-linking time influences the ChIP efficiency; thus, it has to be optimized according to the interactions between PER1 and target genes. Moreover, a different antibody is essential and its specificity has to be proven prior to the immunoprecipitation. On the other hand, this experimental setup of ChIP was developed for the first time at Department of Neurobehavioral Genetics, but it will also be very useful for the future studies performed at the University of Trier.

CHAPTER 5: DISCUSSION

The HPA axis and the circadian clock system mutually interact with each other at multiple levels. In this thesis, we focused on their relationship under baseline conditions and after different types of stress in healthy humans. The interrelation between cortisol as a stress hormone and *PERIOD* transcripts as circadian clock genes was specifically investigated in whole blood.

The study performed under baseline conditions is the first study in humans aiming to investigate the putative natural pulsing of *PERIOD* gene transcripts in peripheral blood. We determined the mRNA levels of *PER1, PER2* and *PER3* genes via real-time PCR and applied spectrum analysis and deconvolution analysis in order to prove or disprove the pulsatile expression of these genes in healthy subjects. Although these techniques had both advantages and disadvantages, they were not sufficient to reveal the putative periodic structure of *PERIOD* gene expression. In addition, we investigated that *PERIOD* gene expression is weakly but significantly correlated with the pulsatile cortisol concentrations in human blood under baseline conditions.

We collected blood and saliva samples at 15-min intervals for 8 h from 17 healthy donors to analyze the expression of *PERIOD* mRNAs. There are several techniques and algorithms with different levels of complexity to analyze the periodicity of time series data. We first applied the method previously described by Wichert et al. (2004) to our gene expression data since the periodogram is a non-parametric method, which is applicable to very short time series (Wichert et al., 2004). Although there was a random periodicity in the gene expression of some donors, the spectral analysis did not reveal any clear periodic structure in the ultradian range of *PERIOD* gene transcripts in general. Moreover, same method was applied on the time series of cortisol concentration data. Spectral analysis of the dominant frequencies revealed a four-hour periodicity in cortisol time series, which was previously shown to be approximately one hour (Veldhuis et al., 1989; Trifonova et al., 2013). Since the spectral analysis could not demonstrate the actual periodic structure of cortisol data, non-pulsatile expression of *PERIOD* genes revealed by spectral analysis became specious.

Secondly, we applied deconvolution analysis, which was used to investigate cortisol pulsatility in plasma and saliva cortisol concentrations in the same cohort previously (Trifonova et al., 2013). The Autodecon algorithm used for this analysis estimated statistically significant pulsatile models for time series of gene expression and predicted quantitative

parameters, such as peak number and half-life of *PERIOD* mRNAs, which need critical discussions. Therefore, putative periodic expression of *PERIOD* genes was not proven by deconvolution analysis properly.

The first critical point is about mRNA half-life of *PERIOD* genes. The Autodecon algorithm revealed mRNA half-life changing between 0.89 min and 271.72 min, which was a wide range among individuals. However, there are studies reporting the mRNA half-life of *PERIOD* genes in the literature. *Per1* mRNA has a short half-life in *Drosophila melanogaster* (Hardin et al., 1992). It has been reported that the half-life of *PER1* mRNA is approximately 3 h in human liver cancer cell line (HepG2) (Koyanagi et al., 2006), whereas it is 44.1 min for *Per1* and 50 min for *Per2* in rat SCN-derived cultured cells (Ogawa et al., 2011). On the other hand, when GCs were administrated in a pulsatile manner, *Per1* mRNA levels remained elevated for 2 h after the final pulse and then decreased to the baseline levels, suggesting a relatively short half-life in rat hippocampus (Conway-Campbell et al., 2010).

The second critical point is about the AIC. AIC is a way of selecting a model from several models for a set of data. The model with a lower AIC represents the best fit model to the true model. After our analysis, the fitted models of almost all donors reached a higher AIC value, indicating that statistical models are not the best fit to the observed data. The software used for the deconvolution analysis was developed for the analysis of hormone pulsatility time series data and requires parameters which are not appropriate for gene expression, such as basal secretion and concentration at time zero. Therefore, the calculated mRNA half-life showed variability and estimated models were significantly different from the true data, suggesting a non-pulsatile expression profiles for *PERIOD* genes.

Plasma and saliva cortisol have similar characteristics (Trifonova et al., 2013); however, they show differences in biologically active cortisol, which can affect the expression of the target genes (Vining & McGinley, 1986). A small, unbound fraction is available in saliva while in plasma 90 % of the cortisol is bound to the corticosteroid-binding globulin, 7 % is bound to the albumin and the rest is free (Westermann et al., 2004). When we took this into account, we used both plasma and saliva cortisol concentrations to determine their correlation to the expression of *PERIOD* genes. Our analysis showed that although weak, the expression of *PERIOD* genes, especially *PER1,* was very significantly associated with both plasma and saliva cortisol. It is worth noting that cortisol regulation of *PER1* has previously been reported in animals, cell culture and human studies (see section 1.3.2). Thus, it is reasonable to see a change in cortisol concentrations accompanied by a change in *PER1* expression. For the

future analysis an alternative method, such as non-linear mixed effect modeling (Jin et al., 2003; Scheff et al., 2012), may be applied to investigate the relationship between *PERIOD* genes and cortisol. Moreover, cross-correlation analysis was computed between the time series of *PERIOD* genes and cortisol concentrations with 15 min time lags since we had 15-min sampling design. While the data from some subjects showed a correlation with the cortisol levels, there was no significant relationship between gene expression and cortisol concentrations in general. However, it might be more informative to run analysis within a time range in which is expected for mRNA translation to occur, like 0 to 4 h.

From the animal and cell culture studies in the literature and from our preliminary statistical analyses, we can speculate that *PERIOD* genes are not pulsatile in blood. Also, there are three points that seem to be important in the ultradian rhythmicity: 1) baseline conditions vs. pulsatile treatment, 2) tissue specificity and 3) mature vs. nascent mRNA expression.

Under baseline conditions, *Per1*, *Per2* and *Per3* transcripts were shown to have a period length of approximately 24 h in the mouse liver (Hughes et al., 2009), suggesting a circadian expression profile. However, it has recently been reported that *Per1*, *Per2* and *Bmal1* have ultradian periods of approximately 3 h in the SCN of the freely moving mice (Ono et al., 2015). On the other hand, pulsatile hormone treatment in adrenalectomized animals and cell lines induced the transcriptional pulsing of *Per1* (Stavreva et al., 2009; Conway-Campbell et al., 2010; George et al., 2017). From these studies, it seems that transcriptional programming is responsive to the acute hormonal changes. We also reported that intravenously administered hydrocortisone causes an acute and temporary induction of the *PER1* and *PER3* mRNA expression in human blood (Yurtsever et al., 2016). However, unlike pulsatile hormone treatment, natural cortisol pulses might not affect the transcriptional pulsing of the genes *in vivo*.

Tissue architecture is important for transcriptional activity (Featherstone et al., 2011). Transcriptional rhythms of the genes are controlled by both local, tissue-specific oscillators and circadian systemic cues. Expression analysis of genes with a period length of approximately 12 h demonstrated that many transcripts return to 24 h periodicity in non-liver tissues (Hughes et al., 2009). Moreover, a glucocorticoid-regulated gene transcript, *Sgk1*, was determined to show pulsatile induction profile in the pituitary such that a significant increase in *Sgk1* mRNA was established in response to each consecutive pulse (George et al., 2017). On the other hand, *Sgk1* showed a non-pulsatile induction profile in the prefrontal cortex, where mRNA levels of *Sgk1* increased rapidly, reached a plateau phase and decreased within

270 min to the baseline levels after pulsatile hormone administration. Therefore, the cellular context affects the transcriptional programming and might lead non-pulsatile expression of *PERIOD* genes in blood.

After pulsatile hormone treatment, the transcriptional pulsing of nascent *Per1* was induced in adrenalectomized animals and cell lines (Stavreva et al., 2009; Conway-Campbell et al., 2010), whereas the mature *Per1* mRNA was continuously released (Stavreva et al., 2009). *Sgk1* was the first transcript shown to be regulated by pulsatile hormone treatment at the mature mRNA level in mouse pituitary (George et al., 2017). In our study, we measured the expression of mature *PERIOD* mRNAs in human blood, a reason why it might not be expected to detect periodic expression profiles in the ultradian range. In the future, the analysis of nascent *PERIOD* mRNA transcripts will help us to better understand pulsatile transcriptional programming in humans.

Transcriptional programming is regulated by rapid dynamics of GR interactions with response elements in chromatin (McNally et al., 2000; Stavreva et al., 2009) and a “hit and run” mechanism has been proposed for these interactions (Rigaud et al., 1991). In this mechanism, the activated receptor binds to chromatin, recruits chromatin remodeling factors and is then lost from the regulatory site. Findings from living cells revealed that hormone-occupied receptor rapidly exchanges with response elements, forming a dynamic interplay in chromatin (McNally et al., 2000). This dynamic interaction between GR and DNA correlates with the binding of the receptor to the GREs in the promoters of GC-responsive genes. *PERIOD* genes have GREs in their promoter regions (see section 1.2.1.1); thus, we expected to detect ultradian expression pattern in *PERIOD* transcripts controlled by pulsatile cortisol *in vivo*.

Pulsatile hormone administration to adrenalectomized rats and cell lines induced cyclic GR-mediated transcriptional regulation (Stavreva et al., 2009; Conway-Campbell et al., 2010; Conway-Campbell et al., 2011), confirming the dynamic interplays between GR and target DNA. However, our preliminary analyses of the baseline study suggested that without any treatment, natural pulsatile cortisol concentrations might not be effective enough to change the expression of *PERIOD* genes. Therefore, these genes might not follow the fluctuations of the cortisol and might not be expressed in an ultradian manner in human blood. Indeed, translational or post-translational modifications regulate the rhythm formation. For that reason, rhythmic gene expression does not correspond to rhythmic proteins inevitably (Mauvoisin et al., 2014). Then, why does a gene have to be expressed in an ultradian manner? Chubb et al. answers “*Pulsing permits greater flexibility in transcriptional decisions—the cell*

is less committed to a particular program if it does not make all the required RNA in one burst (Chubb et al., 2006).” The key point in this answer is the transcriptional decisions. These decisions are controlled by tissue-specific and circadian systemic signals. As it has been reported for the SCN (Ono et al., 2015) and the pituitary (George et al., 2017), a dynamic expression of the genes is expected in the brain more than in the blood since the brain activity is generated by the continuous change.

Limitations of our study are need to be admitted. Several factors, such as sleep-wake cycle, chronotype, light exposure and food intake, can influence the expression of *PERIOD* genes. However, these factors were not controlled in our study since the initial recruitment of the subjects was part of a project that was designed for another purpose. This is a clear weakness and might cause the interindividual variability we faced in this study. Thus, it is important to perform analysis in each participant and treat them as an individual variable in the future.

As a result, *PER1*, *PER2* and *PER3* transcripts might not show ultradian pulsatility in the blood of healthy humans. Furthermore, the expression of *PERIOD* genes, especially *PER1*, was weakly but significantly correlated with the pulsatile cortisol levels under baseline conditions. In order to draw better conclusions, proper statistical analyses have to be applied to the time series of *PERIOD* genes.

The goal of the second study in this thesis was to examine the effect of psychosocial stress on the expression of *PERIOD* genes in humans. Mean cortisol levels were significantly elevated 10 min after stress test; however, *PER1*, *PER2* and *PER3* did not show any significant change during sampling period.

TSST is the most useful and highly standardized protocol to activate HPA axis stress response (Dickerson & Kemeny, 2004); however, interindividual difference in response to the TSST is an important point to be considered. In our study, we observed cortisol responses varying among individuals. However, since the sample size was small, we took the average of all cortisol values for analysis. It has been revealed that mean cortisol levels were increased 1.4 fold 10 min after TSST compared to the levels of pre-test and this elevation was confirmed to be significant via non-parametric test. Therefore, we concluded that psychosocial stress caused a small but significant induction in cortisol levels.

The expression of *PERIOD* transcripts was not affected by psychosocial stress test in our setting. Although there are several reports demonstrating the effect of glucocorticoids on

PERIOD gene expression (Burioka et al., 2005; Charmandari et al., 2011; Cuesta et al., 2015; Fukuoka et al., 2005; Reddy et al., 2009), there might be some explanations for the non-responsiveness we observed. First of all, in the previous investigations, high concentrations of glucocorticoids are administrated but the TSST induced comparably lower cortisol levels, even though the mean change was significant. In our pharmacological study, it has been demonstrated that infusion of low hydrocortisone concentrations did not affect the expression of *PERIOD* transcripts, whereas higher doses caused an acute and temporary induction of *PER1* and *PER3* in human blood (Yurtsever et al., 2016). Thus, low levels of free cortisol could not modulate the gene expression. Secondly, we aimed to investigate a very first reaction to psychological stress; thus, the expression of *PERIOD* genes was observed for 4.5 h, which might be too short to determine large effects.

Participants involved in this study were recruited among students of the University of Trier, who have experience with public speaking and mental arithmetic. This might be one possible explanation for the low cortisol response we observed after stress test. Thus, recruiting nonacademic volunteers from the normal population might increase the activity of the HPA axis sufficiently and give reliable results about gene expression after psychological stress. On the other hand, it is important to get rid of nonspecific response components, which are not dependent on the environment (Fink, 2007, p 292); therefore, two or more stress exposures might improve the understanding of the individual's cortisol responsiveness in the future studies (Kirschbaum et al., 1995).

Limitations are needed to be admitted. Our study has a small number of subjects, which lacks the statistical power to statistically reveal small or medium-sized effects. In order to get an effect size of 0.5 with a statistical power of 0.8, 34 participants need to be recruited for this stress study in the future. Moreover, high interindividual differences in cortisol levels and in gene expression might neutralize effects and result in non-responsiveness. High number of subjects may give us an opportunity to make groups and apply proper statistics to examine large effects of the psychological stress.

As a conclusion, acute and small induction of cortisol caused by the psychosocial stress test cannot regulate the expression of *PERIOD* gene in human blood for a short sampling period, suggesting that humans are able to maintain the homeostasis after an acute stress.

In the third study of this thesis, we investigated the fast transcriptional effects of cortisol on the expression of human *PER1, PER2* and *PER3 in vivo* and to understand the dose-response

relationship between cortisol and *PERIOD* genes. In our study, we demonstrated for the first time the effects of intravenously administered escalating doses of hydrocortisone on the expression of *PERIOD* genes in human subjects. Moderate cortisol concentrations affect *PER1* and *PER3* expression within 45 and 30 minutes, respectively, whereas *PER2* did not show significant changes in our study.

The escalating cortisol doses chosen in our study exhibited a physiological conceivable value in saliva (Schilling et al., 2013) and plasma (Yurtsever et al., 2016). At the lowest dose (3 mg) of hydrocortisone no significant response in the expression of *PERIOD* genes was detected. *PER1* and *PER3* mRNA expressions were induced at the moderate dose (6 mg). The expression of *PER1* remained elevated for 45 minutes, whereas *PER3* mRNA expression leveled out within 30 minutes. In the 12 mg receiving group, the effects of cortisol decreased for both genes and even delayed in *PER3* mRNAs. For the highest dose, the effects of cortisol on *PER3* were disappeared, whereas *PER1* mRNAs showed a delayed increase which leveled out within 15 minutes. Responsiveness of *PER1* and *PER3* was reduced with escalating doses of the hormone. Physical and psychological stressful events induce a rise in the plasma levels of glucocorticoids and induce the expression of *PER1* both in mice (Tahara et al., 2015; Yamamoto et al., 2005) and in humans (Cuesta et al., 2015; Fukuoka et al., 2005). In our *in vivo* study, we mimicked these events and generated a cortisol concentration pattern different from the endogenous circadian cortisol rhythm. Our finding suggests that the pharmacological stress causes an acute and temporary induction of the *PER1* and *PER3* mRNA expression in whole blood.

The acute induction of *PER1* mRNA observed in the group that had received moderate amounts of hormone might be crucial in other cellular and physiological mechanisms, such as cell division, apoptosis and malignant growth (Gery et al., 2006), as well as in driving the circadian gene expression. In a study of cultured hepatic cells (HepG2), it was shown that prednisolone modulates the mRNA levels of circadian genes such as *PER2*, *CRY1*, *DBP*, *REV-ERB* and *BMAL1*; however, this modulation was abolished when cells were pretreated with a *PER1* antisense oligodeoxynucleotide (Koyanagi et al., 2006). Moreover, after being treated by low-dose glucocorticoids, *PER1* triggers the expression of other circadian genes like *CRY*s several hours later in lung epithelial cell line. Then, increased expression of *CRY1* and *CRY2* occur simultaneously with the down-regulation of *PER1*, *PER2* and *BMAL1* (Reddy et al., 2009). This repression is caused by murine *Cry* genes, which interact with GR to repress glucocorticoid-mediated gene transcription, thereby forming a feedback inhibition

(Lamia et al., 2011). Together, transient induction of *PER1* by glucocorticoids regulates the expression of circadian rhythm genes and these circadian genes oppose the glucocorticoid-mediated activation.

PER2 was not modulated by cortisol in our experiment. Supporting our result, Fukuoka et al. (2005) and Cuesta et al. (2015) reported that glucocorticoids increased *PER1* mRNA level, but not *PER2* mRNA level in human PBMCs a short time after administration. Moreover, *PER3* showed an expression profile which is similar to *PER1*. *Per3* knockout has subtle effects on the circadian rhythm in mice (Shearman et al., 2000) and an association of the interindividual differences in *PER3* expression with human sleep regulation has recently been identified (Archer et al., 2008; Viola et al., 2007). Cuesta and colleagues reported a resetting of *PER3* after six days Cortef (hydrocortisone tablet) administration with a phase shift but no acute change in gene expression probably due to the sampling at longer time intervals (Cuesta et al., 2015). The acute and temporary induction of *PER3* after hormone administration, which we determined in this study, might be caused by tissue-specific functions of *Per3* (Pendergast et al., 2012a), a variable-number of tandem-repeats in the coding region of this gene (Archer et al., 2003) or other polymorphism, which were not investigated in our setting. As a result, the above mentioned glucocorticoid action might indicate a mechanism of *PER3* transcription in non-circadian systems, in addition to the circadian clock-related function of *PER3* in the periphery.

In previous reports, the effects of glucocorticoids have been studied at longer time intervals. With the 15 min-sampling design, we determined the fast genomic effects of cortisol on the expression of *PERIOD* genes. Steroid hormones modulate the expression of their target genes a few hours after stimulation, although immediate early genes are expressed within one hour after for example aldosterone stimulation (Verrey, 1998). Immediate early genes are genes, which are rapidly and transiently transcribed after a cellular stimulus. Murine *Per1* and *Per2* are shown to be immediate early genes in the SCN activated shortly after light exposure (Shearman et al., 1997; Shigeyoshi et al., 1997). In our study, *PER1* and *PER3* act as immediate early genes in whole blood and respond to cortisol within 45 and 30 minutes, respectively, after hormone administration. Therefore, the sampling at shorter time intervals revealed the rapid genomic action of cortisol, which was missed in the literature previously.

The actions of glucocorticoids are mediated by GR. Thus, we next intended to verify the possible role of GR on *PER1*, *PER2* and *PER3* expression. When whole blood was treated with hydrocortisone and RU486 *ex vivo*, *PER1* and the positive control *GILZ* showed an

expected increase in the expression after hormone administration and a decrease in the expression after antagonist administration. The induction of *PER1* mRNA expression was reduced by RU486 treatment but not completely blocked. RU486 has both glucocorticoid antagonist and agonist activity. In order to exert its glucocorticoid antagonistic action, it occupies the GRs and competes with agonist-bound receptors to block agonist-induced transcriptional activation (Sartor & Cutler, 1996). RU486 at a dose of 20 mg/kg caused an incomplete suppression of the corticotropic releasing hormone-induced ACTH secretion, which supports the partial agonistic effect of RU486 on the GR (Laue et al., 1988). The incomplete blockade, which we observed in our study, might be produced by this agonistic effect of RU486. Nevertheless, we could confirm that *PER1* expression is GR-dependent due to the lower increase in the presence of GR antagonist. Furthermore, *PER2* showed no significant change after hydrocortisone treatment. Consistent with the *in vivo* study, this might be caused by short time treatment of hydrocortisone in the blood. In addition, *PER3* was not responsive to the hormone treatment in the *ex vivo* study. This non-responsiveness of *PER3* might be caused by high hormone concentration which was also observed *in vivo* study at 24 mg hormone receiving group. Comparisons between *in vivo* and *in vitro* conditions are always complicated due to the higher complexity in the *in vivo* settings. However, we can still make relative comparisons such that the plasma cortisol concentrations reached their highest levels (15 minutes after infusion) with 32.86 µg/dl and 45.63 µg/dl in the groups that had received 12 and 24 mg of hormone, respectively, and the application of 10 µM (36.25 µg/dl) hydrocortisone is also a high dose for *in vitro* experiments.

Pharmacological stress was performed at a certain time of the day in our setting. However, when we think about the treatment methods for several psychological and immunological disorders, optimization the time of administration could improve the positive effects and reduce the side effects of these hormones. Glucocorticoids could be administrated at different time of the day to reveal the specific time at which clock system is affected at minimum level by a hormone treatment in human subjects. Optimizing the time of administration could prevent the modulation of clock genes by glucocorticoids at pharmacological doses.

Several limitations needed to be admitted. This report was a part of another study that was designed for another purpose. Therefore, interindividual differences in daily rhythms were not controlled, which is a clear weakness of the study. However, we assume that in a short-term design the phases of the subjects might play a minor role whereas a phase shift in response to glucocorticoid administration has been shown in long-term experiments (Cuesta et al., 2015).

Moreover, our study has the small sample size of the administrated subgroups that lacks the statistical power to statistically reveal small or medium-sized effects. Analysis with uncorrected values showed a dose- and time-dependence of *PER1*. However, after corrections for multiple testing, most of the values did not remain significant because of high interindividual differences in stress sensitivity. Two of the factors affecting this sensitivity are high endogenous cortisol levels and high mRNA levels of *PERIOD* genes causing either no effects or dampened effects of the exogenous glucocorticoid stimuli. Therefore, replication of our results is necessary and interpretation has to be taken with caution.

In conclusion, our data showed the fast transcriptional effects of cortisol on the expression of human *PER1* and *PER3 in vivo*. These effects on the mRNA levels are gene-specific and disappear with high concentrations of cortisol. In addition, cortisol-modulation of these genes is controlled by either GR-dependent or GR-independent mechanisms resulting in differential expression patterns for *PER1*, *PER2* and *PER3*. Especially *PER1* was regulated in a GR-dependent manner in whole blood. Therefore, our findings suggest that the expressions of *PER1* and *PER3* are temporarily regulated by acute stress in human blood.

In the last study of this thesis, we aimed to investigate genes regulated by glucocorticoid-induced *PER1*. Human liver cancer cell line, HepG2, was used and chromatin immunoprecipitation was performed to determine target genes of PER1 as a transcription factor. PER1 plays an important role in the circadian clock system as well as in other cellular and physiological mechanisms, such as cell division, apoptosis and malignant growth (Gery et al., 2006). Therefore, downstream genes controlled by stress-activated PER1 can shed light on pathways involved in human well-being.

ChIP is a method used to investigate the interactions between proteins and DNA in the cells. There are several steps in the ChIP protocol, including cross-linking of the cells, shearing the DNA-protein complexes, immunoprecipitation of the target protein associated with the target DNA fragments and purification of the target DNA fragments. All steps have to be optimized in order to have high quality DNA samples for consecutive analyses.

Cross-linking of the cells with formaldehyde is the first step which needs to be optimized. The duration of the fixation influence the ChIP efficiency. Thus, each cell type has to be fixed for an appropriate time. The protein of interest in our experiment was PER1, which has been reported to bind to ~4600 sites including the intergenic, promoter and intronic regions of genes in the mouse genome (Koike et al., 2012). However, PER1 is missing the basic region,

which is crucial for the DNA binding, and cannot bind directly to the DNA (Kucera et al., 2012). This was the first challenging step in our study. Non-direct binding to DNA might affect the cross-linking efficiency of PER1 to chromatin since protein-protein complexes have to be formed before binding to the DNA, which takes more time than a direct binding. Therefore, in the future experiments, the cross-linking time should be increased and/or protein-protein cross-linking reagents might be added before formaldehyde treatment in order to improve cross-linking efficiency (Carey et al., 2009).

The shearing of the protein-DNA complexes is another step affecting the success of the ChIP. Sonication performed to shear DNA is influenced by sonicator type, cell type and number, volume of the cell lysate and temperature. In our study, we showed that even microcentrifuge tubes used for sonication change the shearing efficiency of the DNA. Although proper size of the DNA fragments was evaluated after sonication by agarose gel electrophoresis, immunoprecipitation step determined the final result of ChIP experiment.

Antibody used for immunoprecipitation is one of the key components of ChIP. If the antibody is not efficient to precipitate protein from the fixed and native samples, the ChIP experiment will fail. Therefore, precipitation efficiency of the target antibody has to be determined by Western blot analysis or enzyme-linked immunosorbent assay before using it for immunoprecipitation. Commercially available anti-PER1 antibody used in our study was not tested for the specificity and resulted in poor DNA samples after immunoprecipitation. Specificity of the antibody should be investigated in the future studies. In addition, more antibodies and/or more incubation time might also improve the immunoprecipitation results (Carey et al., 2009). Although anti-PER1 antibody concentration was changed in our experiment, it resulted in poor DNA precipitation, suggesting non-efficiency of the antibody. Thus, instead of commercially available antibody, another antibody might be generated or identified in the future. Furthermore, beads are other players of the immunoprecipitation influencing the ChIP efficiency. Beads have a proper binding range which determines the specificity of binding to antibody. Protein A/G magnetic beads, for example, were used in this ChIP experiment do not bind murine IgA and IgM antibodies (Thermo Scieintific, Pierce protein A/G magnetic beads instructions). In order to improve these results, another beat-antibody combination might be required.

As a conclusion, ChIP analysis has been standardized and widely used; however, it is inevitable to optimize key parameters in order to obtain valuable ChIP data. In general, one of the major limitations is the antibody specificity, which leads an efficient precipitation of the

target protein (Carey et al., 2009). In this thesis, ChIP was failed because of several reasons discussed previously. Downstream genes controlled by stress-activated PER1 still remained undiscovered and might be examined via an antibody, recognizing the target protein with a high specificity. Investigation of possible target genes regulated by glucocorticoid-induced PER1 might improve our understanding about the consequences of the interactions between stress and circadian clock genes.

Several drawbacks in human studies of this thesis need critical discussions. First important point is the interindividual variation in gene expression patterns of the participants. Humans have different genetic backgrounds and confront changing environmental exposures, leading variability among donors recruited for gene expression studies. Microarray analysis of whole blood samples revealed that individual variations in gene expression reflect pathological and/or physiological conditions (Whitney et al., 2003). Moreover, the peak timing of the clock gene rhythms in the peripheral blood is a crucial factor contributing the inter-subject variation since it has been shown to be significantly different among individuals (Archer et al., 2008). In one study, the peak timing of *PER1* and *PER2* expression was early after wake-time and that of *BMAL1* expression was the middle of the wake period (James et al., 2007), whereas in another study *BMAL1* fluctuated with roughly the same phase as *PER2* in PBMCs (Teboul et al., 2005). Moreover, clock gene rhythms might be affected by polymorphisms in these genes. For example, a variable number of tandem repeats in *PER3* is associated with chronotype. Neither peak timing nor polymorphisms were investigated in our experiments, which is a clear weakness of our studies. In contrast to other studies, we did not apply any protocol to entrain the participants to a sleep-wake schedule (James et al., 2007; Kusanagi et al., 2008; Teboul et al., 2005). It seems that naturally occurring interindividual differences are inevitable and the most critical limitation in human studies. In order to minimize these differences, genotyping and grouping according to a specific polymorphism and/or questionnaires, entraining to a light-dark cycle as well as gender factors have to be taken into account when subjects are recruited.

Second drawback of our studies was the small sample size, lacking the statistical power to statistically reveal small or medium-sized effects. Unfortunately, small sample size reduces the probability of detecting a correct effect and reproducibility of findings as well as reveals statistically significant results which do not reflect the correct effect (Button et al., 2013). On the other hand, studies with small sample size can be designed when we take the technical, ethical and financial reasons into account in humans (Faber & Fonseca, 2014). The small

sample size in human studies can be overcome by collaborating with other colleagues from different departments or organizations, and/or using the data from population-based studies reported by other groups.

Relative quantification of the target gene expression was another point, which needs critical discussions. Comparative Ct ($2^{-\Delta\Delta Ct}$) method was used in the baseline study since it is a broadly used method, presenting the relative gene expression (Schmittgen & Livak, 2008a), whereas $2^{-\Delta Ct}$ method was used in the stress studies. In $2^{-\Delta\Delta Ct}$ method, a housekeeping gene as an internal control and a calibrator as a reference sample are required, while $2^{-\Delta Ct}$ method gives individual data points, independent from a calibrator. The calibrator is actually based on the gene expression experiment and shows the expression of the transcript at time zero (Livak & Schmittgen, 2001). The choice of sample as the calibrator might yield different results. Moreover, the comparative Ct method could generally cause inaccurate results, when the amplification efficiencies of the target gene and internal control gene are not equal and the target gene is expressed at low levels (Liu & Saint, 2002). The criterion for the PCR efficiency was between 90% and 110% in our experiments. Although this range is broad, it has been shown to give reliable results for quantitative real-time PCR (Broeders et al., 2014); thus, the relative gene expression measured and calculated in this thesis is valid.

The understanding about the interplay between the HPA axis and the circadian clock is improving and this thesis provides new insight into the human research. However, several important questions are left open until today and need to be answered in the future. For example, one can think that most of the core clock genes of the circadian system have been discovered. Nevertheless, two research groups (Anafi et al., 2014; Goriki et al., 2014) recently identified a new clock gene in mice, called *Chrono* (computationally highlighted repressor of network oscillator or ChIP-derived repressor of network oscillator). The expression profile of *CHRONO* under baseline conditions and after stress can be investigated in humans. This can open a new dimension in the interplay between stress and clock genes.

Interindividual variation in gene expression patterns of the human subjects was one of the limitations challenging the stress response in our studies. It would be very interesting to investigate individual genotypes and polymorphisms in clock genes, involved in the stress response and contributing the interindividual differences. In addition, polymorphisms in clock genes affecting the protein structure can also be determined. This may improve our understanding of factors affecting phenotypes.

It has been reported that humans show gender-dependent differences in stress response, although the response of the HPA axis is stressor dependent (Kudielka & Kirschbaum, 2005). Effects of sex differences on the clock gene expression can be of interest after stress test. Moreover, the effects of age on clock gene expression can be examined in multiple age groups. Both gender- and age-dependent outcomes in clock gene transcripts might show differences between healthy subjects and clinical patients. This understanding may help to promote human well-being.

Clock genes are under the control of epigenetic mechanisms. Phosphorylation, methylation, acetylation and ubiquitination are genome wide mechanisms involved in epigenetic control. There are already studies showing the importance of histone acetylation and methylation for clock function (Etchegaray et al., 2003; Etchegaray et al., 2006). Investigation of these epigenetic mechanisms regulating the glucocorticoid-responsive clock genes will provide information about how the environmental changes, e.g. stress, controls the circadian response.

Keeping the basal and stress-related homeostasis is essentially contributed by glucocorticoids, which are one of the most pervasive hormones reaching all organs in the body (Chrousos & Kino, 2007). These hormones activate 12 % and repress 9 % of the genes expressed in human leukocyte genome (Galon et al., 2002) through interactions with GRs. Glucocorticoids, as the end-product of the HPA axis, also play important roles in molecular, cellular and physiological pathways. On the other hand, circadian clocks regulate several functions and up to 10 % of the transcriptome is under the control of circadian system in a tissue-specific manner (Panda et al., 2002). The research presented in this thesis is a small piece of these extremely broad networks of biological interactions. We provided better understanding of transcriptional programming regulated by pulsatile cortisol at standard conditions, while our findings revealed fast and temporary response of clock genes to acute stress in human blood, suggesting two important key points, which are dose-dependent: 1) the involvement of acute regulatory mechanisms and 2) the stability of the circadian rhythms against acute stress. Moreover, our results help us to understand the non-circadian functions of clock genes regulated by glucocorticoids, e.g. cortisol, in the periphery. These non-circadian functions are different from resetting of the circadian time and may be fundamental in physiological and cellular pathways involved in stress response. The experimental evidence presented in this thesis proposed *PER1* and *PER3* as stress makers in humans.

The HPA axis communicates with the circadian clock system at multiple levels. Abnormalities and impairments in the regulation of these systems lead similar consequences.

Metabolic syndrome, depression, Cushing's syndrome and Alzheimer's disease can be given as example caused by altered circadian cortisol regulation (Dickmeis, 2009). Although there is a growing research in this field, it is still not clear when these alterations are reasons or consequences of the diseases (Dickmeis, 2009). Therefore, we, as biologists, have to understand these mechanisms in order to optimize treatment strategies as well as to promote human well-being.

CHAPTER 6: SUMMARY

The circadian clock system and the HPA axis are complicated systems and made up of extremely broad networks of biological interactions. These interactions are well described in rodents. However, findings obtained from animal studies are not always easy to translate to humans since animals and humans have different rest-activity cycles and hormonal regulation. Therefore, human studies are inevitable to explore the underlying regulatory mechanisms. This thesis aimed to shed light on these mechanisms, connecting stress response to the circadian system in healthy humans.

We compared the interplays between cortisol as an end-product of the HPA axis and *PERIOD* (*PER1*, *PER2* and *PER3*) transcripts as circadian clock genes under baseline conditions and after stress exposure in the blood. *PERIOD* genes might not oscillate in a pulsatile manner and especially *PER1* was weakly but significantly correlated with pulsatile cortisol concentrations under baseline conditions. Psychological stress caused a small but significant increase in the cortisol levels but not affected the gene expression, whereas pharmacological stress produced an acute and temporary induction of *PER1* and *PER3* mRNA levels at moderate doses, whereas *PER2* was not responsive to the hormone administration.

Previous studies were designed to reveal the effects of glucocorticoids at longer time intervals. With the 15-min sampling, we investigated the fast genomic effects of cortisol and rapid response of *PER1* and *PER3*, acting as immediate early genes in whole blood. Responsiveness of *PER1* to glucocorticoid treatment has been shown by several animal and cell culture studies. Our results confirmed those findings and also took attention to *PER3* as a stress-responsive gene in the peripheral tissues.

As a conclusion, acute stress can cause small and transient effects on circadian clock genes in dose-dependent manner in short sampling settings, while endogenous cortisol may not seem to modulate the gene expression at standard conditions. Interindividual stress sensitivity is an important factor, shaping these molecular mechanisms in humans. Our results suggest that there are protective mechanisms which prevent stress from altering circadian rhythms. It is meaningful not to be easily affected by stress; and to keep homeostasis and rhythms stable in human body.

CHAPTER 7: REFERENCES

Abe H, Honma S, Namihira M, Masubuchi S, Honma K (2001). Behavioural rhythm splitting in the CS mouse is related to clock gene expression outside the suprachiasmatic nucleus. Eur J Neurosci. 14:1121–8.

Akaike H (1974). A new look at the statistical model identification. IEEE Trans Autom Control. 19: 716–23.

Akashi M, Soma H, Yamamoto T, Tsugitomi A, Yamashita S, Yamamoto T et al. (2010). Noninvasive method for assessing the human circadian clock using hair follicle cells. Proc Natl Acad Sci U S A. 107:15643–8.

Akashi M, Tsuchiya Y, Yoshino T, Nishida E (2002). Control of intracellular dynamics of mammalian period proteins by casein kinase I epsilon (CKIepsilon) and CKIdelta in cultured cells. Mol Cell Biol. 22:1693–703.

Albrecht U, Sun ZS, Eichele G, Lee CC (1997). A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. Cell. 91:1055–64.

Anafi RC, Lee Y, Sato TK, Venkataraman A, Ramanathan C, Kavakli IH et al. (2014). Machine learning helps identify CHRONO as a circadian clock component. PLoS Biol. 12:e1001840.

Archer SN, Carpen JD, Gibson M, Lim GH, Johnston JD, Skene DJ et al. (2010). Polymorphism in the PER3 promoter associates with diurnal preference and delayed sleep phase disorder. Sleep. 33:695–701.

Archer SN, Robilliard DL, Skene DJ, Smits M, Williams A, Arendt J et al. (2003). A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference. Sleep. 26:413–5.

Archer SN, Viola AU, Kyriakopoulou V, Schantz M von, Dijk D-J (2008). Inter-individual differences in habitual sleep timing and entrained phase of endogenous circadian rhythms of BMAL1, PER2 and PER3 mRNA in human leukocytes. Sleep. 31:608–17.

Aserinsky E, Kleitman N (1953). Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. Science. 118:273–4.

Ashall L, Horton CA, Nelson DE, Paszek P, Harper CV, Sillitoe K et al. (2009). Pulsatile stimulation determines timing and specificity of NF-kappaB-dependent transcription. Science. 324:242–6.

Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, Kreppel F et al. (2008). SIRT1 regulates circadian clock gene expression through PER2 deacetylation. Cell. 134:317–28.

Ashwell JD, Lu FW, Vacchio MS (2000). Glucocorticoids in T cell development and function*. Annu Rev Immunol. 18:309–45.

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR (2001). Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron. 30:525–36.

Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM et al. (2000). Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science. 289:2344–7.

Balsalobre A, Damiola F, Schibler U (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell. 93:929–37.

Bamberger CM, Bamberger AM, Castro M de, Chrousos GP (1995). Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. J Clin Invest. 95:2435–41.

Bartlang MS, Neumann ID, Slattery DA, Uschold-Schmidt N, Kraus D, Helfrich-Förster C et al. (2012). Time matters. Pathological effects of repeated psychosocial stress during the active, but not inactive, phase of male mice. J Endocrinol. 215:425–37.

Bjarnason GA, Jordan RC, Wood PA, Li Q, Lincoln DW, Sothern RB et al. (2001). Circadian expression of clock genes in human oral mucosa and skin. Association with specific cell-cycle phases. Am J Pathol. 158:1793–801.

Blum ID, Zhu L, Moquin L, Kokoeva MV, Gratton A, Giros B et al. (2014). A highly tunable dopaminergic oscillator generates ultradian rhythms of behavioral arousal. eLife. 3.

Boivin DB, James FO, Wu A, Cho-Park PF, Xiong H, Sun ZS (2003). Circadian clock genes oscillate in human peripheral blood mononuclear cells. Blood. 102:4143–5.

Born J, Lange T, Hansen K, Mölle M, Fehm HL (1997). Effects of sleep and circadian rhythm on human circulating immune cells. J Immunol. 158:4454–64.

Bose S, Boockfor FR (2010). Episodes of prolactin gene expression in GH3 cells are dependent on selective promoter binding of multiple circadian elements. Endocrinology. 151:2287–96.

Brazis PW, Masdeu JC, Biller J (2011). Localization in clinical neurology. 6th ed. Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins Health.

Brodsky VY (2014). Circahoralian (ultradian) metabolic rhythms. Biochemistry. Biokhimiia. 79:483–95.

Broeders S, Huber I, Grohmann L, Berben G, Taverniers I, Mazzara M, Roosens N, Morisset D (2014). Guidelines for validation of qualitative real-time PCR methods. Trends Food Sci Tech. 37:113–26.

Brown SA, Zumbrunn G, Fleury-Olela F, Preitner N, Schibler U (2002). Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Current biology: CB. 12:1574–83.

Buckley TM, Schatzberg AF (2005). On the interactions of the hypothalamic-pituitary-adrenal (HPA) axis and sleep. Normal HPA axis activity and circadian rhythm, exemplary sleep disorders. J Clin Endocrinol Metab. 90:3106–14.

Buhr ED, Yoo S-H, Takahashi JS (2010). Temperature as a universal resetting cue for mammalian circadian oscillators. Science. 330:379–85.

Buijs RM, Kalsbeek A (2001). Hypothalamic integration of central and peripheral clocks. Nat Rev Neurosci. 2:521–6.

Buijs RM, Wortel J, van Heerikhuize, J J, Feenstra MG, Ter Horst, G J, Romijn HJ et al. (1999). Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. Eur J Neurosci. 11:1535–44.

Buijs RM, Wortel J, van Heerikhuize, J J, Kalsbeek A (1997). Novel environment induced inhibition of corticosterone secretion: physiological evidence for a suprachiasmatic nucleus mediated neuronal hypothalamo-adrenal cortex pathway. Brain Res. 758:229–36.

Burioka N, Fukuoka Y, Takata M, Endo M, Miyata M, Chikumi H et al. (2007). Circadian rhythms in the CNS and peripheral clock disorders. Function of clock genes: influence of medication for bronchial asthma on circadian gene. J Pharmacol Sci. 103:144–9.

Burioka N, Takata M, Okano Y, Ohdo S, Fukuoka Y, Miyata M et al. (2005). Dexamethasone influences human clock gene expression in bronchial epithelium and peripheral blood mononuclear cells in vitro. Chronobiol Int. 22:585–90.

Button KS, Ioannidis JPA, Mokrysz C, Nosek BA, Flint J, Robinson ESJ et al. (2013). Power failure. Why small sample size undermines the reliability of neuroscience. Nat Rev Neurosci. 14:365–76.

Cardone L, Hirayama J, Giordano F, Tamaru T, Palvimo JJ, Sassone-Corsi P (2005). Circadian clock control by SUMOylation of BMAL1. Science. 309:1390–4.

Carey MF, Peterson CL, Smale ST (2009). Chromatin immunoprecipitation (ChIP). Cold Spring Harbor protocols. 2009:pdb.prot5279.

Charmandari E, Chrousos GP, Lambrou GI, Pavlaki A, Koide H, Ng, Sinnie Sin Man et al. (2011). Peripheral CLOCK regulates target-tissue glucocorticoid receptor transcriptional activity in a circadian fashion in man. PLoS One. 6:e25612.

Cheng MY, Bullock CM, Li C, Lee AG, Bermak JC, Belluzzi J et al. (2002). Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. Nature. 417:405–10.

Cheon S, Park N, Cho S, Kim K (2013). Glucocorticoid-mediated Period2 induction delays the phase of circadian rhythm. Nucleic Acids Res. 41:6161–74.

Chrousos GP (2009). Stress and disorders of the stress system. Nature reviews. Endocrinology. 5:374–81.

Chrousos GP, Gold PW (1998). A healthy body in a healthy mind--and vice versa--the damaging power of "uncontrollable" stress. J Clin Endocrinol Metab. 83:1842–5.

Chrousos GP, Kino T (2007). Glucocorticoid action networks and complex psychiatric and/or somatic disorders. Stress. 10:213–9.

Chubb JR, Trcek T, Shenoy SM, Singer RH (2006). Transcriptional pulsing of a developmental gene. Curr Biol. 16:1018–25.

Chyun YS, Kream BE, Raisz LG (1984). Cortisol decreases bone formation by inhibiting periosteal cell proliferation. Endocrinology. 114:477–80.

Conway-Campbell BL, George CL, Pooley JR, Knight DM, Norman MR, Hager GL et al. (2011). The HSP90 molecular chaperone cycle regulates cyclical transcriptional dynamics of the glucocorticoid receptor and its coregulatory molecules CBP/p300 during ultradian ligand treatment. Mol Endocrinol. 25:944–54.

Conway-Campbell BL, Sarabdjitsingh RA, McKenna MA, Pooley JR, Kershaw YM, Meijer OC et al. (2010). Glucocorticoid ultradian rhythmicity directs cyclical gene pulsing of the clock gene period 1 in rat hippocampus. J Neuroendocrinol. 22:1093–100.

Cooper CL (op. 2005). Handbook of stress medicine and health. 2nd ed. Boca Raton: CRC Press.

Cuesta M, Cermakian N, Boivin DB (2015). Glucocorticoids entrain molecular clock components in human peripheral cells. FASEB J. 29:1360–70.

Dallmann R, Touma C, Palme R, Albrecht U, Steinlechner S (2006). Impaired daily glucocorticoid rhythm in $Per1^{Brd}$ mice. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 192:769–75.

Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 14:2950–61.

Dickerson SS, Kemeny ME (2004). Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. Psychol Bull. 130:355–91.

Dickmeis T (2009). Glucocorticoids and the circadian clock. J Endocrinol. 200:3–22.

Ding JM, Chen D, Weber ET, Faiman LE, Rea MA, Gillette MU (1994). Resetting the biological clock. Mediation of nocturnal circadian shifts by glutamate and NO. Science. 266:1713–7.

Doane LD, Kremen WS, Eaves LJ, Eisen SA, Hauger R, Hellhammer D et al. (2010). Associations between jet lag and cortisol diurnal rhythms after domestic travel. Health Psychol. 29:117–23.

Doi M, Hirayama J, Sassone-Corsi P (2006). Circadian regulator CLOCK is a histone acetyltransferase. Cell. 125:497–508.

Dressendorfer RA, Kirschbaum C, Rohde W, Stahl F, Strasburger CJ (1992). Synthesis of a cortisol-biotin conjugate and evaluation as a tracer in an immunoassay for salivary cortisol measurement. J Steroid Biochem Mol Biol. 43:683–92.

Dumbell R, Matveeva O, Oster H (2016). Circadian Clocks, Stress, and Immunity. Front Endocrinol. 7:37.

Eide EJ, Woolf MF, Kang H, Woolf P, Hurst W, Camacho F et al. (2005). Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. Mol Cell Biol. 25:2795–807.

Engeland WC, Yoder JM, Karsten CA, Kofuji P (2016). Phase-Dependent Shifting of the Adrenal Clock by Acute Stress-Induced ACTH. Front Endocrinol. 7:81.

Engler D, Pham T, Fullerton MJ, Ooi G, Funder JW, Clarke IJ (1989). Studies of the secretion of corticotropin-releasing factor and arginine vasopressin into the hypophysial-portal circulation of the conscious sheep. I. Effect of an audiovisual stimulus and insulin-induced hypoglycemia. Neuroendocrinology. 49:367–81.

Engler D, Pham T, Liu JP, Fullerton MJ, Clarke IJ, Funder JW (1990). Studies of the regulation of the hypothalamic-pituitary-adrenal axis in sheep with hypothalamic-pituitary disconnection. II. Evidence for in vivo ultradian hypersecretion of proopiomelanocortin peptides by the isolated anterior and intermediate pituitary. Endocrinology. 127:1956–66.

Etchegaray J-P, Lee C, Wade PA, Reppert SM (2003). Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature. 421:177–82.

Etchegaray J-P, Yang X, DeBruyne JP, Peters AHFM, Weaver DR, Jenuwein T et al. (2006). The polycomb group protein EZH2 is required for mammalian circadian clock function. J Biol Chem. 281:21209–15.

Faber J, Fonseca LM (2014). How sample size influences research outcomes. Dental Press J Orthod. 19:27–9.

Featherstone K, Harper CV, McNamara A, Semprini S, Spiller DG, McNeilly J et al. (2011). Pulsatile patterns of pituitary hormone gene expression change during development. J Cell Sci. 124:3484–91.

Fink G (2007). Encyclopedia of stress. 2nd ed. San Diego, Calif: Elsevier.

Fonken LK, Weber MD, Daut RA, Kitt MM, Frank MG, Watkins LR et al. (2016). Stress-induced neuroinflammatory priming is time of day dependent. Psychoneuroendocrinology. 66:82–90.

Fukuoka Y, Burioka N, Takata M, Ohdo S, Miyata M, Endo M et al. (2005). Glucocorticoid administration increases hPer1 mRNA levels in human peripheral blood mononuclear cells in vitro or in vivo. J Biol Rhythms. 20:550–3.

Galon J, Franchimont D, Hiroi N, Frey G, Boettner A, Ehrhart-Bornstein M et al. (2002). Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. FASEB J. 16:61–71.

George CL, Birnie MT, Flynn BP, Kershaw YM, Lightman SL, Conway-Campbell BL (2017). Ultradian glucocorticoid exposure directs gene-dependent and tissue-specific mRNA expression patterns in vivo. Mol Cell Endocrinol. 439:46–53.

Gerkema MP, Groos GA, Daan S (1990). Differential elimination of circadian and ultradian rhythmicity by hypothalamic lesions in the common vole, Microtus arvalis. J Biol Rhythms. 5:81–95.

Gerkema MP, van der Leest F (1991). Ongoing ultradian activity rhythms in the common vole, Microtus arvalis, during deprivations of food, water and rest. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 168:591–7.

Gery S, Komatsu N, Baldjyan L, Yu A, Koo D, Koeffler HP (2006). The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. Mol Cell. 22:375–82.

Gibbs J, Ince L, Matthews L, Mei J, Bell T, Yang N et al. (2014). An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action. Nat Med. 20:919–26.

Goldstein DS, Kopin IJ (2007). Evolution of concepts of stress. Stress. 10:109–20.

Gomez-Abellan P, Diez-Noguera A, Madrid JA, Lujan JA, Ordovas JM, Garaulet M (2012). Glucocorticoids affect 24 h clock genes expression in human adipose tissue explant cultures. PLoS One. 7:e50435.

Goriki A, Hatanaka F, Myung J, Kim JK, Yoritaka T, Tanoue S et al. (2014). A novel protein, CHRONO, functions as a core component of the mammalian circadian clock. PLoS Biol. 12:e1001839.

Gower WR (1993). Mechanism of glucocorticoid action. J Fla Med Assoc. 80:697–700.

Granados-Fuentes D, Saxena MT, Prolo LM, Aton SJ, Herzog ED (2004). Olfactory bulb neurons express functional, entrainable circadian rhythms. Eur J Neurosci. 19:898–906.

Guillaumond F, Dardente H, Giguere V, Cermakian N (2005). Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. J Biol Rhythms. 20:391–403.

Hall, JE, Guyton AC (2011). Guyton and Hall textbook of medical physiology. 12th ed. Philadelphia, PA: Saunders Elsevier.

Han D-H, Lee Y-J, Kim K, Kim C-J, Cho S (2014). Modulation of glucocorticoid receptor induction properties by core circadian clock proteins. Mol Cell Endocrinol. 383:170–80.

Hardin PE, Hall JC, Rosbash M (1992). Circadian oscillations in period gene mRNA levels are transcriptionally regulated. Proc Natl Acad Sci U S A. 89:11711–5.

Harmar AJ (2003). An essential role for peptidergic signalling in the control of circadian rhythms in the suprachiasmatic nuclei. J Neuroendocrinol. 15:335–8.

Hennig S, Strauss HM, Vanselow K, Yildiz O, Schulze S, Arens J et al. (2009). Structural and functional analyses of PAS domain interactions of the clock proteins Drosophila PERIOD and mouse PERIOD2. PLoS Biol. 7:e94.

Henry JT, Crosson S (2011). Ligand-binding PAS domains in a genomic, cellular, and structural context. Annu Rev Microbiol. 65:261–86.

Hirata H, Bessho Y, Kokubu H, Masamizu Y, Yamada S, Lewis J et al. (2004). Instability of Hes7 protein is crucial for the somite segmentation clock. Nat Genet. 36:750–4.

Hirata H, Yoshiura S, Ohtsuka T, Bessho Y, Harada T, Yoshikawa K et al. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. Science. 298:840–3.

Hirayama J, Sahar S, Grimaldi B, Tamaru T, Takamatsu K, Nakahata Y et al. (2007). CLOCK-mediated acetylation of BMAL1 controls circadian function. Nature. 450:1086–90.

Hughes ME, DiTacchio L, Hayes KR, Vollmers C, Pulivarthy S, Baggs JE et al. (2009). Harmonics of circadian gene transcription in mammals. PLoS Genet. 5:e1000442.

Ishida A, Mutoh T, Ueyama T, Bando H, Masubuchi S, Nakahara D et al. (2005). Light activates the adrenal gland. Timing of gene expression and glucocorticoid release. Cell Metab. 2:297–307.

Isomura A, Kageyama R (2014). Ultradian oscillations and pulses: coordinating cellular responses and cell fate decisions. Development. 141:3627–36.

Ixart G, Barbanel G, Nouguier-Soule J, Assenmacher I (1991). A quantitative study of the pulsatile parameters of CRH-41 secretion in unanesthetized free-moving rats. Exp Brain Res. 87:153–8.

James FO, Boivin DB, Charbonneau S, Bélanger V, Cermakian N (2007). Expression of clock genes in human peripheral blood mononuclear cells throughout the sleep/wake and circadian cycles. Chronobiol Int. 24:1009–34.

Jefferies WM (1991). Cortisol and immunity. Med Hypotheses. 34:198–208.

Jin JY, Almon RR, DuBois DC, Jusko WJ (2003). Modeling of corticosteroid pharmacogenomics in rat liver using gene microarrays. J Pharmacol Exp Ther. 307:93-109

Jin X, Shearman LP, Weaver DR, Zylka MJ, Vries GJ de, Reppert SM (1999). A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell. 96:57–68.

Johnson EO, Kamilaris TC, Chrousos GP, Gold PW (1992). Mechanisms of stress. A dynamic overview of hormonal and behavioral homeostasis. Neurosci Biobehav Rev. 16:115–30.

Johnson ML, Pipes L, Veldhuis PP, Farhy LS, Boyd DG, Evans WS (2008). AutoDecon, a deconvolution algorithm for identification and characterization of luteinizing hormone secretory bursts: description and validation using synthetic data. Anal Biochem. 381: 8–17.

Kalinyak JE, Dorin RI, Hoffman AR, Perlman AJ (1987). Tissue-specific regulation of glucocorticoid receptor mRNA by dexamethasone. J Biol Chem. 262:10441–4.

Kalogeras KT, Nsieman LK, Friedman TC, Doppman JL, Cutler GB, JR, Chrousos GP et al. (1996). Inferior petrosal sinus sampling in healthy subjects reveals a unilateral corticotropin-releasing hormone-induced arginine vasopressin release associated with ipsilateral adrenocorticotropin secretion. J Clin Invest. 97:2045–50.

Kalsbeek A, Buijs RM, van Heerikhuize, J J, Arts M, van der Woude, T P (1992). Vasopressin-containing neurons of the suprachiasmatic nuclei inhibit corticosterone release. Brain Res. 580:62–7.

Karlsson BH, Knutsson AK, Lindahl BO, Alfredsson LS (2003). Metabolic disturbances in male workers with rotating three-shift work. Results of the WOLF study. International archives of occupational and environmental health. 76:424–30.

Kino T, Chrousos GP (2004). Glucocorticoid and mineralocorticoid receptors and associated diseases. Essays in biochemistry. 40:137–55.

Kino T, Chrousos GP (2011). Circadian CLOCK-mediated regulation of target-tissue sensitivity to glucocorticoids. Implications for cardiometabolic diseases. Endocr Dev. 20:116–26.

Kino T, Nordeen SK, Chrousos GP (1999). Conditional modulation of glucocorticoid receptor activities by CREB-binding protein (CBP) and p300. J Steroid Biochem Mol Biol. 70:15–25.

Kirschbaum C, Kudielka BM, Gaab J, Schommer NC, Hellhammer DH (1999). Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. Psychosom Med. 61:154–62.

Kirschbaum C, Prussner JC, Stone AA, Federenko I, Gaab J, Lintz D et al. (1995). Persistent high cortisol responses to repeated psychological stress in a subpopulation of healthy men. Psychosom Med. 57:468–74.

Ko CH, Takahashi JS (2006). Molecular components of the mammalian circadian clock. Hum Mol Genet. 15 Spec No 2:R271-7.

Koike N, Yoo S-H, Huang H-C, Kumar V, Lee C, Kim T-K et al. (2012). Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science. 338:349–54.

Koyanagi S, Okazawa S, Kuramoto Y, Ushijima K, Shimeno H, Soeda S et al. (2006). Chronic treatment with prednisolone represses the circadian oscillation of clock gene expression in mouse peripheral tissues. Mol Endocrinol. 20:573–83.

Kramer A, Yang FC, Snodgrass P, Li X, Scammell TE, Davis FC et al. (2001). Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. Science. 294:2511–5.

Kubo T, Fujino Y, Nakamura T, Kunimoto M, Tabata H, Tsuchiya T et al. (2013). An industry-based cohort study of the association between weight gain and hypertension risk among rotating shift workers. J Occup Environ Med. 55:1041–5.

Kucera N, Schmalen I, Hennig S, Ollinger R, Strauss HM, Grudziecki A et al. (2012). Unwinding the differences of the mammalian PERIOD clock proteins from crystal structure to cellular function. Proc Natl Acad Sci U S A. 109:3311–6.

Kudielka BM, Kirschbaum C (2005). Sex differences in HPA axis responses to stress: a review. Biol Psychol. 69:113–32.

Kudryavtseva NN, Bakshtanovskaya IV, Koryakina LA (1991). Social model of depression in mice of C57BL/6J strain. Pharmacol Biochem Behav. 38:315–20.

Kusanagi H, Hida A, Satoh K, Echizenya M, Shimizu T, Pendergast JS et al. (2008). Expression profiles of 10 circadian clock genes in human peripheral blood mononuclear cells. Neurosci Res. 61:136–42.

Lamia KA, Papp SJ, Yu RT, Barish GD, Uhlenhaut NH, Jonker JW et al. (2011). Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. Nature. 480:552–6.

Lamont EW, Robinson B, Stewart J, Amir S (2005). The central and basolateral nuclei of the amygdala exhibit opposite diurnal rhythms of expression of the clock protein Period2. Proc Natl Acad Sci U.S.A. 102:4180–4.

Laue L, Chrousos GP, Loriaux DL, Barnes K, Munson P, Nieman L et al. (1988). The antiglucocorticoid and antiprogestin steroid RU 486 suppresses the adrenocorticotropin response to ovine corticotropin releasing hormone in man. J Clin Endocrinol Metab. 66:290–3.

Leliavski A, Shostak A, Husse J, Oster H (2014). Impaired glucocorticoid production and response to stress in Arntl-deficient male mice. Endocrinology. 155:133–42.

Lev Bar-Or R, Maya R, Segel LA, Alon U, Levine AJ, Oren M (2000). Generation of oscillations by the p53-Mdm2 feedback loop. A theoretical and experimental study. Proc Natl Acad Sci U S A. 97:11250–5.

Lightman SL (2008). The neuroendocrinology of stress: a never ending story. J Neuroendocrinol. 20:880–4.

Lightman SL, Conway-Campbell BL (2010). The crucial role of pulsatile activity of the HPA axis for continuous dynamic equilibration. Nat Rev Neurosci. 11:710–8.

Liu J, Malkani G, Mankani G, Shi X, Meyer M, Cunningham-Runddles S et al. (2006). The circadian clock Period 2 gene regulates gamma interferon production of NK cells in host response to lipopolysaccharide-induced endotoxic shock. Infect Immun. 74:4750–6.

Liu W, Saint DA (2002). A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal Biochem. 302:52–9.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25:402–8.

Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR et al. (2000). Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science. 288:483–92.

Lu NZ, Cidlowski JA (2005). Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. Mol Cell. 18:331–42.

Lupien SJ, Maheu F, Tu M, Fiocco A, Schramek TE (2007). The effects of stress and stress hormones on human cognition. Implications for the field of brain and cognition. Brain Cogn. 65:209–37.

Mauvoisin D, Wang J, Jouffe C, Martin E, Atger F, Waridel P et al. (2014). Circadian clock-dependent and -independent rhythmic proteomes implement distinct diurnal functions in mouse liver. Proc Natl Acad Sci U S A. 111:167–72.

McKenna NJ, Lanz RB, O'Malley BW (1999). Nuclear receptor coregulators. Cellular and molecular biology. Endocr Rev. 20:321–44.

McMaster A, Jangani M, Sommer P, Han N, Brass A, Beesley S et al. (2011). Ultradian cortisol pulsatility encodes a distinct, biologically important signal. PLoS One. 6:e15766.

McNally JG, Müller WG, Walker D, Wolford R, Hager GL (2000). The glucocorticoid receptor. Rapid exchange with regulatory sites in living cells. Science. 287:1262–5.

Mershon JL, Sehlhorst CS, Rebar RW, Liu JH (1992). Evidence of a corticotropin-releasing hormone pulse generator in the macaque hypothalamus. Endocrinology. 130:2991–6.

Moore RY, Silver R (1998). Suprachiasmatic nucleus organization. Chronobiol Int. 15:475–87.

Moore RY, Speh JC, Leak RK (2002). Suprachiasmatic nucleus organization. Cell Tissue Res. 309:89–98.

Morf J, Rey G, Schneider K, Stratmann M, Fujita J, Naef F et al. (2012). Cold-inducible RNA-binding protein modulates circadian gene expression posttranscriptionally. Science. 338:379–83.

Morris CJ, Yang JN, Scheer FAJL (2012). The impact of the circadian timing system on cardiovascular and metabolic function. Prog Brain Res. 199:337–58.

Nader N, Chrousos GP, Kino T (2009). Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications. FASEB J. 23:1572–83.

Nader N, Chrousos GP, Kino T (2010). Interactions of the circadian CLOCK system and the HPA axis. Trends Endocrinol Metab. 21:277–86.

Nakahata Y, Kaluzova M, Grimaldi B, Sahar S, Hirayama J, Chen D et al. (2008). The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. Cell. 134:329–40.

Nussey S, Whitehead SA (2001). Endocrinology. An integrated approach. Oxford, UK: BIOS.

Obrietan K, Impey S, Storm DR (1998). Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. Nat Neurosci. 1:693–700.

Ogawa Y, Koike N, Kurosawa G, Soga T, Tomita M, Tei H (2011). Positive autoregulation delays the expression phase of mammalian clock gene Per2. PLoS One. 6:e18663.

O'Neill JS, Reddy AB (2011). Circadian clocks in human red blood cells. Nature. 469:498–503.

O'Neill JS, Reddy AB (2012). The essential role of cAMP/Ca2+ signalling in mammalian circadian timekeeping. Biochem Soc Trans. 40:44–50.

Ono D, Honma K-i, Honma S (2015). Circadian and ultradian rhythms of clock gene expression in the suprachiasmatic nucleus of freely moving mice. Sci Rep. 5:12310.

Oster H, Damerow S, Hut RA, Eichele G (2006). Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes. J Biol Rhythms. 21:350–61.

Pacak K, Palkovits M (2001). Stressor specificity of central neuroendocrine responses. Implications for stress-related disorders. Endocr Rev. 22:502–48.

Pan A, Schernhammer ES, Sun Q, Hu FB (2011). Rotating night shift work and risk of type 2 diabetes. Two prospective cohort studies in women. PLoS medicine. 8:e1001141.

Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M et al. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. Cell. 109:307–20.

Partonen T, Treutlein J, Alpman A, Frank J, Johansson C, Depner M et al. (2007). Three circadian clock genes Per2, Arntl, and Npas2 contribute to winter depression. Ann Med. 39:229–38.

Pendergast JS, Niswender KD, Yamazaki S (2012a). Tissue-specific function of Period3 in circadian rhythmicity. PLoS One. 7:e30254.

Pendergast JS, Oda GA, Niswender KD, Yamazaki S (2012b). Period determination in the food-entrainable and methamphetamine-sensitive circadian oscillator(s). Proc Natl Acad Sci U S A. 109:14218–23.

Pratt WB (1990). Glucocorticoid receptor structure and the initial events in signal transduction. Prog Clin Biol Res. 322:119–32.

Presul E, Schmidt S, Kofler R, Helmberg A (2007). Identification, tissue expression, and glucocorticoid responsiveness of alternative first exons of the human glucocorticoid receptor. J Mol Endocrinol. 38:79–90.

Razzoli M, Karsten C, Yoder JM, Bartolomucci A, Engeland WC (2014). Chronic subordination stress phase advances adrenal and anterior pituitary clock gene rhythms. Am J Physiol Regul Integr Comp Physiol. 307:R198-205.

Reddy TE, Pauli F, Sprouse RO, Neff NF, Newberry KM, Garabedian MJ et al. (2009). Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. Genome Res. 19:2163–71.

Refinetti R (2010). The circadian rhythm of body temperature. Front Biosci. 15:564–94.

Rigaud G, Roux J, Pictet R, Grange T (1991). In vivo footprinting of rat TAT gene. Dynamic interplay between the glucocorticoid receptor and a liver-specific factor. Cell. 67:977–86.

Ripperger JA, Shearman LP, Reppert SM, Schibler U (2000). CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. Genes Dev. 14:679–89.

Roybal K, Theobold D, Graham A, DiNieri JA, Russo SJ, Krishnan V et al. (2007). Mania-like behavior induced by disruption of CLOCK. Proc Natl Acad Sci U.S.A. 104:6406–11.

Ruby NF, Brennan TJ, Xie X, Cao V, Franken P, Heller HC et al. (2002). Role of melanopsin in circadian responses to light. Science. 298:2211–3.

Rygula R, Abumaria N, Flügge G, Hiemke C, Fuchs E, Rüther E et al. (2006). Citalopram counteracts depressive-like symptoms evoked by chronic social stress in rats. Behav Pharmacol. 17:19–29.

Sartor O, Cutler GB (1996). Mifepristone: treatment of Cushing's syndrome. Clin Obstet Gynecol. 39:506–10.

Savory JG, Hsu B, Laquian IR, Giffin W, Reich T, Hache RJ et al. (1999). Discrimination between NL1- and NL2-mediated nuclear localization of the glucocorticoid receptor. Mol Cell Biol. 19:1025–37.

Scheer FAJL, Hilton MF, Mantzoros CS, Shea SA (2009). Adverse metabolic and cardiovascular consequences of circadian misalignment. Proc Natl Acad Sci U S A. 106:4453–8.

Scheff JD, Calvano SE, Lowry SF, Androulakis IP (2012). Transcriptional implications of ultradian glucocorticoid secretion in homeostasis and in the acute stress response. Physiol Genomics. 44:121–9.

Scheiermann C, Kunisaki Y, Frenette PS (2013). Circadian control of the immune system. Nat Rev Immunol. 13:190–8.

Scheinman RI, Gualberto A, Jewell CM, Cidlowski JA, Baldwin AS (1995). Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. Mol Cell Biol. 15:943–53.

Schilling TM, Kölsch M, Larra MF, Zech CM, Blumenthal TD, Frings C et al. (2013). For whom the bell (curve) tolls: cortisol rapidly affects memory retrieval by an inverted U-shaped dose-response relationship. Psychoneuroendocrinology. 38:1565–72.

Schmittgen TD, Livak KJ (2008). Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 3:1101–8.

Schote AB, Turner JD, Schiltz J, Muller CP (2007). Nuclear receptors in human immune cells: expression and correlations. Mol Immunol. 44:1436–45.

Seifter J, Sloane D, Ratner A (2005). Concepts in medical physiology. Philadelphia, PA: Lippincott Williams & Wilkins.

Shearman LP, Jin X, Lee C, Reppert SM, Weaver DR (2000). Targeted disruption of the mPer3 gene: subtle effects on circadian clock function. Mol Cell Biol. 20:6269–75.

Shearman LP, Zylka MJ, Weaver DR, Kolakowski LF, Reppert SM (1997). Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. Neuron. 19:1261–9.

Shigeyoshi Y, Taguchi K, Yamamoto S, Takekida S, Yan L, Tei H et al. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. Cell. 91:1043–53.

So AY-L, Bernal TU, Pillsbury ML, Yamamoto KR, Feldman BJ (2009). Glucocorticoid regulation of the circadian clock modulates glucose homeostasis. Proc Natl Acad Sci U S A. 106:17582–7.

Stavreva DA, Wiench M, John S, Conway-Campbell BL, McKenna MA, Pooley JR et al. (2009). Ultradian hormone stimulation induces glucocorticoid receptor-mediated pulses of gene transcription. Nature cell biology. 11:1093–102.

Stocksley M (2001). Abdominal ultrasound. 1. publ. London [u.a.]: Greenwich Medical Media.

Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M (2001). Entrainment of the circadian clock in the liver by feeding. Science. 291:490–3.

Sujino M, Furukawa K, Koinuma S, Fujioka A, Nagano M, Iigo M et al. (2012). Differential entrainment of peripheral clocks in the rat by glucocorticoid and feeding. Endocrinology. 153:2277–86.

Tahara Y, Shiraishi T, Kikuchi Y, Haraguchi A, Kuriki D, Sasaki H et al. (2015). Entrainment of the mouse circadian clock by sub-acute physical and psychological stress. Sci Rep. 5:11417.

Tamiya H, Ogawa S, Ouchi Y, Akishita M (2016). Rigid Cooperation of Per1 and Per2 proteins. Sci Rep. 6:32769.

Teboul M, Barrat-Petit M-A, Li XM, Claustrat B, Formento J-L, Delaunay F et al. (2005). Atypical patterns of circadian clock gene expression in human peripheral blood mononuclear cells. J Mol Med. 83:693–9.

Toh KL, Jones CR, He Y, Eide EJ, Hinz WA, Virshup DM et al. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science. 291:1040–3.

Travnickova-Bendova Z, Cermakian N, Reppert SM, Sassone-Corsi P (2002). Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. Proc Natl Acad Sci U S A. 99:7728–33.

Trifonova ST, Gantenbein M, Turner JD, Muller CP (2013). The use of saliva for assessment of cortisol pulsatile secretion by deconvolution analysis. Psychoneuroendocrinology. 38:1090–101.

Tsang AH, Barclay JL, Oster H (2014). Interactions between endocrine and circadian systems. J Mol Endocrinol. 52:R1-16.

Tsigos C, Chrousos GP (2002). Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res. 53:865–71.

Tu BP, Kudlicki A, Rowicka M, McKnight SL (2005). Logic of the yeast metabolic cycle. Temporal compartmentalization of cellular processes. Science. 310:1152–8.

Ueda HR, Hayashi S, Chen W, Sano M, Machida M, Shigeyoshi Y et al. (2005). System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat Genet. 37:187–92.

Ulrich-Lai YM, Arnhold MM, Engeland WC (2006). Adrenal splanchnic innervation contributes to the diurnal rhythm of plasma corticosterone in rats by modulating adrenal sensitivity to ACTH. Am J Physiol Regul Integr Comp Physiol. 290:R1128-35.

Ulrich-Lai YM, Herman JP (2009). Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci. 10:397–409.

Veldhuis JD, Iranmanesh A, Lizarralde G, Johnson ML (1989). Amplitude modulation of a burstlike mode of cortisol secretion subserves the circadian glucocorticoid rhythm. Am J Physiol. 257:E6-14.

Verrey F (1998). Early aldosterone effects. Exp Nephrol. 6:294–301.

Vetter C, Devore EE, Wegrzyn LR, Massa J, Speizer FE, Kawachi I et al. (2016). Association between rotating night shift work and risk of coronary heart disease Among Women. JAMA. 315:1726–34.

Vgontzas AN, Bixler EO, Lin HM, Prolo P, Mastorakos G, Vela-Bueno A et al. (2001). Chronic insomnia is associated with nyctohemeral activation of the hypothalamic-pituitary-adrenal axis. Clinical implications. J Clin Endocrinol Metab. 86:3787–94.

Vgontzas AN, Tsigos C, Bixler EO, Stratakis CA, Zachman K, Kales A et al. (1998). Chronic insomnia and activity of the stress system. A preliminary study. J Psychosom Res. 45:21–31.

Vining RF, McGinley RA (1986). Hormones in saliva. Crit Rev Clin Lab Sci. 23:95–146.

Viola AU, Archer SN, James LM, Groeger JA, Lo, June C Y, Skene DJ et al. (2007). PER3 polymorphism predicts sleep structure and waking performance. Curr Biol. 17:613–8.

Vyas MV, Garg AX, Iansavichus AV, Costella J, Donner A, Laugsand LE et al. (2012). Shift work and vascular events. Systematic review and meta-analysis. BMJ. 345:e4800.

Walker JJ, Terry JR, Lightman SL (2010). Origin of ultradian pulsatility in the hypothalamic-pituitary-adrenal axis. Proc Biol Sci. 277:1627–33.

Westermann J, Demir A, Herbst V (2004). Determination of cortisol in saliva and serum by a luminescence-enhanced enzyme immunoassay. Clin Lab. 50:11–24.

Whitney AR, Diehn M, Popper SJ, Alizadeh AA, Boldrick JC, Relman DA et al. (2003). Individuality and variation in gene expression patterns in human blood. Proc Natl Acad Sci U.S.A. 100:1896–901.

Wichert S, Fokianos K, Strimmer K (2004). Identifying periodically expressed transcripts in microarray time series data. Bioinformatics (Oxford, England). 20:5–20.

Windle RJ, Wood SA, Shanks N, Lightman SL, Ingram CD (1998). Ultradian rhythm of basal corticosterone release in the female rat: dynamic interaction with the response to acute stress. Endocrinology. 139:443–50.

Xu Y, Padiath QS, Shapiro RE, Jones CR, Wu SC, Saigoh N et al. (2005). Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome. Nature. 434:640–4.

Yagita K, Tamanini F, Yasuda M, Hoeijmakers JHJ, van der Horst GTJ, Okamura H (2002). Nucleocytoplasmic shuttling and mCRY-dependent inhibition of ubiquitylation of the mPER2 clock protein. Embo J. 21:1301–14.

Yamamoto T, Nakahata Y, Tanaka M, Yoshida M, Soma H, Shinohara K et al. (2005). Acute physical stress elevates mouse period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. J Biol Chem. 280:42036–43.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M et al. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. Science. 288:682–5.

Yang S, Liu A, Weidenhammer A, Cooksey RC, McClain D, Kim MK et al. (2009). The role of mPer2 clock gene in glucocorticoid and feeding rhythms. Endocrinology. 150:2153–60.

Yurtsever T, Schilling TM, Kölsch M, Turner JD, Meyer J, Schächinger H et al. (2016). The acute and temporary modulation of PERIOD genes by hydrocortisone in healthy subjects. Chronobiol Int. 33:1222–34.

Zhang J, Wu Z, Zhou L, Li H, Teng H, Dai W et al. (2011). Deficiency of antinociception and excessive grooming induced by acute immobilization stress in Per1 mutant mice. PLoS One. 6:e16212.

Zhu L, Zee PC (2012). Circadian rhythm sleep disorders. Neurologic clinics. 30:1167–91.

Zhu X, Gleiberman AS, Rosenfeld MG (2007). Molecular physiology of pituitary development. Signaling and transcriptional networks. Physiol Rev. 87:933–63.

Zylka MJ, Shearman LP, Weaver DR, Reppert SM (1998). Three period homologs in mammals. Differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. Neuron. 20:1103–10.

CHAPTER 8: APPENDIX

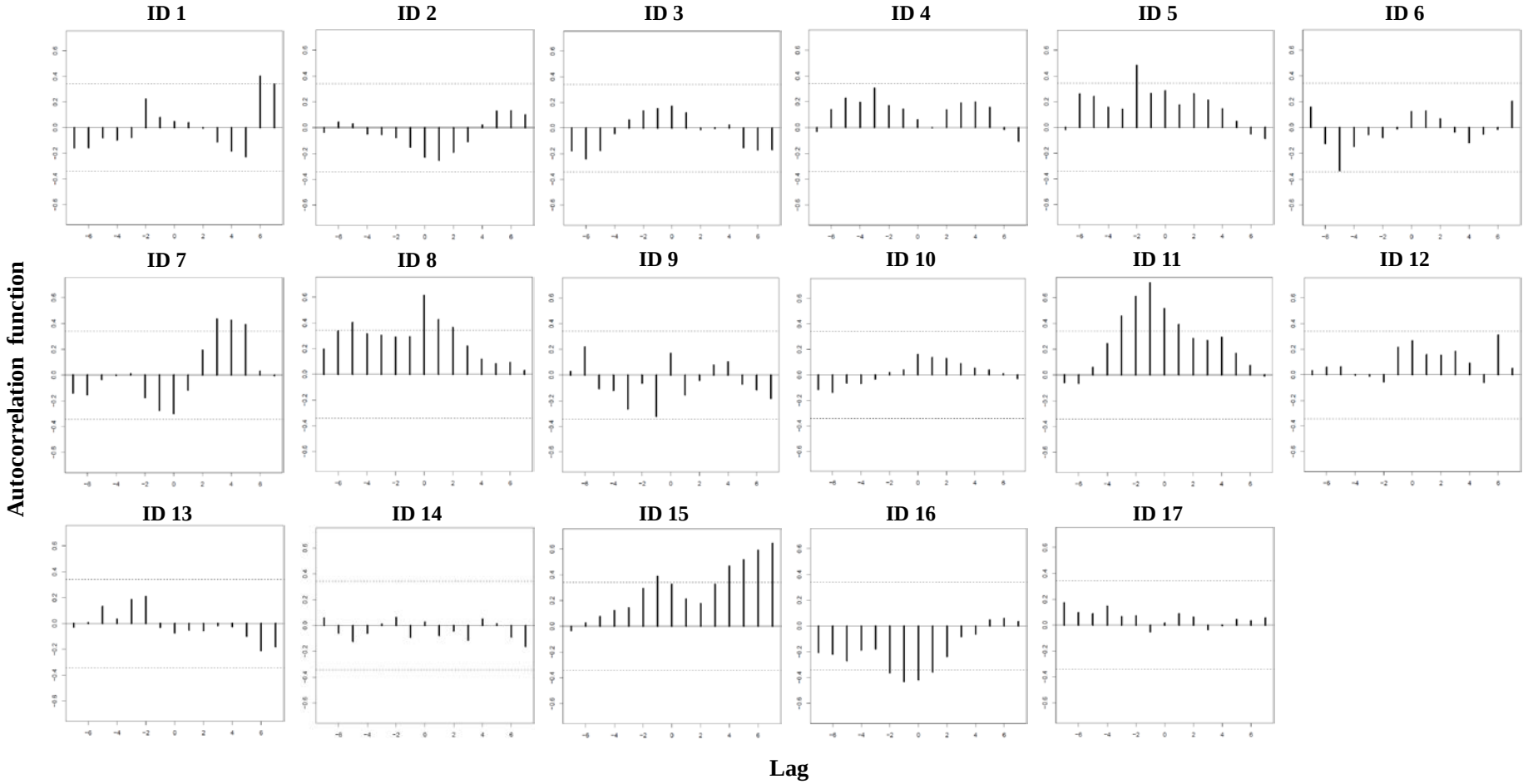

Figure 27: Cross-correlation analysis between the time series of *PER1* and saliva cortisol concentrations with 15 min time lags.

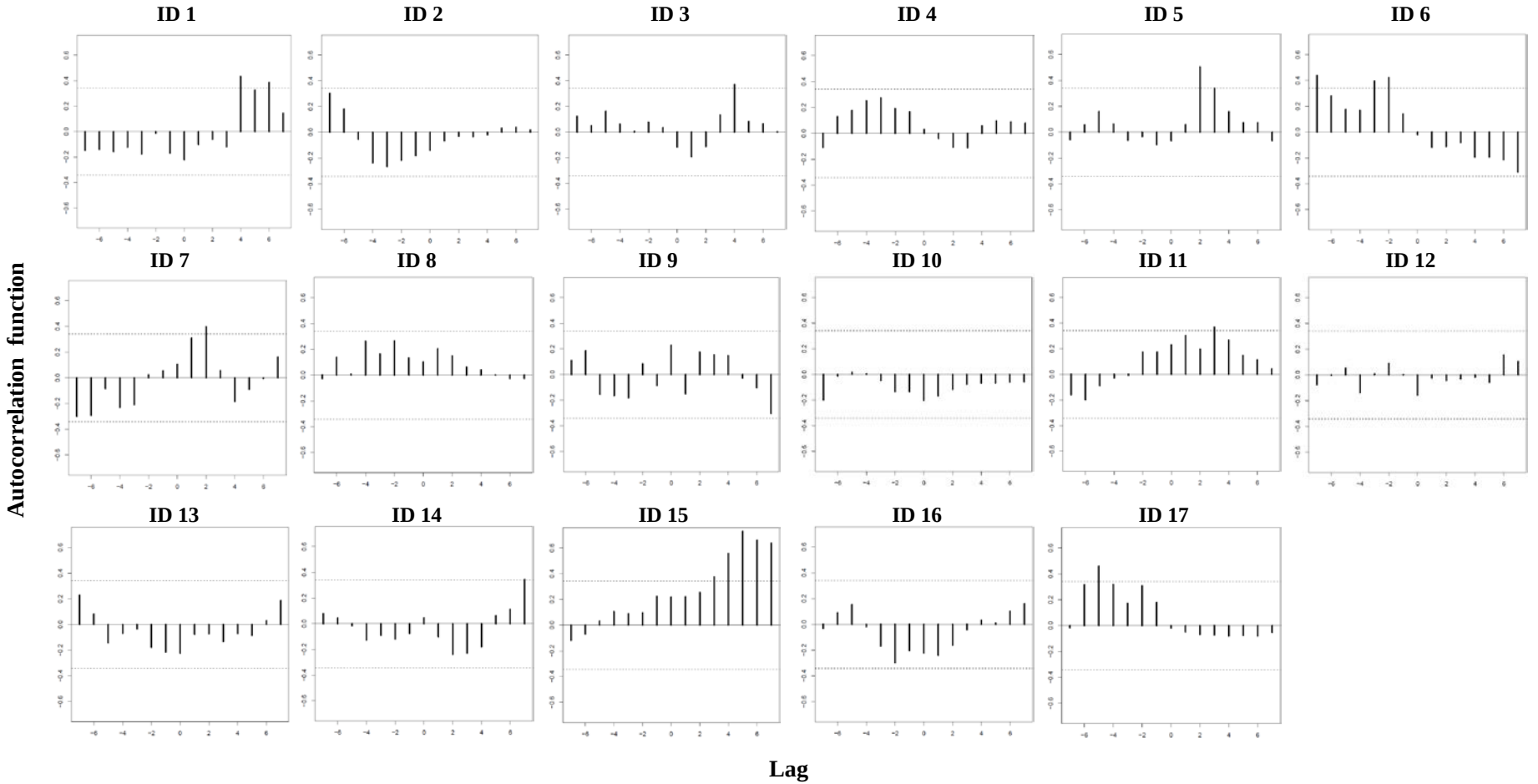

Figure 28: Cross-correlation analysis between the time series of *PER2* and saliva cortisol concentrations with 15 min time lags.

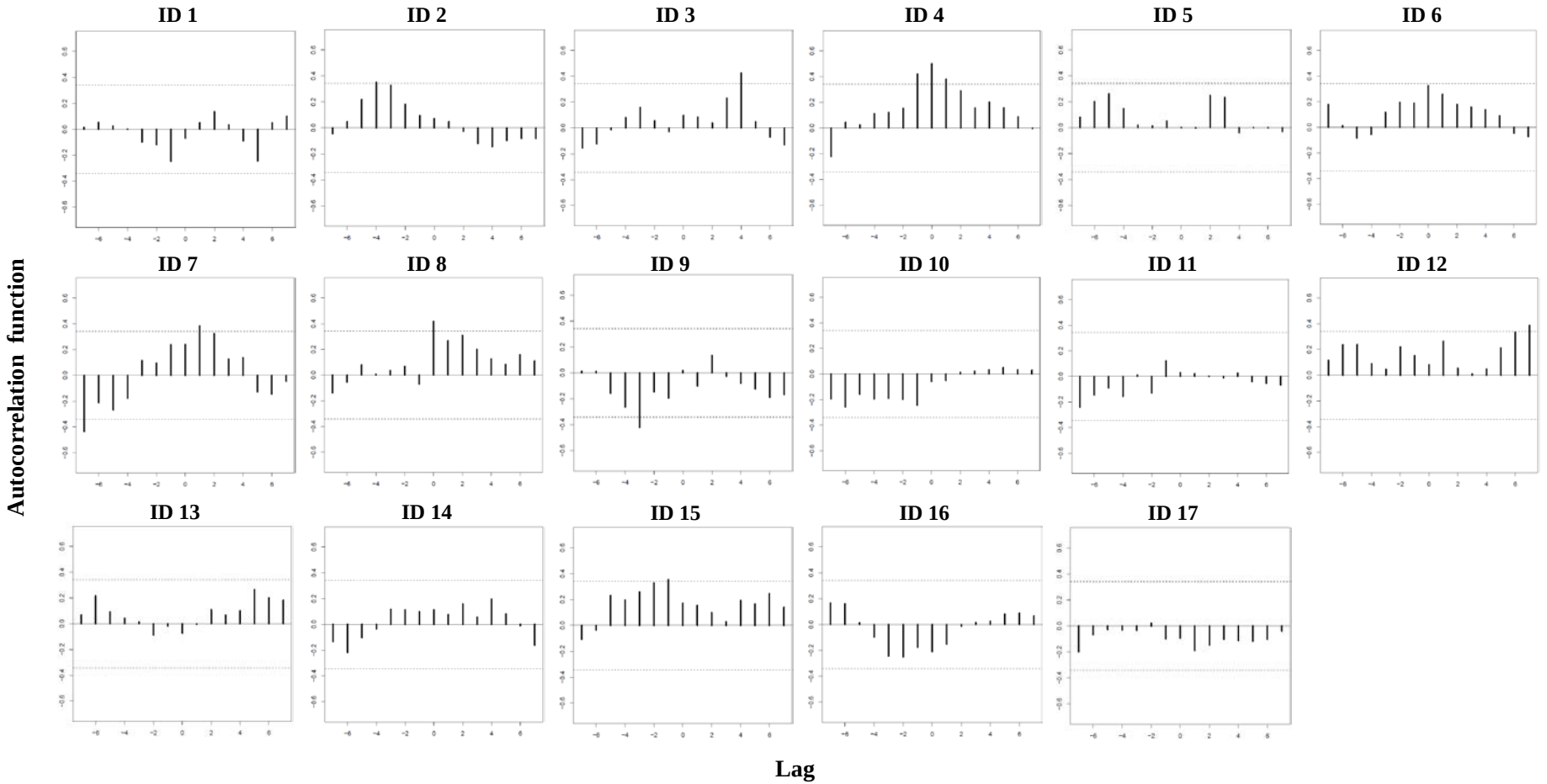

Figure 29: Cross-correlation analysis between the time series of *PER3* and saliva cortisol concentrations with 15 min time lags.

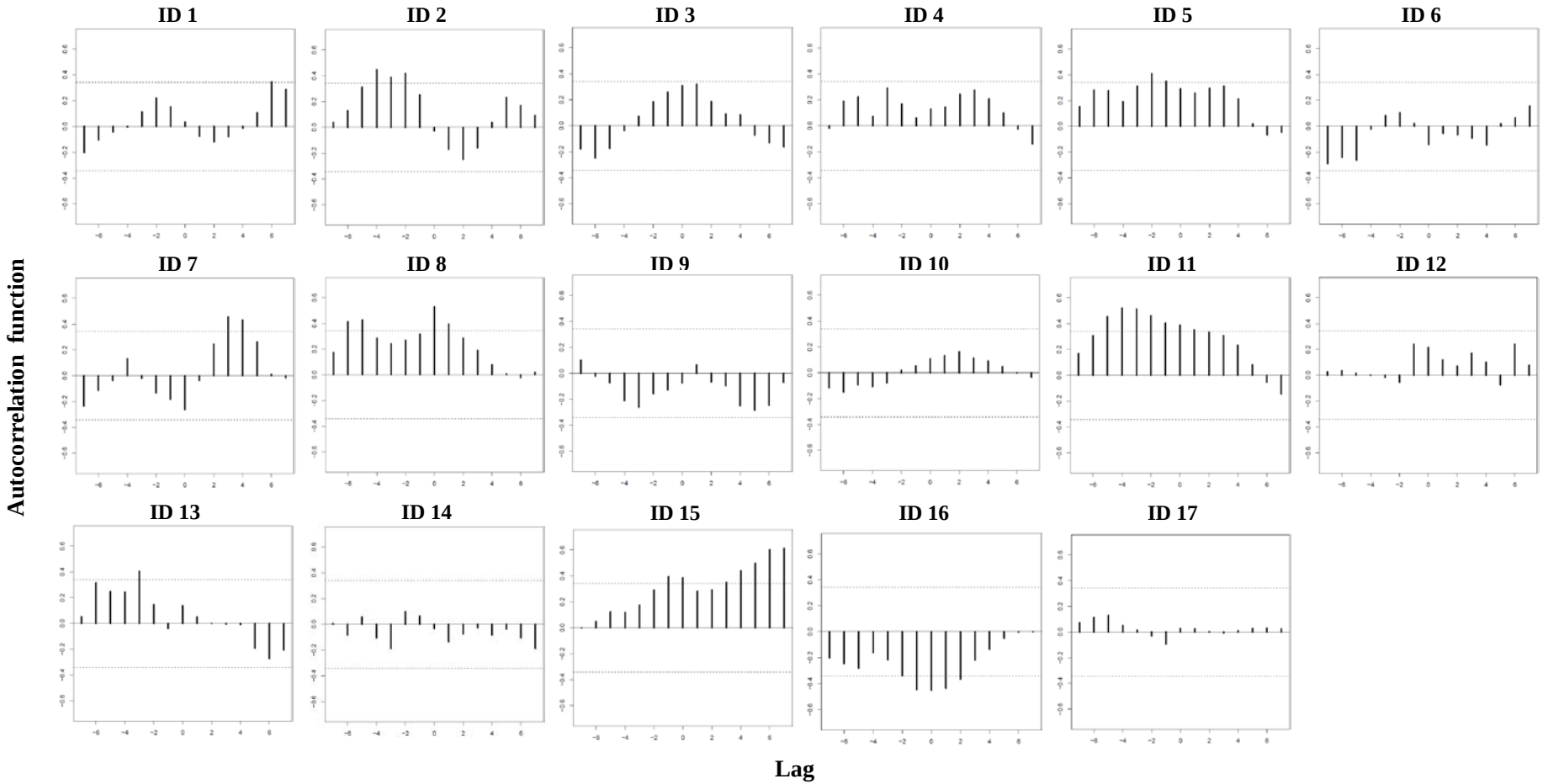

Figure 30: Cross-correlation analysis between the time series of *PER1* and plasma cortisol concentrations with 15 min time lags.

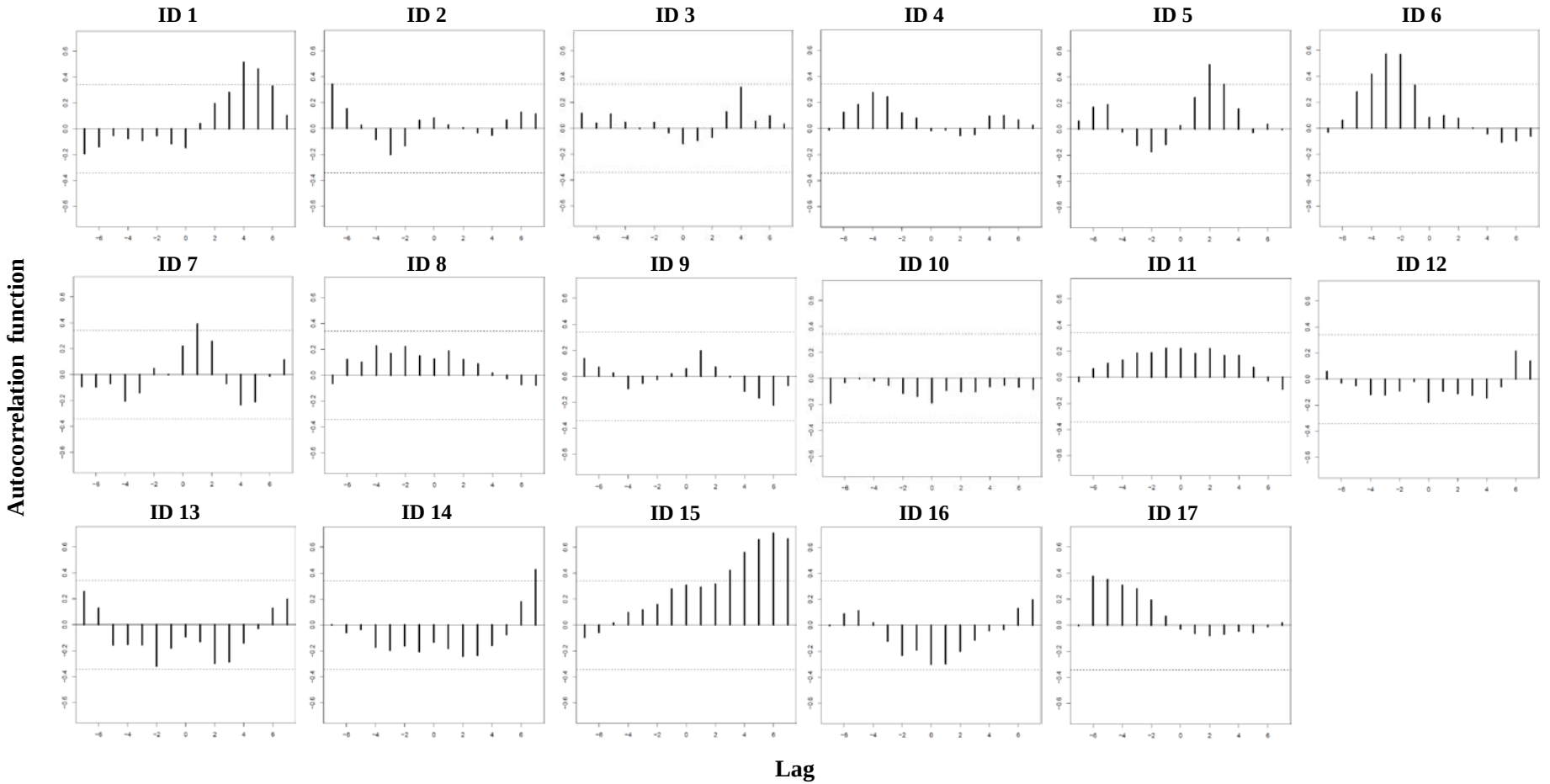

Figure 31: Cross-correlation analysis between the time series of *PER2* and plasma cortisol concentrations with 15 min time lags.

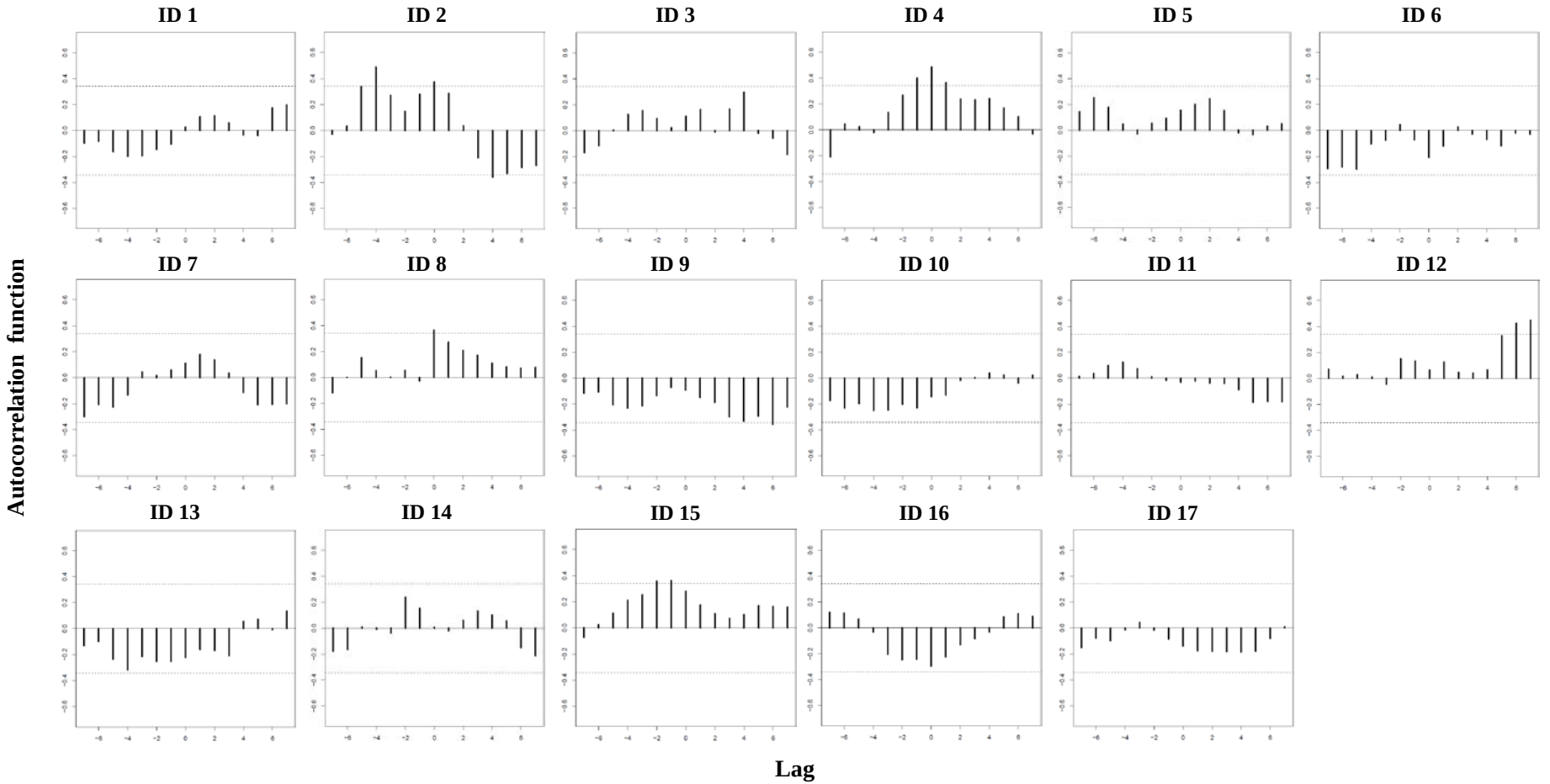

Figure 32: Cross-correlation analysis between the time series of *PER3* and plasma cortisol concentrations with 15 min time lags.

Erklärung

nach § 9, Abs. 1 der Promotionsordnung des Fachbereichs I der Universität Trier vom 13.11.2008.

Hiermit versichere ich, dass ich die vorliegende Arbeit selber verfasst und keine außer den angegebenen Hilfsmitteln und Referenzen benutzt habe. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Trier, August 2017

Türkan Yurtsever