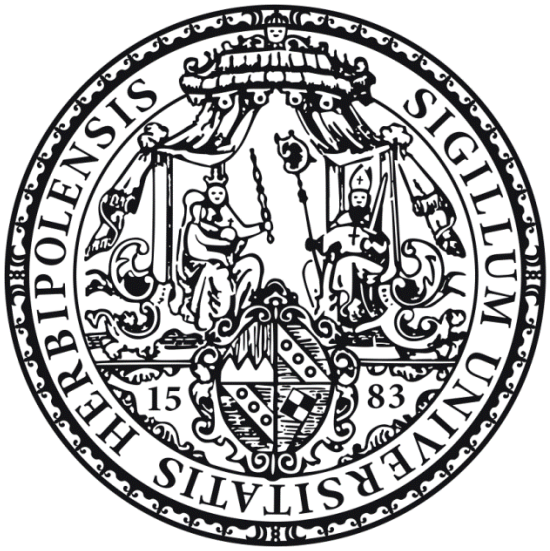
Timing is everything: The interaction of psychosocial stress and the circadian clock in male C57BL/6 mice

Auf das richtige Timing kommt es an: Die Interaktion zwischen psychosozialem Stress und der inneren Uhr in männlichen C57BL/6 Mäusen



DOCTORAL THESIS FOR A DOCTORAL DEGREE
AT THE GRADUATE SCHOOL OF LIFE SCIENCES,
JULIUS-MAXIMILIANS-UNIVERSITÄT WÜRZBURG,
SECTION NEUROSCIENCE

submitted by

Manuela Slavica Bartlang

from Munich

Würzburg, October 2014

CC BY NC SA

Submitted on: ……

Office stamp

Members of the Thesis Committee (Promotionskomitee):

Chairperson: **Prof. Dr. Thomas Dandekar**

Primary Supervisor: **Prof. Dr. Charlotte Förster**

Supervisor (Second): **PD Dr. Angelika Schmitt**

Supervisor (Third): **PD Dr. Gabriella Lundkvist**

Date of Public Defense: ……

Date of Receipt of Certificates: ……

"Das schönste Glück des denkenden Menschen ist, das Erforschliche erforscht zu haben und das Unerforschliche zu verehren."

(Johann Wolfgang von Goethe)

ZUSAMMENFASSUNG VII

SUMMARY XI

LIST OF PUBLICATIONS XV

CHAPTER 1 – GENERAL INTRODUCTION 1

1.1. Adaptation to predictable environmental changes – The circadian system 2

1.1.1. The mammalian circadian clock 3

1.1.1.1. The central clock 6

1.1.1.2. Peripheral clocks 16

1.1.1.3. Rhythm disturbances and link to human diseases 18

1.2. Adaptation to unpredictable environmental changes – The stress system 21

1.2.1. The sympathetic nervous system 22

1.2.2. The hypothalamic-pituitary-adrenal axis 25

1.2.3. Acute vs repeated/chronic stress 29

1.2.4. Psychosocial stress 32

1.3. Interactions between the circadian and the stress system 35

1.3.1. The circadian release of glucocorticoids 35

1.3.1.1. Suprachiasmatic nucleus control over the hypothalamic-pituitary-adrenal axis 38

1.3.1.2. The autonomic nervous system – an alternative pathway of circadian control 39

1.3.1.3. The adrenal peripheral clock 41

1.3.2. Stress affects the circadian clock 42

1.3.2.1. Stress effects on the master clock 43

1.3.2.2. Glucocorticoid receptors – a checkpoint for the interaction between glucocorticoids and the circadian system at peripheral target tissues 45

1.4. Concluding remarks 47

1.5. Aim of the thesis 48

CHAPTER 2 – MATERIAL AND METHODS 51

2.1. Material 52

2.1.1. Antibodies for protein and immunohistochemical analysis 52

2.1.1.1. Primary antibodies 52

2.1.1.2. Secondary antibodies 53

2.1.2. Protein extraction and western blotting 53

2.1.3. Neuroendocrine analysis 54

2.1.4. In situ hybridization 54

2.1.5. Chemicals, enzymes, reagents and equipment 55

2.1.6. Software 55

2.2. Methods 57

2.2.1. Animals 57

2.2.1.1. Mice and housing conditions 57

2.2.1.2. Social defeat paradigm 58

2.2.1.3. Body weight development and food intake 59

2.2.1.4. Microdialysis 60

2.2.2. Tissue extraction and preparation 62

2.2.2.1. Trunk blood collection and analysis of plasma ghrelin, leptin, corticosterone, and adrenocorticotropic hormone 62

2.2.2.2. Removal of different brain regions 63

2.2.2.3. Determination of body, pituitary, and adrenal weight 64

2.2.3. Protein techniques 64

2.2.3.1. Preparation of total cell lysates 64

2.2.3.2. Quantitative determination of protein concentrations 65

2.2.3.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) 66

2.2.3.4. Western blotting 67

2.2.4. In situ hybridization of hypothalamic CRH and AVP mRNA 71

2.2.5. Oil-red lipid staining in adrenal tissue 72

2.2.6. Statistics 73

CHAPTER 3 – RESULTS 75

3.1. Time matters: pathological effects of repeated psychosocial stress during the active, but not inactive, phase of male mice (Paper 1) 76

3.2. Repeated psychosocial stress at night, but not day, affects the central molecular clock (Paper 2) 78

3.3. Repeated psychosocial stress at night, but not day, delays the entrained activity rhythm of male mice (Paper 3, submitted) 80

3.4. Established techniques 82

3.4.1. Assessment of the corticosterone rhythm in the hippocampus by *in vivo* microdialysis 82

3.4.2. Assessment of *in vitro* corticosterone secretion by adrenal explants.. 83

3.5. Effects of repeated social defeat on different metabolic parameters 85

3.5.1. Basal plasma ghrelin and leptin levels 85

3.5.2. Body weight development 86

3.5.3. Food intake 88

3.6. Effects of repeated social defeat on hypothalamic-pituitary-adrenal axis activity 89

3.6.1. Hypothalamus 89

3.6.1.1. CRH mRNA expression in the suprachiasmatic nucleus, paraventricular nucleus, and supraoptic nucleus 89

3.6.1.2. AVP mRNA expression in the suprachiasmatic nucleus, paraventricular nucleus, and supraoptic nucleus 90

3.6.2. Pituitary 91

3.6.2.1. Pituitary weight and plasma adrenocorticotropic hormone 91

3.6.2.2. CRH-R1 and AVP-R1b protein expression 92

3.6.3. Adrenal glands 93

3.6.3.1. Adrenal gland weight and plasma corticosterone 93

3.6.3.2. Mc2r and SR-B1 protein expression 94

3.6.3.3. LDLR and HSL protein expression 96

3.6.3.4. HMGCR protein expression 97

3.6.3.5. Adrenal cortical lipids 98

3.6.4. Hippocampal free corticosterone rhythm 99

CHAPTER 4 – GENERAL DISCUSSION 101

4.1. Discussion of the present results 102

4.1.1. Metabolism and physiology 102

4.1.2. Hypothalamic-pituitary-adrenal axis 109

4.1.3. Final conclusions 120

4.2. Established techniques – Performance and analysis 122

4.2.1. Assessment of the corticosterone rhythm in the hippocampus by *in vivo* microdialysis 122

4.2.2. Assessment of *in vitro* corticosterone secretion by adrenal explants 124

REFERENCES 127

FULL TEXT PAPER 1 152

FULL TEXT PAPER 2 165

FULL TEXT PAPER 3 (MANUSCRIPT, SUBMITTED) 177

ABBREVIATIONS 212

ACKNOWLEDGEMENTS 217

CURRICULUM VITAE 220

DECLARATIONS 222

ZUSAMMENFASSUNG

Aufgrund der Bewegung der Erde in unserem Sonnensystem sind alle Lebewesen auf unserem Planeten seit mehr als 3,5 Milliarden Jahren tagesperiodischen Veränderungen der Umweltbedingungen ausgesetzt. In Anpassung an diese zeitlichen Abläufe haben sich im Laufe der Evolution bei fast allen Organismen – vom Bakterium bis hin zum Menschen – und auf verschiedenen Ebenen – von der Zellebene bis zum Verhalten – biologische Rhythmen entwickelt, die von endogenen Uhren im Körper und äußeren Zeitgebern gesteuert werden. Bei vielzelligen Organismen besitzen nahezu alle Zelltypen ihren eigenen Oszillator. In Säugetieren ist das zirkadiane System hierarchisch strukturiert. Der zentrale Schrittmacher der inneren Uhr befindet sich im bilateralen suprachiasmatischen Nukleus (SCN) des Hypothalamus, während untergeordnete periphere Taktgeber in beinahe jedem Gewebe und Organ oszillieren.

Im Gegensatz zu den oben erwähnten regelmäßig wiederkehrenden Veränderungen der Umweltbedingungen sind die meisten Lebewesen ebenso unvorhersehbaren und raschen Umweltveränderungen ausgesetzt. In Anpassung an derartig plötzlich wechselnde Reizbedingungen ist die kurzfristige Aktivierung des Stress-Systems, bestehend aus der Hypothalamus-Hypophysen-Nebennieren-Achse (*hypothalamic-pituitary-adrenal axis, HPA axis*) und dem sympathischen Nervensystem, für eine adaptive Reaktion essentiell und sogar lebensnotwendig. Im Gegensatz dazu zählt eine andauernde/chronische Aktivierung des Stress-Systems zu den Risikofaktoren für eine Reihe von somatischen und affektiven Erkrankungen.

Obwohl das zirkadiane System und das Stress-System auf den ersten Blick zwei verschiedene körperliche Anpassungssysteme darstellen, kommt es auf mehreren

Ebenen zum wechselseitigen Einfluss. Es wurde bereits in früheren Arbeiten gezeigt, dass eine Stressorexposition zu unterschiedlichen Tageszeiten verschiedene Effekte hervorruft, wobei die Natur des Stressors dabei eine entscheidende Rolle spielt. Des Weiteren konnten Veränderungen im SCN und peripheren zirkadianen Uhren als Folge einer Stressorexposition aufgezeigt werden.

In Zusammenarbeit mit verschiedenen Kollegen wurde im Rahmen dieser Doktorarbeit untersucht, ob die endogene Uhr die Stressempfindlichkeit tageszeitabhängig moduliert und ob wiederholter psychosozialer Stress die innere Uhr in Abhängigkeit von der Tageszeit der Stressorexposition beeinflusst. Männliche C57BL/6 Mäuse wurden daher entweder zu Beginn der inaktiven/Licht-Phase (SDL Mäuse) oder der aktiven/Dunkel-Phase (SDD Mäuse) wiederholt einem psychosozialem Stressor ausgesetzt.

Im Anschluss wurden verschiedene Verhaltensweisen sowie physiologische/endokrine und immunologische/inflammatorische Konsequenzen untersucht. Es konnte gezeigt werden, dass die Effekte wiederholter Stressorexposition auf das Verhalten, die Physiologie und die Immunologie deutlich von der Tageszeit der Stressorexposition abhängt. Die gewonnenen Ergebnisse zeigen, dass wiederholte Stressorexposition während der aktiven/Dunkel-Phase negativere Konsequenzen nach sich zieht als die Stressorexposition während der inaktiven/Licht-Phase. Wurden C57BL/6 Mäuse dem psychosozialen Stressor während ihrer aktiven Phase ausgesetzt, führte dies zu typischen Symptomen von depressiven Patienten wie z.B. einer Verringerung der Aktivität und des sozialen Erkundungsverhaltens, Entzündungserscheinungen, sowie Veränderungen in hormonalen Rhythmen im Plasma. Im Gegensatz dazu wiesen C57BL/6 Mäuse, die dem Stressor in ihrer inaktiven Phase begegneten, geringfügige physiologische Veränderungen auf,

welche die Entstehung der oben genannten negativen Konsequenzen verhindern und somit positive Adaptationen darstellen könnten.

Des Weiteren wurden in dieser Arbeit die Effekte wiederholter Stressorexposition zu unterschiedlichen Tageszeiten auf verschiedene Ebenen des zirkadianen Systems untersucht. Es konnte eine erhöhte Expression des PERIOD2 (PER2) Proteins, das einen essentiellen Bestandteil des zirkadianen Uhrenmechanismus darstellt, im SCN nach wiederholter Stressorexposition während der aktiven Phase festgestellt werden. Die Veränderung im zentralen Schrittmacher spiegelte sich auch in der Tagesrhythmik verschiedener Hormone sowie im rhythmischen Verhalten der Tiere wider. SDD Mäuse zeigten dabei einen verschobenen oder fehlenden Rhythmus in den Hormonen Corticosteron und Leptin. Des Weiteren war die Aktivität nach 19-tägiger Stressorexposition zu Beginn der aktiven Phase deutlich nach hinten verschoben. Dabei kommt dem *Period (Per)* Gen eine zentrale Bedeutung zu, da SDD *Per1/Per2* Doppelmutanten keinen veränderten Aktivitätsrhythmus aufwiesen. Eine verfrühte Phasenlage der peripheren Uhr in der Nebenniere zeigte sich hingegen in C57BL/6 Mäusen, die dem Stressor während ihrer inaktiven Tageszeit ausgesetzt wurden. Diese Phasenverschiebung nach vorne könnte für die Aufrechterhaltung der Rhythmik im Verhalten und in der Hormonausschüttung eine Rolle spielen.

Vorangehende Arbeiten wiesen bereits darauf hin, dass die HPA-Achsen-Aktivierung infolge einer Stressorexposition zu unterschiedlichen Tageszeiten davon abhängt, ob der Stressor von physischer oder psychologischer Natur ist. Die Ergebnisse der vorliegenden Arbeit erweitern die bestehenden Erkenntnisse insofern, als dass die HPA-Achsen-Antwort auch von psychosozialen Stressoren tageszeitabhängig beeinflusst wird. Die HPA-Achsen-Analyse dieser Arbeit zeigte eine verringerte

Aktivität der Stressachse nach wiederholter Stressorexposition zu Beginn der aktiven Phase. Mit großer Wahrscheinlichkeit stellt diese Verringerung der basalen HPA-Achsen-Aktivität eine dysfunktionale Überadjustierung dar, die zu den negativen Konsequenzen in Folge der Stressorexposition während der aktiven Phase beitragen könnte.

Zusammenfassend lässt sich sagen, dass die endogene Uhr in Mäusen die Stressempfindlichkeit tageszeitabhängig moduliert und dass wiederholter psychosozialer Stress die innere Uhr in Abhängigkeit von der Tageszeit der Stressorexposition beeinflusst. Dabei zieht die Stressorexposition während der aktiven Phase weitaus negativere Konsequenzen nach sich als die Stressor-exposition in der inaktiven Phase. Aus den Daten kann geschlossen werden, dass die Wechselwirkung von der inneren Uhr und dem Stress-System einen komplexen Sachverhalt darstellt, der gewährleisten soll, dass die innere Uhr nicht beliebig aus dem Takt geraten kann.

SUMMARY

Due to the rotation of the earth in the solar system all inhabitants of our planet are exposed to regular environmental changes since more than 3.5 billion years. In order to anticipate these predictable changes in the environment, evolutionarily conserved biological rhythms have evolved in most organisms – ranging from ancient cyanobacteria up to human beings – and also at different levels of organization – from single cells up to behavior. These rhythms are endogenously generated by so called circadian clocks in our body and entrained to the 24 h cycle by external timing cues. In multi-cellular organisms the majority of the cells in the body is equipped with such an oscillator. In mammals, the circadian system is structured in a hierarchical fashion: A central pacemaker resides in the bilateral suprachiasmatic nucleus (SCN) of the hypothalamus, while subsidiary peripheral clocks exist in nearly every tissue and organ.

In contrast to the aforementioned recurrent environmental changes most organisms are also exposed to unpredictable changes in the environment. In order to adapt to these sudden alterations the acute activation of the stress response system, involving the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system, displays a fundamental survival mechanism. However, if activation of the stress system becomes chronic, devastating somatic and affective disorders might be the consequence.

At first glance, the circadian and the stress system seem to represent two separate bodily control systems that are involved in adaptation to predictable and unpredictable stimuli, respectively. However, both systems are fundamental for survival, and thus, communicate with each other at various levels. Early studies

already demonstrated that stressor exposure at different times of the diurnal cycle generates different stress effects, whereupon the type of stressor plays a pivotal role. Moreover, alterations in the SCN and peripheral circadian clocks could be shown following stressor exposure.

In cooperation with various co-workers, I investigated whether the stress responsiveness is modulated by the endogenous clock in a diurnal fashion and whether repeated psychosocial stress impacts the circadian clock depending on the time of day of stressor exposure. Therefore, male C57BL/6 mice were repeatedly exposed to a psychosocial stressor, either at the beginning of the inactive/light phase (SDL mice) or active/dark phase (SDD mice).

Subsequently, different behavioral, physiological/endocrine and immunological/ inflammatory consequences were assessed. It could be shown that the effects of repeated psychosocial stressor exposure strongly depend on the time of day of stressor exposure. The present results demonstrate that repeated daily stressor exposure has a more negative outcome when applied during the active/dark phase compared to the inactive/light phase. Stressor exposure during the active phase resulted in a loss of general activity, decreased interest in an unfamiliar conspecific, a shift towards a more pro-inflammatory body milieu, and rhythm disturbances in plasma hormones, all representing well-accepted hallmarks of depression. In contrast, C57BL/6 mice exposed to the stressor in their inactive phase exhibited minor physiological alterations that might prevent the formation of the maladaptive consequences mentioned above, thus representing beneficial adaptations.

The second focus of this thesis was put on the investigation of the effects of repeated psychosocial stressor exposure at different times of the light-dark cycle on various levels of the circadian system. An increased expression of the PERIOD2

(PER2) protein, which represents an essential core clock component, could be found in the SCN of mice repeatedly exposed to the stressor during their active phase. In consistence with the alterations in the central circadian pacemaker, the daily rhythm of different hormones and the activity rhythm were considerably affected by SDD. Mice exposed to the psychosocial stressor in their active phase showed a shifted, or absent, rhythm of the hormones corticosterone and leptin. Moreover, their activity was found to be phase-delayed, which seems to be attributable to the *Period (Per)* gene since *Per1/Per2* double-mutants still exhibited their normal activity rhythm following 19 days of stressor exposure during the active phase. In contrast, a phase-advance in the peripheral adrenal gland clock could be seen in C57BL/6 mice subjected to the stressor during their inactive phase. This phase-shift might be required for maintaining the normal rhythmicity in hormonal release and activity.

It has previously been suggested that activation of the HPA axis upon stressor exposure at different times of the light-dark cycle is depending on whether the stressor is of physical or psychological nature. Data from the HPA axis analysis now refine previous findings, indicating that psychosocial stressors also modulate HPA axis responses based on the time of day of stressor presentation. The present results demonstrate that HPA axis activity was reduced following repeated stressor exposure during the active phase. It is reasonable to speculate that this reduced basal activity of the stress system represents a failure in HPA axis adjustment, which could contribute to the negative consequences of repeated psychosocial stressor exposure during the dark phase.

Taken together, it can be concluded that the endogenous clock in mice modulates the stress responsiveness in a circadian fashion and that repeated psychosocial

stressor exposure affects the biological clock depending on the time of day of stressor presentation. Thereby, stressor exposure during the active phase results in a more negative outcome as compared to stressor experience during the inactive phase. It is assumed that the interaction between the circadian clock and the stress system is a complex issue that might ensure that the endogenous clock does not get out of synchrony in any order.

LIST OF PUBLICATIONS

Paper 1 **Bartlang MS**, Neumann ID, Slattery DA, Uschold-Schmidt N, Kraus D, Helfrich-Förster C, Reber SO. 2012. Time matters: pathological effects of repeated psychosocial stress during the active, but not inactive, phase of male mice. J Endocrinol 215(3):425-37

Paper 2 **Bartlang MS**, Savelyev SA, Johansson AS, Reber SO, Helfrich-Förster C, Lundkvist GB. 2014. Repeated psychosocial stress at night, but not day, affects the central molecular clock. Chronobiol Int 31(9):996-1007

Paper 3 (submitted) **Bartlang MS**, Oster H, Helfrich-Förster C, Repeated psychosocial stress at night, but not day, delays the entrained activity rhythm of male mice.

CHAPTER 1

GENERAL INTRODUCTION

1.1. ADAPTATION TO PREDICTABLE ENVIRONMENTAL CHANGES – THE CIRCADIAN SYSTEM

In our universe, we are exposed to several natural rhythms that guide all our actions and accompany us throughout the whole life. In general, a rhythm is defined as a recurrent change that occurs with a defined probability and period. The movements of earth in the solar system, its yearly orbit around the sun, and daily rotation around its own axis give rise to regular changes in the environment for all organisms inhabiting the planet (Dickmeis et al., 2013). Thereby, the most conspicuous rhythms on earth are the daily changes of illumination and ambient temperature that result from the earth's rotation around its own axis. In order to anticipate these predictable environmental changes, organisms have evolved an evolutionarily conserved internal time-keeping system. This system, referred to as the circadian clock, autonomously generates cellular rhythms of roughly 24 hours (h) (Kwon et al., 2011) and organizes the behavior and physiology of an individual in a proactive rather than a responsive manner (Dibner et al., 2010). Circadian clocks exist in a wide spectrum of *phyla* and species, ranging from ancient cyanobacteria, fungi, and plants up to human beings (Dunlap, 1999). Moreover, they have been discovered at different levels of organization, from single cells and the specific activity of enzymes up to behavior and the release of hormones (Pittendrigh, 1993).

As insinuated by their name, circadian clocks (from the latin *circa diem*, literally meaning "approximately one day"; first introduced by Franz Halberg in 1959) are inaccurate clocks that do not run with a period of exactly 24 h. Thus, they have to be periodically synchronized in order to stay tuned to geophysical time, a process called entrainment. Thereby, the periodic daily change in light intensity is the key

environmental *Zeitgeber* (time giver) for most organisms that relays time information to circadian oscillators in order to reset their phase and to match the external conditions (Dibner et al., 2010). Besides light, other *Zeitgebers*, such as temperature, nutrition, humidity, and social factors exist, which might in certain situations dominate the solar cycle and contribute to a precise temporal allocation of resources and physiological economies (Hastings et al., 1995; Golombek and Rosenstein, 2010). In the absence of such cues the clock "free runs" according to its endogenous period.

Despite huge differences in complexity among species, circadian clocks from all organisms have the following fundamental intrinsic properties: (1) they can entrain to environmental stimuli and even re-entrain when the *Zeitgeber* cycles become shifted (Hall, 1990; Ruby et al., 1999); (2) the rhythms persist in the absence of external time cues, i.e. they are self-sustained (Reppert and Weaver, 2002); and (3) the rhythms are independent of the environmental temperature, i.e. they are temperature compensated (Hastings et al., 2008).

In the following sections I will focus on the mammalian circadian system.

1.1.1. The mammalian circadian clock

The mammalian circadian system is composed of nearly as many clocks as cells exist in the body. This has led to the crucial question of how all these clocks are synchronized in order to provoke a coherent rhythmic output (Albrecht, 2012). Early lesion and transplantation studies of the suprachiasmatic nucleus (SCN) located in the anterior hypothalamus provided first insights into the hierarchical organization of the mammalian circadian system with the SCN being on top of it. Following

selective disruption of the SCN, circadian rhythmicity of locomotor activity, drinking behavior, body temperature, and glucocorticoid (GC) secretion was completely absent (Moore and Eichler, 1972; Stephan and Zucker, 1972; Meyer-Bernstein et al., 1999). Despite the lack of neuronal connections between the grafted SCN and the host brain, transplantation of donor SCN tissue into hosts with lesioned SCN remarkably restored these rhythms, suggesting that a diffusible secreted factor might be responsible for transmitting the circadian signal from the SCN (Aguilar-Roblero et al., 1994; Silver et al., 1996). However, while circadian rhythms of locomotor activity, drinking behavior, and body temperature could be restored by SCN transplants, circadian GC secretion was not, implicating that, in addition to secreted factors, neuronal efferents are required for generation of certain circadian rhythms (Meyer-Bernstein et al., 1999; Cho et al., 2005).

Besides the central component residing in the SCN, subsidiary peripheral clocks have been identified in extra-SCN brain regions and peripheral tissues, such as the hippocampus, the liver or the adrenal glands (Stratmann and Schibler, 2006) (see Fig. 1). These peripheral clocks are believed to possess tissue- or organ-specific roles in controlling circadian rhythms. Together, the SCN and the subordinated peripheral clocks build up a multi-oscillatory network that receives timing information via external *Zeitgebers* and, in turn, coordinates behavioral and physiological output rhythms in accordance with the environment. While the SCN is entrained to the environment by light or other non-photic cues, downstream peripheral clocks are mainly synchronized by timing cues emanating from the SCN. In general, the SCN can be considered as a conductor that orchestrates the peripheral clocks to ensure temporally coordinated physiology (Koch et al., 2009; Albrecht, 2012). The

peripheral clocks, in turn, may be connected among themselves and may even feed back to the master pacemaker (Schibler and Sassone-Corsi, 2002).

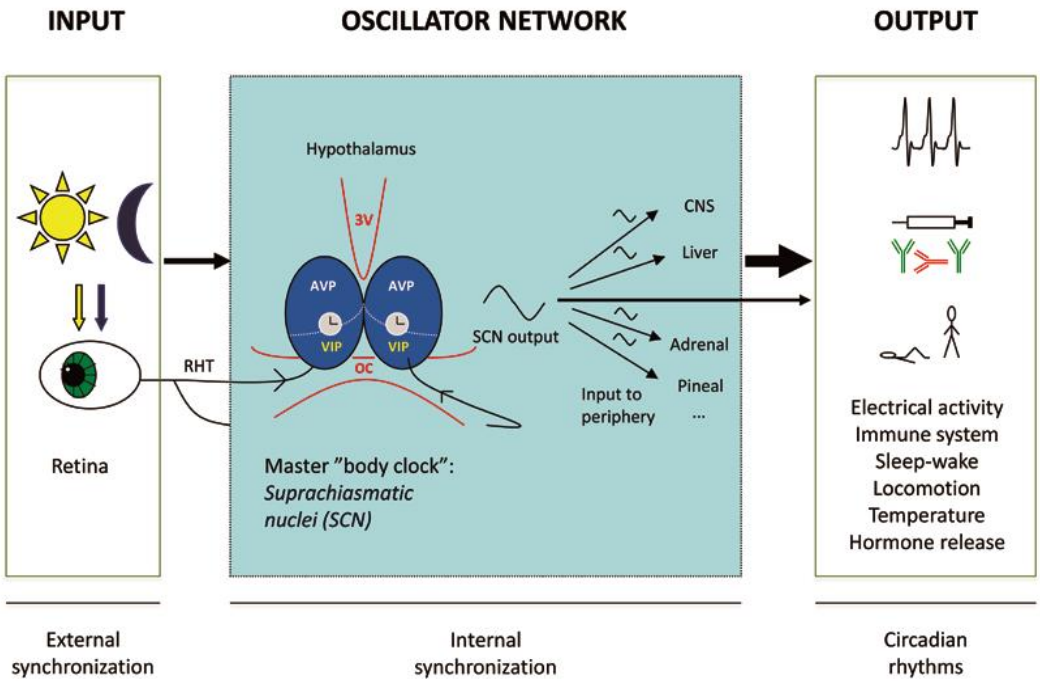


Figure 1: Organization of the mammalian circadian system. The circadian system consists of three major components: the inputs (*Zeitgebers*), the rhythm generator, and the outputs. Furthermore, it is organized in a hierarchical fashion with the SCN being the central pacemaker on top of it and subsidiary peripheral clocks functioning as slave clocks. The SCN is synchronized by the external 24 h cycle or other non-photic *Zeitgebers* and produces sustained and synchronous cellular rhythmicity in both central and peripheral tissues. This, in turn, coordinates behavioral and physiological outputs. The multi-oscillator network is synchronized through several lines of communication. While light is the primary input for the SCN, peripheral oscillators are synchronized by timing cues from the SCN (SCN output), which regulate local circadian physiology. Abbreviations: AVP, arginine-vasopressin; CNS, central nervous system; OC, optic chiasm; RHT, retinohypothalamic tract; VIP, vasoactive intestinal peptide; 3V, third ventricle. [taken and adapted from (Lundkvist, 2014)]

Below, the central and peripheral circadian clocks are discussed in further detail, with the main focus directed on the former.

1.1.1.1. The central clock

Architecture

The SCN is a bilaterally paired tiny nucleus located above the optic chiasm near the third ventricle in the anterior hypothalamus (see Fig. 2A). Depending on the species, each of the bilateral SCN is formed by a heterogeneous network of 10,000 – 50,000 single neurons. These neuronal oscillators are coupled to each other by synaptic interaction in order to become a circadian pacemaker (Guldner, 1983; Swaab et al., 1985). According to peptide expression, neuronal morphology, and projection patterns, each nucleus can be divided into two main subdivisions: A dorsomedial/ shell and a ventrolateral/core region (Morin, 2007). The ventrolateral SCN receives most of the input from the retina and brain regions that get photic input, as well as innervation from the median raphe nucleus. In contrast, the dorsomedial SCN is mainly innervated by limbic areas, the hypothalamus, and the core region (Golombek and Rosenstein, 2010). Core neurons produce primarily vasoactive intestinal peptide (VIP), while arginine-vasopressin (AVP) is mainly synthesized in the shell region (Moore, 1996) (see Fig. 2B). In contrast to AVP, which is mainly implicated in mediating efferent signals, VIP seems to be critically involved in maintaining intra-SCN synchrony (Tonsfeldt and Chappell, 2011). Although the precise position of peptide expression varies between different species, the overall separation of VIP and AVP is maintained among rodents (Morin and Allen, 2006). In addition, the majority of SCN neurons expresses the inhibitory transmitter γ-aminobutyric acid (GABA) (Moore and Speh, 1993), which is important for both intracellular SCN signaling and SCN efferent projections (Strecker et al., 1997). In rats, AVP-containing neurons comprise about 37 % of all SCN neurons, while approximately 25 % of SCN neurons express VIP (Moore et al., 2002). Neurons

expressing gastrin-releasing peptide and calretinin are located medially and constitute about 14 % of SCN neurons each. Additionally, neurons containing calbindin, somatostatin or neurotensin are also found in the SCN but since these only make up 10 %, they are not considered as main neuroactive substances of the SCN (Abrahamson and Moore, 2001; Moore et al., 2002). Individual SCN neurons show daily rhythms in firing rate and, due to the paracrine and synaptic communication between single oscillators within the master clock, the SCN oscillators are protected from desynchronization in the absence of external cues *in vivo* and *in vitro* (Dibner et al., 2010).

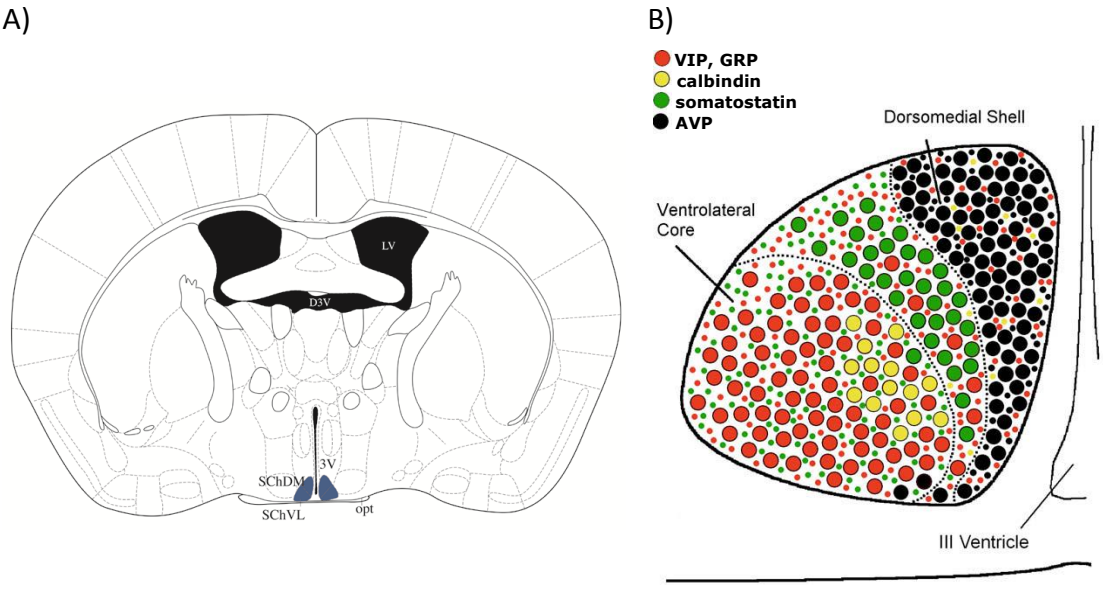


Figure 2: Mouse coronal brain slice (A) and cellular organization of the master clock in the hamster (B). A: The bilateral suprachiasmatic nucleus (SCN; labelled in blue) is located above the optic chiasm (opt) near the third ventricle (3V) in the anterior hypothalamus. The central pacemaker can be divided into two subdivisions, namely the dorsomedial (SChDM) and the ventrolateral (SChVL) region (Paxinos and Franklin, 1997) [adapted and printed with kind permission of Elsevier]. B: Only the SCN of the left brain hemisphere is shown. Each hamster SCN can be divided into a ventrolateral core and a dorsomedial shell region, both densely packed with somata of small neurons (7-11 µm). The main neuropeptide transmitters of these areas are vasoactive intestinal peptide (VIP), gastrin-releasing peptide (GRP), calbindin, somatostatin, and arginine-vasopressin (AVP). Most neurons additionally contain γ-aminobutyric acid (GABA). The somata of the neurons are shown as larger circles, their projections as small circles (Helfrich-Förster, 2004) [adapted and printed with kind permission of Springer Science + Business Media].

Besides the different neuronal cell types, glial cells constitute a large portion of the total cell number within the SCN. Although playing an essential role in most functions of the central nervous system (CNS), glia cell function in this context is largely unknown. However, is it believed that astroglial cells are crucially involved in the alteration of circadian rhythms by immune activation (Duhart et al., 2013).

Rhythm generation

In order to achieve circadian regulation, a transcriptional-translational auto-regulatory loop generates recurrent molecular oscillations in messenger ribonucleic acid (mRNA) and protein levels of so called clock genes at the cellular level. This, in turn, coordinates the expression of pathways involved in metabolism, physiology, and behavior (Roenneberg and Merrow, 2003; Asher and Schibler, 2011). Mutations in clock components or their regulators might lead to asynchrony from the cellular to the organ-system level and could manifest in devastating health problems (see section 1.1.1.3).

The positive limb of this auto-regulatory loop is composed by the transcription factors CIRCADIAN LOCOMOTOR OUTPUT CYCLES KAPUT (CLOCK) (or NEURONAL PER-ARNT-SIM DOMAIN PROTEIN 2 in brain tissue) and BRAIN AND MUSCLE ARYL HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR-LIKE PROTEIN 1 (BMAL1), which form a heterodimer that binds to the E-box promoter elements of their target clock and clock-controlled genes (CCGs) at the beginning of a circadian day (independent of the period of day during which an organism is active). The clock genes *Period* (*Per*; isoforms *Per1* and *Per2; Per3* plays a negligible role in the core clock machinery) and *Cryptochrome* (*Cry;* isoforms *Cry1* and *Cry2*), in turn, constitute the negative limb (see Fig. 3). In detail, their mRNA is translated into proteins in the cytoplasm of the cell over the course of the day. Upon reaching a

certain threshold, the protein products form hetero- and homodimers that feed back to the nucleus by binding to the CLOCK:BMAL1 protein complex to auto-repress the expression of their own genes at the beginning of the circadian night (Albrecht, 2012). Afterwards, constitutive degradation decreases the PER and CRY protein levels and when these levels fall below a certain threshold that is necessary for efficient auto-repression, a new transcriptional cycle can start (Stratmann and Schibler, 2006).

Post-translational modifications, such as phosphorylation, histone acetylation, methylation, and ubiquitination, affecting mainly the negative limb, seem to be required for the delay between transcriptional activation and repression that is important for a precise and functional circadian rhythm (Reppert and Weaver, 2002).

Besides the central loop, accessory regulatory loops contribute to a proper clock function. One of those is formed by nuclear receptors from the reverse erythroblastosis virus (REV-ERB) and retinoic acid receptor-related orphan receptor (ROR) families, which are transcriptionally regulated by the positive limb and, in turn, activate (ROR) or repress (REV-ERB) transcription of *Bmal1* (Reppert and Weaver, 2002). Robust oscillations in antiphase with those of *Per* and *Cry* genes are evident for *Bmal1* transcripts (Shearman et al., 2000), whereas *Clock* is constitutively expressed in most mammalian tissues, including the SCN (Preitner et al., 2003; Sato et al., 2004). The PER2 protein fine-tunes this process by interacting with REV-ERB to synchronize the negative and positive limbs of these transcriptional translational feedback loops.

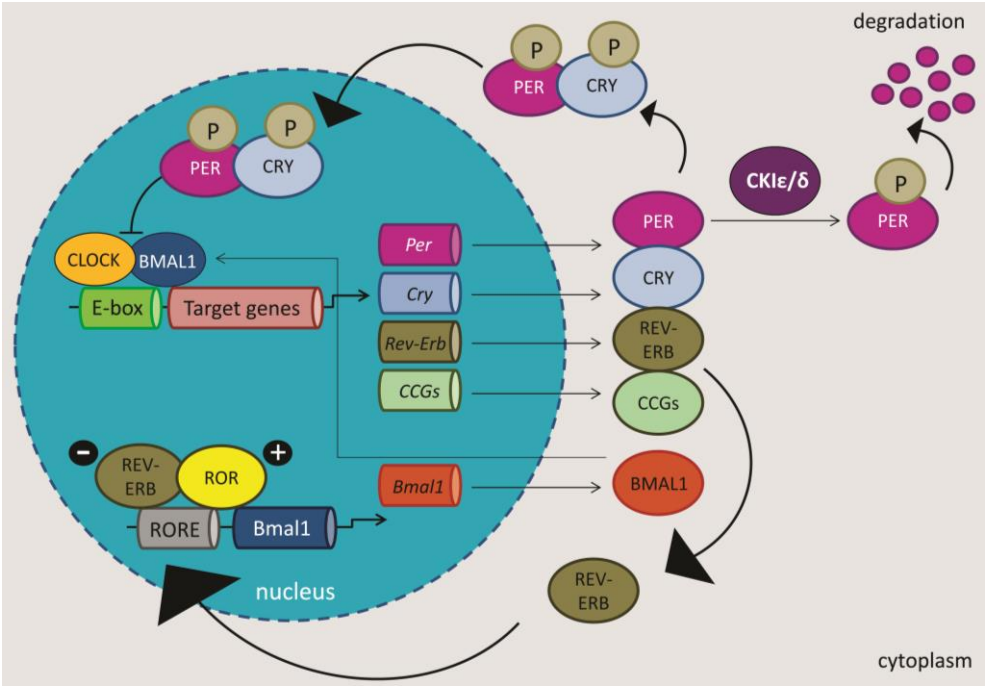


Figure 3: A simplified view of the molecular circadian clock. The mammalian clock is supposed to rely on two interlocked transcriptional and translational feedback loops. The positive limb is formed by the transcription factors CLOCK and BMAL1 that bind to E-box sequences in the promoters of clock genes like *Per*, *Cry*, and *Rev-Erb*, thus activating their transcription at the beginning of a circadian day. As a result, PER proteins accumulate in the cytoplasm but become phosphorylated (P) by kinases like casein kinases (CKI) ε and δ and glycogen synthase kinase 3β. These phosphorylated forms of PER proteins are unstable and are degraded. Late in the subjective day, CRY accumulates in the cytoplasm and promotes the formation of stable CKI/PER/CRY complexes that enter the nucleus at the beginning of a circadian night and repress CLOCK:BMAL1-mediated transcription. To initiate a new circadian cycle, PER and CRY proteins get degraded. The interacting positive and negative feedback loops of circadian genes warrant low levels of PER and CRY and, concomitantly, high levels of BMAL1 at the beginning of a new circadian day. The CLOCK:BMAL1 heterodimer can further inhibit *Bmal1* transcription. Following transcription and translation, the REV–ERB protein enters the nucleus to suppress the transcription of the *Bmal1* gene during day. At night, REV-ERB protein levels are low, allowing *Bmal1* transcription to take place. ROR, another nuclear receptor, competes with the REV-ERB for the RORE binding site and activates the transcription of *Bmal1*. Abbreviations: BMAL1, brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein; CCGs, clock-controlled genes; CKI, casein kinase; CLOCK, circadian locomotor output cycles kaput; Cry, Cryptochrome; E-box, Enhancer box; P, phosphorylated; Per, Period; REV-ERB, reverse erythroblastosis virus; ROR, retinoic acid receptor-related orphan receptor; RORE, retinoic acid receptor-related orphan receptor response element. Italic notation for genes, plain and capital notation for respective proteins. [taken and adapted with kind permission by (Golombek and Rosenstein, 2010)]

The basic clock components are not only expressed in the central pacemaker, but also throughout the whole body, where they also show oscillating expression (Balsalobre, 2002). Noteworthy, these rhythms persist in organ cultures as revealed by real-time imaging of such cultures from transgenic bioluminescent rats and mice carrying clock gene reporter constructs (Yoo et al., 2004). In contrast to peripheral clocks, the phase of clock gene expression in the central clock does not differ between nocturnal and diurnal animals. Accordingly, PER2 protein in the SCN is high at the actual or projected light-to-dark transition in both species (Field et al., 2000; Smale et al., 2003). Based on the common molecular clockwork, it is assumed that signals emanating from the SCN are interpreted contrarily and, as a consequence, downstream actions of output factors differ between night- and day-active species (Mrosovsky et al., 2001; Caldelas et al., 2003).

Clock Input

To optimally adapt metabolic processes, physiology, and behavior of an organism to the natural environment, information from outside has to reach the body where time-related information can be integrated and, in turn, is relayed to various tissues (Eskin, 1979; Albrecht, 2012). The master clock in the SCN has the capacity to function as a relay between the external and internal world. Thereby, three major input pathways to the central clock are known: the retinohypothalamic tract (RHT), the geniculohypothalamic tract (GHT), and serotonergic input from the raphe nuclei. While the RHT provides photic information to the SCN, the latter two transmit non-photic (behavioral) information. Interestingly, the temporal responsiveness of the SCN to photic and non-photic stimuli differs from each other (Dibner et al., 2010).

Mammals receive information about external lighting conditions exclusively through the retina of the eye. The principal light detecting systems are not the visual photoreceptors of the retina, the rods and cones, but rather a subset of retinal ganglion cells that express the photopigment melanopsin (Foster et al., 1991; Foster and Hankins, 2007). These ganglion cells are directly light responsive and project through the RHT to the ventrolateral core region of the SCN where retino-recipient cells expressing VIP are located. Admittedly, it is not fully understood to which extent the classical photoreceptor system, consisting of rods and cones, contributes to circadian photoreception (Reppert and Weaver, 2002). The ganglion cell-SCN circuit uses pituitary adenylate cyclase-activating protein (PACAP), aspartate, and glutamate as neurotransmitters, whereas the latter is considered to be the main photic signal for the circadian clock. Upon photic stimulation, glutamate is released at the synaptic terminals of the RHT. This leads to a calcium influx in the SCN neurons, which initiates the activation of calcium-dependent kinases, proteases, and transcription factors (Moore and Lenn, 1972; Moore et al., 1995). Consequently, phosphorylation of cyclic adenosine monophosphate responsive elements binding protein (CREB) in SCN neurons is induced. Phosphorylated CREB binds to cyclic adenosine monophosphate responsive elements (CRE) located in the promoter region of clock genes and, thus, resets their phase independent of the CLOCK:BMAL1-controlled E-box activation mentioned before. This resetting is rapidly established since the individual SCN oscillators are tightly coupled to each other. However, light can elicit phase-shifts by activating clock gene transcription only during early or late night (activity period in rodents), as revealed by phase response curve analyses. Interestingly, virtually all species show similar phase-dependent resetting responses to light, regardless of the period of the day during

which an organism is active. In contrast, non-photic cues are able to reset clock gene expression mainly during day (resting period in rodents), when light is ineffective (Dibner et al., 2010). Among the mammalian *Per* genes, *Per1* and *Per2* mRNAs in the SCN are inducible by light, whereas *Per3* is not (Takumi et al., 1998). Trophic factors might also be involved in photic signaling in the SCN. Indeed, brain-derived neurotrophic factor (BDNF) has been proposed to play a role in photic entrainment since BDNF mutant mice exhibit impaired responses to light and mice targeted for the BDNF receptor tropomyosin-related kinase B (TrkB) also show decreased phase-shifts in response to light pulses (Golombek and Rosenstein, 2010).

In addition to this direct monosynaptic connection to the SCN, the RHT also projects to the intergeniculate leaflets (IGL), from which the GHT signals to the master clock. Thus, the GHT provides indirect light information to the SCN by releasing neuropeptide Y and GABA (Johnson et al., 1989).

The third afferent projection to the SCN originates in the raphe nuclei (in rats both the medial and dorsal raphe nucleus). Like for the RHT, these fibers also terminate in the core region of the SCN and provide non-photic (i.e. behavior-induced) input to the circadian pacemaker (Ruby et al., 2009).

Of note, the IGL is stimulated also by the dorsal raphe nucleus and, thus, allows the integration of photic as well as non-photic signals to entrain the SCN (Meyer-Bernstein and Morin, 1996; Marchant et al., 1997).

Clock Output

In order to function as a pacemaker and synchronizer, the intrinsic time-keeping signal from the SCN has to be transmitted to the rest of the body. Besides AVP and VIP, numerous SCN output factors, including neuronal and hormonal signals, have been proposed to act as timing cues for peripheral oscillators in order to control diverse behavioral and physiological functions (Pezuk et al., 2012). Amongst the blood-borne timing factors, GCs are strong candidates since the synthetic GC dexamethasone induces circadian gene expression in fibroblasts and is a potent phase-shifting cue in peripheral organs (Balsalobre et al., 2000). Although an involvement of GCs in the regulation of peripheral clocks is obvious, the effects are tissue-specific and the exact mechanisms remain to be elucidated. In general, it is assumed that behavioral rhythms rely on humoral outputs of the SCN, whereas endocrine rhythms also require neuronal projections from the SCN to endocrine targets, as aforementioned (Guo et al., 2005).

Efferent pathways of the master clock within the brain include few thalamic brain areas, such as the IGL and several hypothalamic brain regions like the medial preoptic area, concerned with thermoregulation, and the dorsomedial hypothalamus (DMH) and arcuate nucleus (ARC), important for modulating food intake (Takahashi et al., 2008). The major hypothalamic efferent target of the SCN is the paraventricular nucleus of the hypothalamus (PVN), one of the most important integrative control centers involved in neuroendocrine and autonomic processes (Ferguson et al., 2008).

Moreover, the SCN coordinates peripheral tissues more indirectly, e.g. via daily rest-activity rhythms and, as a consequence, feeding-fasting rhythms (Stratmann and Schibler, 2006). Noteworthy, different subdivisions of the SCN project to designated

areas and diverse tissues might require different resetting cues or combinations of several factors. The peptide prokineticin 2, for instance, has been indicated as SCN output signal to regulate circadian locomotor activity (Tonsfeldt and Chappell, 2011), while AVP seems to be involved in the regulation of circadian hormone rhythms (Li et al., 2012). Interestingly, environmental cues are capable of adjusting the output of the clock acting directly upon overt rhythms, an effect termed “masking”. This might account for a fast and proper response to potentially threatening stimuli.

1.1.1.2. Peripheral clocks

Although equipped with a molecular make-up similar to SCN neurons, thus, capable of generating rhythmic periodicity, peripheral oscillators differ from the master pacemaker in a number of aspects. Circadian gene expression in peripheral tissues is approximately 4 – 6 h phase-delayed in comparison with the SCN and, therefore, peripheral clocks are often referred to as slave clocks (Yamamoto et al., 2004). Moreover, peripheral oscillations dampen out after a few days in culture, as shown by genetic coupling of a luciferase reporter to the *Per1* gene promoter (Yamazaki et al., 2000). This is due to the fact that individual cells have slightly different periods and become desynchronized over time unless they are efficiently synchronized to each other. Since peripheral oscillators are not coupled to each other by paracrine signals, this has led to the hypothesis that the master clock in the SCN coordinates and synchronizes individual oscillators in peripheral tissues. Indeed, daily resetting cues from the SCN (directly or indirectly) are required for phase coherence of peripheral tissue clocks (Stratmann and Schibler, 2006). By this, the SCN coordinates the activity of tissues and organs of the body relative to one another, thereby maintaining homeostasis (Kriegsfeld and Silver, 2006).

Unlike the central pacemaker in the SCN, subsidiary peripheral clocks are not directly entrained by light, except for the adrenal (Ishida et al., 2005) and pineal gland (Fukuhara et al., 2000), as assessed *in vivo* and *in vitro*, respectively. Thus, the aforementioned output signals from the SCN are required for transmitting temporal information to the periphery (Welsh et al., 2010). In addition, feeding rhythms are strong *Zeitgebers* for many peripheral organs including the liver and kidney. Normally, feeding-fasting cycles are in accordance with rest-activity rhythms.

However, restricted daytime feeding of nocturnal animals inverts the phase of peripheral clock gene expression and, thus, uncouples the peripheral clocks from the central clock. To correlate daily rhythms of physiology and behavior with the food availability rhythm, hormones secreted upon feeding and fasting, such as leptin and ghrelin, respectively, represent possible entrainment cues (Dibner et al., 2010).

But what is the biological sense of having autonomous peripheral clocks? If the SCN coordinates all cell populations, why do individual cells need their own intrinsic clocks? The purpose of peripheral clocks is mainly to provide responsiveness to local conditions and temporal fine-tuning in a local population (Kriegsfeld and Silver, 2006). In particular, these local clocks optimize cellular physiology by sequestering chemically incompatible reactions to different time windows (e.g. opposite temporal regulation of glycolytic and gluconeogenic enzymes) (Schibler, 2007). As stated above, peripheral oscillators can even become uncoupled from the SCN if dictated by their specific needs, as occurs in the liver or kidney after entrainment by food (Yamazaki et al., 2000).

Furthermore, it becomes increasingly evident that feedback from peripheral clocks to the central clock is essential to maintain robustness and stability of certain circadian rhythms, such as GC release from the adrenal gland (Barclay et al., 2012).

1.1.1.3. Rhythm disturbances and link to human diseases

Once evolved to adapt to our natural environment, circadian rhythms become progressively disrupted due to the masking of reliable external markers by artificial cues. In other words, we are prepared for a world that does not exist anymore (Golombek and Rosenstein, 2010). Since we have received access to electricity our activity gradually shifted into the night period that naturally was intended to be the rest period. As a result, "social jet lag", i.e. a discrepancy between social and biological timing, occurs (Wittmann et al., 2006). The impact of an acute dis-adjustment between internal and external temporal cues is evident to anyone who has experienced "jet lag" after a long flight across different meridian zones. In our modern 24/7 society, nocturnal lifestyle, rotating shift-work, and frequent travelling across multiple time zones are in order of the day. The resulting disturbed biological rhythms are strongly associated with somatic and affective disorders and, thus, may have profound impact on human health. In animal models, tumor growth following disturbances of the circadian system could be observed (Filipski et al., 2006) and human epidemiological studies have shown that rotating-shift nurses exhibit a higher risk to suffer from breast cancer compared to day-shift nurses (Schernhammer et al., 2001). Likewise, shift-work experience is associated with a higher incidence of major depressive disorder (MDD) (Cole et al., 1990; Scott et al., 1997).

Vice versa, abnormal body temperature, plasma cortisol, norepinephrine (NE), melatonin, and social rhythms are some of the major hallmarks seen in affective diseases, such as MDD and bipolar disorder (Souetre et al., 1989; Monk et al., 1994; Grandin et al., 2006; McClung, 2007). Given that diverse peripheral rhythms are

perturbed in depressed patients, it is likely that this is centrally mediated involving the central circadian pacemaker and/or the molecular circadian clock machinery. Indeed, human genetic studies strongly associate polymorphisms of clock genes with mood disorders. For example, variations in *Clock*, *Bmal1*, and *Rev-Erbα* have all been linked to bipolar disorder (Nievergelt et al., 2006; Benedetti et al., 2007; Kishi et al., 2008), and mice bearing a mutation in the *Per2* gene exhibit increased vulnerability to MDD and seasonal depression (Partonen et al., 2007; Lavebratt et al., 2010). Furthermore, virtually all of the successful treatments for mood disorders seem to affect circadian rhythms and it appears that stabilization and/or resetting of these rhythms, produced by the treatments, are crucial for therapeutic efficacy (McClung, 2007). For instance, the effects of seasonal affective disorder, a mood disorder that correlates with the extremely shortened daily light period during the winter season, can be alleviated by bright light therapy. Amongst abnormal levels of the dark phase hormone melatonin, these patients are phase-delayed in their chronobiological cycle (Danilenko et al., 1994; Murray et al., 2005; Winkler et al., 2005). Since clock genes, especially *Per1* and *Per2*, are inducible by light in the SCN, early morning light can phase-advance behavioral and endocrine rhythms, thus alleviating depressive-like symptoms (Lewy et al., 1987; Yan and Silver, 2002). Moreover, the melatonergic antidepressant Agomelatine has recently been implicated in the treatment of seasonal affective disorder (Pjrek et al., 2007) and MDD (Srinivasan et al., 2012), based on its resynchronization properties. Further, sleep deprivation has antidepressant-like effects and might improve the synchronization between single oscillators, further highlighting the link between mood disorders and the circadian system.

Somatic disorders have also been linked to a disturbed circadian clock as it was shown that a single nucleotide polymorphism in the human *Per3* gene is associated with inflammatory bowel disease (Mazzoccoli et al., 2012). Although an implication of circadian rhythm disturbances in the development of the above-mentioned diseases is obvious, it still remains unclear whether rhythm disturbances are the underlying cause of such diseases or simply symptoms of the disease process. In any case, it is very likely that a disruption of the circadian clock can at least modify the severity of the disease (Albrecht, 2010). The question to what extent the course and development of such diseases can be ameliorated by re-creating circadian rhythms is a current line of inquiry.

In general, the health compromising potential of the above described discrepancies between social and biological timing and the resulting dis-adjustment between internal and external temporal cues are not surprising. Exactly these processes, resulting in a disruption of the internal homeostasis of an organism, are generally referred to as stressors and the potential of chronic, in particular chronic psychosocial stressors to promote somatic and affective disorders is well known and accepted. In the following, the stress system will be explained in detail.

1.2. ADAPTATION TO UNPREDICTABLE ENVIRONMENTAL CHANGES – THE STRESS SYSTEM

All living organisms maintain a complex dynamic equilibrium, or homeostasis, in order to retain a constant internal environment in response to external environmental changes (Cannon, 1929). Any disruption of this equilibrium state by a threatening stimulus ("stressor") evokes the activation of the sympathoadrenal system, promoting the "fight or flight" response in order to restore the internal milieu (Cannon, 1929; Sorrells et al., 2009). The reaction of an organism aimed to regain homeostasis is called "stress response" (Chrousos, 2009). In the year 1936, Hans Selye defined the term "stress" as "the non-specific response of the body to any physical demand" (Selye, 1936). He further described the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) in response to threatening situations (Selye, 1950). More recently, it was postulated that stress should be considered as a process that includes the stimulus, the perceptual processing of the input and the behavioral and physiological output (Koolhaas et al., 2011).

Stressors can be classified according to quality (positive vs negative), type (psychological vs physiological), duration (acute vs chronic), and controllability/predictability. In the present thesis, the word "stress" will be used as synonym for the term "stressor". Central and peripheral functions of the stress response include facilitation of arousal and attention and increase of metabolism, oxygenation, and cardiovascular tone with concomitant inhibition of vegetative functions. Together with the SNS, the HPA axis is the primary system for maintaining or reinstating homeostasis during stress. Although both systems

interact to coordinate the adaptive responses of an organism to any stressor, they differ from each other with regard to their temporal patterns and their effector hormones (Ulrich-Lai and Herman, 2009). In the following sections, the SNS and the HPA axis are explained in detail.

1.2.1. The sympathetic nervous system

The SNS, together with the parasympathetic and enteric nervous system constituting the autonomic nervous system (ANS), provides an immediate response to stressful stimuli and controls cardiovascular, gastrointestinal, respiratory and further somatic systems (Chrousos, 1998).

The central control station of the SNS is the locus coeruleus (LC) in the brain stem that contains the largest cluster of NE-containing neurons in the brain and is a major nucleus involved in neuronal pathways controlling autonomic function. The LC was shown to be activated by diverse stressors, such as restraint (Abercrombie et al., 1988), noise (Britton et al., 1992) or social ones (Valentino and Van Bockstaele, 2008), most likely via direct projections arising from the PVN (Reyes et al., 2005). Importantly, the LC projects to widespread areas within the CNS and these projections can result in both excitatory and inhibitory effects via stimulation of different adrenergic receptors (Jones, 2005). As a major premotor autonomic nucleus the LC directly projects to sympathetic preganglionic neurons located in the intermediolateral column (IML) at the thoracic and lumbar region of the spinal cord (Nygren and Olson, 1977; Westlund et al., 1983). Axons of these preganglionic neurons leave the spinal cord in the ventral branches of the spinal nerves and synapse on postganglionic sympathetic neurons mostly on paravertebral ganglia next to the spinal cord (i.e. the sympathetic trunk) or, to a lesser extent, at

prevertebral ganglia (celiac ganglion, superior mesenteric ganglion, inferior mesenteric ganglion) located in front of the vertebrae (see Fig. 4A). The postganglionic neurons, in turn, innervate peripheral effector organs, resulting in energy mobilization, vasoconstriction of blood vessels, stimulation of sweat glands, and increased heart and breathing rate, representing the classical “fight or flight” response (Abboud, 2010). Additionally, some preganglionic fibers pass through the sympathetic trunk without synapsing and continue as splanchnic nerves. For instance, the greater thoracic splanchnic nerve can innervate the adrenal medulla directly, without synapsing on neurons in the sympathetic ganglia, in order to stimulate the release of catecholamines from adrenal chromaffin cells (Holgert et al., 1998) (see Fig. 4B).

While preganglionic neurons are cholinergic and use acetylcholine as neurotransmitter, noradrenergic postganglionic neurons release NE onto their target tissues (Holgert et al., 1998). Importantly, chromaffin cells are considered as modified postganglionic sympathetic neurons due to their embryological and anatomical homology to sympathetic ganglia. Thus, upon stimulation, adrenal chromaffin cells also secrete NE (about 20 %), but to a higher portion epinephrine (about 80 %), directly into the blood stream, thus assigning the adrenal medulla an endocrine gland (Elenkov et al., 2000). The catecholamines NE and epinephrine exert their physiological effects via two main adrenoreceptor classes, namely the α- and β-adrenoreceptors, which can be further divided into several subtypes. Of note, these receptors are expressed in an organ-specific manner and, thus, mediate different physiological functions by either stimulation or inhibition of different downstream signaling pathways (Molinoff, 1984).

A) B)

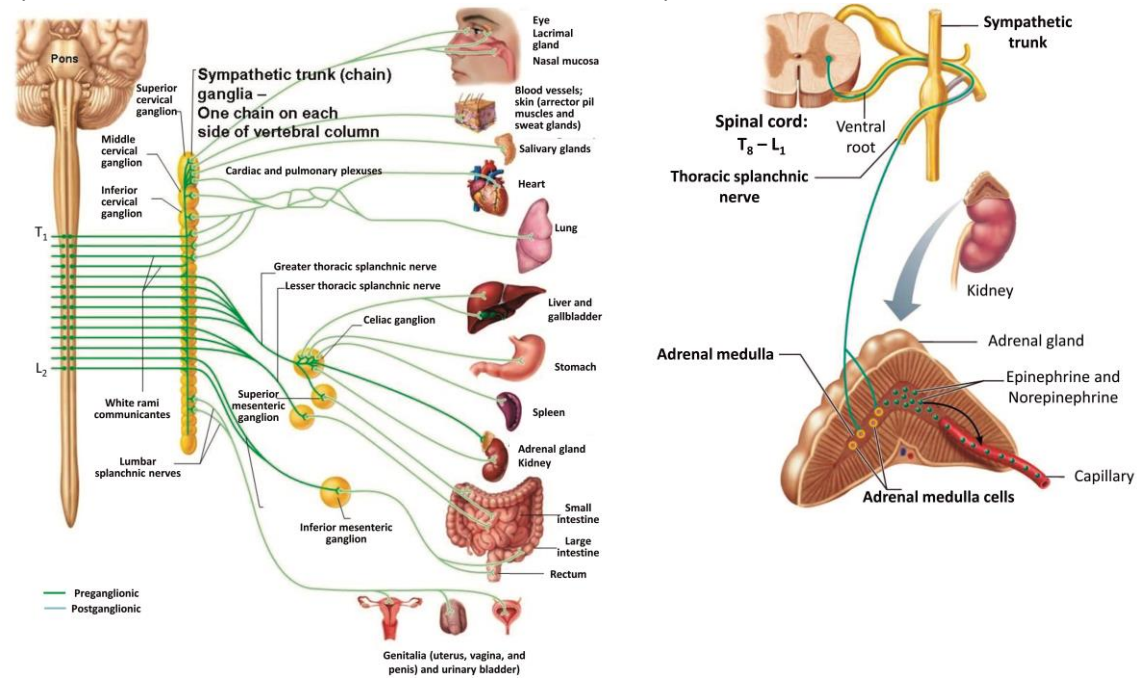


Figure 4: (A) Schematic representation of the sympathetic nervous system (SNS) and (B) sympathetic innervation of the adrenal medulla. A: Preganglionic neurons of the SNS originate from the first thoracic segment (T1) and extend to the second lumbar segment (L2) of the spinal cord. The preganglionic neurons synapse on postganglionic neurons either on the paravertebral ganglia (i.e. the sympathetic trunk) next to the spinal cord or at the prevertebral ganglia (celiac ganglion, superior mesenteric ganglion, inferior mesenteric ganglion) located in front of the vertebrae. From there, the postganglionic neurons affect their different target organs. The greater thoracic splanchnic nerve innervates adrenal medulla cells directly, without synaptic contact in the sympathetic ganglia. B: The adrenal gland is an endocrine organ consisting of an outer layer (adrenal cortex) and an inner layer (adrenal medulla). When stimulated by preganglionic sympathetic fibers from the 8. thoracic segment (T8) to the first lumbar segment (L1) of the spinal cord, adrenal medulla cells secrete large quantities of the catecholamines norepinephrine and epinephrine into nearby capillaries. Once released into the blood, these hormones amplify all of the "fight of flight" stuff in order to provide more energy. [taken and adapted with kind permission by

http://antranik.org/wp-content/uploads/2011/11/sympathetic.jpg?5a311c;
http://antranik.org/wp-content/uploads/2011/11/the-adrenal-medulla-of-the-adrenal-gland-epinephrine-norepinephrine-splanchic-nerves.jpg?5a311c]

While the neuronal innervation of end organs through the SNS provides an immediate response to stressor exposure, activation of the HPA axis resulting in GC release from the adrenal cortex is slower and more persistent in its actions (Kyrou and Tsigos, 2009).

1.2.2. The hypothalamic-pituitary-adrenal axis

Activation of the HPA axis in response to stressful stimuli is a major physiological event that contributes to the maintenance of homeostasis. In addition, another characteristic of the HPA axis is fundamental to homeostatic regulation in mammals: A circadian rhythm in basal activity (Sage et al., 2001). In most vertebrates there is a prominent circadian rhythm in plasma GC secretion with peak levels corresponding to the onset of the active phase of the daily cycle (Keller-Wood and Dallman, 1984) (see section 1.3.1). Importantly, GC secretion driven by stressful stimuli superimpose upon the existing circadian tone (Buckingham, 2006).

The hypothalamic PVN is considered a principal integrator of stress input and receives signals of homeostatic imbalance from ascending brain stem systems and limbic forebrain structures like the hippocampus, the prefrontal cortex, and the amygdala. In response to stressful stimuli, parvocellular neurons of the PVN secrete releasing hormones, such as corticotropin-releasing hormone (CRH) and AVP into the portal circulation at the median eminence (Ulrich-Lai and Herman, 2009) (see Fig. 5). By binding to their respective receptors, CRH type 1 receptor (CRH-R1) and AVP type 1b receptor (AVP-R1b), on corticotrope cells, these releasing hormones act synergistically on the anterior pituitary to trigger the secretion of adrenocorticotropic hormone (ACTH) from pre-formed granules into the peripheral circulation (Lightman, 2008; Aguilera, 2011). ACTH acts on the adrenal cortex via the melanocortin-2 receptor (Mc2r) to initiate the synthesis of GC hormones. Upon ACTH-induced activation of the Mc2r, intracellular cyclic adenosine monophosphate (cAMP) levels rise. This, in turn, increases adrenal cholesterol mobilization from cortical lipid droplets by promoting hormone sensitive lipase (HSL)-mediated hydrolyzation of cholesterol esters. Furthermore, it accelerates subsequent

shuttling of free cholesterol into the mitochondria, which is considered the rate-limiting step in steroidogenesis and involves the steroidogenic acute regulatory protein (StAR) as well as the translocator protein (Ulrich-Lai et al., 2006a; Rupprecht et al., 2010).

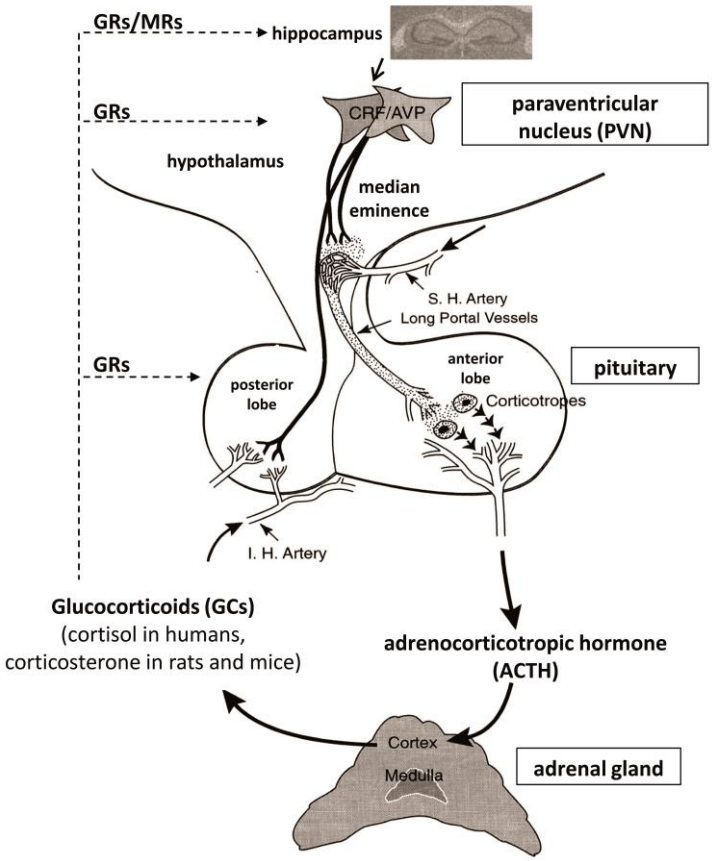


Figure 5: Functional anatomy of the hypothalamic-pituitary adrenal (HPA) axis. The hypothalamic paraventricular nucleus (PVN) receives homeostatic/stress inputs from the brain stem and limbic areas. Upon activation, parvocellular CRH (corresponding to CRF)/AVP-secreting neurons project to the median eminence where they terminate in close proximity to a capillary plexus. CRH and AVP are directly secreted into these vessels and transported via the hypophyseal portal system to corticotropes in the anterior pituitary gland. Thereupon, adrenocorticotropic hormone (ACTH) is released from pre-formed granules into the venous circulation. When ACTH reaches the adrenal cortex, it activates the synthesis and secretion of glucocorticoids (GCs) which, in turn, act on different levels of the HPA axis via negative feedback (dashed arrows). Abbreviations: AVP, arginine-vasopressin; CRF/CRH, corticotropin-releasing factor (hormone); GRs, glucocorticoid receptors; I.H., inferior hypophysial; MRs, mineralocorticoid receptors; S.H., superior hypophysial. [taken and adapted with kind permission by (Turnbull and Rivier, 1999)]

Notably, cholesterol can further be derived from three different sources: (1) it can selectively be taken up from plasma high density and low density lipoproteins (LDL) via the scavenger receptor class B type 1 (SR-B1), (2) it can be recruited from LDL taken up via the LDL receptor (LDLR) and (3) it can be synthetized *de novo* within the adrenal gland from acetyl coenzyme A via the rate-limiting enzyme hydroxymethylglutaryl coenzyme A reductase (HMGCR) (Kraemer, 2007).

Subsequently, GC hormones are immediately released by diffusion from the medial zone (zona fasciculata) and inner zone (zona reticularis) of the adrenal cortex into the systemic circulation (Rosol et al., 2001; Gorrigan et al., 2011). Although mainly stimulated by ACTH, GC release is also promoted by other hormones produced in the adrenal medulla, such as epinephrine (Bremner et al., 1996). GCs belong to the family of steroid hormones and are small lipophilic substances, i.e. they can cross the blood-brain-barrier by passive diffusion through endothelial cells (Oren et al., 2004). Of note, GC efficacy is regulated by corticosteroid binding globulin (CBG) activity. CBG is a plasma carrier protein that binds GCs and, thus, regulates the amount of free GCs acting on target tissues. Consequently, alterations in the CBG level might affect the availability of GCs to enter the brain (Gardill et al., 2012).

The principal endogenous GCs are cortisol and corticosterone (CORT). Even though both steroid hormones are produced by most mammalian organisms, the ratio in which they are secreted varies from species to species. In humans, cortisol is the predominant GC (ratio cortisol to CORT 13:1), while rodents almost exclusively produce CORT. Once released, circulating GCs modulate the expression of approximately 10 % of our genes and exert widespread actions in the body which are essential for the maintenance of homeostasis and enable an organism to prepare for, respond to, and cope with physical and emotional stress (Sapolsky et

al., 2000; Buckingham, 2006). For instance, they are implicated in the mobilization of energy stores as well as immune and cardiovascular functions (Sapolsky et al., 2000). Furthermore, they potentiate numerous sympathetically mediated effects like peripheral vasoconstriction (Ulrich-Lai and Herman, 2009).

GCs exert their actions principally via intracellular receptors which belong to the nuclear receptor superfamily and regulate gene transcription. According to their affinity for GCs and their distribution, two distinct receptor types, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) have been characterized (Buckingham, 2006). Both receptors are expressed in the brain and periphery with some overlap. While the MR is mainly expressed in limbic brain structures, the GR is distributed all over the brain and most abundant in the hypothalamus and pituitary. By binding to these receptors, secreted GCs exert negative feedback inhibition, intended to reset the activated HPA axis and restore homeostasis (Nader et al., 2010). The major sites for this GC-mediated negative feedback loop are the PVN (Weiser et al., 2011), the anterior pituitary (Miller et al., 1992), and the hippocampus (Feldman and Weidenfeld, 1999) (see Fig. 5). In contrast, the SCN is one of the rare brain areas that does not seem to express either the GR or the MR (Cintra et al., 1994; Kalsbeek et al., 2011) (see section 1.3.2.1). The MR has an approximately 10-fold higher affinity than the GR and, thus, is bound even at low levels of circulating GCs, such as those that occur during the circadian nadir of GC secretion. As GC levels rise, either during stress responses or as a function of the HPA axis circadian cycle, MRs are saturated and GRs become primarily occupied (Reul and de Kloet, 1985). In their inactive state, these receptors exist in the cytosol as part of a multiprotein complex, which consists of one receptor molecule and several heat shock proteins. GCs passively diffuse through the cellular membrane

and upon binding to their receptors a conformational change of the receptor takes place, promoting the translocation from the cytoplasm into the nucleus (Picard and Yamamoto, 1987). Within the nucleus, these ligand-activated receptors either interact as monomers with other transcription factors or bind to the DNA on glucocorticoid-responsive elements (GRE) on target genes as monomers or homo-/heterodimers, thus affecting gene transcription (Raison and Miller, 2003). These biological actions are generally slow in onset and persist for some time after the steroid has been cleared from the circulation. Although GC actions are effected mainly by changes in gene transcription, some rapid GC-mediated effects within seconds to minutes cannot be explained this way. This implies the existence of non-genomic signaling, which probably involves the action at the target cell membrane. So far, it is not fully elucidated whether these effects are mediated via the common MR and GR or by a yet unidentified membrane GC receptor (Buckingham, 2006; Ulrich-Lai and Herman, 2009).

1.2.3. Acute vs repeated/chronic stress

Usually, the term stress is associated with negative feelings and reactions that accompany threatening and challenging situations. Paradoxically, a certain amount of stress is actually crucial for survival. An acute stress response results in various alterations concerning behavior (e.g. increased attention) and physiology (e.g. accelerated heart rate, inhibition of digestive activity), which improve the ability of an organism to adjust homeostasis in the face of a challenge and increase its chance for survival (Tsigos and Chrousos, 2002). Therefore, the term “allostasis” was coined by Sterling and Eyer in 1988 to describe the active processes of the body to reinstate homeostasis, mediated by activation of the SNS and HPA axis (McEwen,

2000). These processes are generally considered as beneficial and adaptive and increase the biological fitness of an individual.

As outlined in the beginning of section 1.2, stress can be classified into acute and chronic stress, whereupon predictability and controllability as well as duration and intensity of the stressor are a matter of concern. According to Dhabhar, acute stress is defined to last for a period of minutes to hours, while chronic stress persists for days to months (Dhabhar, 2000). Chronic stress may result from continuously applied stressors or repeated intermittent application of an acute stressor over a longer time period (e.g. restraint once daily for 7 days). Intermittently applied stressors may be further classified as predictable (i.e. the same stressor at each interval) or unpredictable (variable stressors) (Everds et al., 2013). Acute stressor exposure evokes a strong increase in plasma catecholamine concentration reaching peak levels within 1 – 5 minutes, whereas peak GC levels in the blood occur within 15 – 30 minutes and decline slowly to pre-stress levels 60 – 90 minutes later (Armario et al., 2004; de Kloet et al., 2005; Keeney et al., 2006). In contrast, sustained elevated plasma CORT levels were reported following chronic social defeat (SD) stress (Bartolomucci et al., 2005; Keeney et al., 2006), repeated foot shock (Kerr et al., 1991) or restraint stress (Zelena et al., 1999). Such prolonged elevations in plasma GC levels may have detrimental effects on health (Chrousos, 2009; Karatsoreos et al., 2010) and may contribute to the course and onset of somatic disorders, such as cardiovascular disease (Manenschijn et al., 2013) and breast cancer (van der Pompe et al., 1996). Additionally, a hyperactive HPA axis, characterized by increased concentrations of cortisol in plasma, urine, and cerebrospinal fluid, exaggerated adrenocortical response to exogenous ACTH, and

enlarged pituitary as well as adrenal glands, is one of the most consisting findings in depressed patients (Buckingham, 2006).

Yet, plasma GC levels do not necessarily increase after repeated or chronic stressor exposure. Repeated exposure to the same (homotypic) stressor is often associated with a gradual decline of the magnitude of HPA axis response and sympathetic adrenomedullary activity to the stimulus, a process called habituation or desensitization. Many facets of the stressful stimulus can modify HPA axis habituation, such as stressor controllability and predictability (Grissom et al., 2007). The decline in physiological responses to a familiar innocuous stimulus protects an organism from the potentially health-threatening effects of hypercorticism (Armario et al., 2004), and is consistently observed in animals exposed to repeated restraint (Natelson et al., 1988), cold (Bhatnagar and Meaney, 1995), or noise stress (Armario et al., 1984). Generally, habituation seems to depend upon the characteristics of the stressor and only occurs if the homotypic stressor is of non-social nature (Tornatzky and Miczek, 1993). Importantly, prior habituation to a homotypic stressor does not reduce the responsiveness to a different stressor (heterotypic stimulus), an important feature enabling an adequate response to a novel and potentially life-threatening challenge (Scribner et al., 1993; Ma and Lightman, 1998). So far, the mechanism underlying the adaptation of HPA axis responses to familiar, and sensitization to novel, stressors are poorly understood, but they may result from changes at the level of the adrenal gland (Uschold-Schmidt et al., 2012), altered negative feedback on HPA activity, or alterations in pituitary CRH-R1 and AVP-R1b (Aguilera, 1994).

The consequences of repeated or chronic stressor exposure in general, and habituation of HPA axis activity in particular, can vary based on individual

differences in both animals and humans. It is well established that individuals differ from each other with regard to the perception and psychological consequences of adverse life events (Gillespie et al., 2009). In particular, it is assumed that environmental variables, such as stressful experiences and predisposing genetic factors interact and influence the resistance/vulnerability to develop psychiatric disorders by mediating adaptive or maladaptive stress-coping strategies (Gillespie et al., 2009; Razzoli et al., 2011). For instance, an interaction between early life stress and a single nucleotide polymorphism in the human BDNF gene was shown to predict increased anxiety and elevated neuroticism (Gatt et al., 2009). Moreover, by using a knock-in mouse model of this particular polymorphism, an increased stress response has been associated with this specific DNA sequence variation (Yu et al., 2012).

1.2.4. Psychosocial stress

One problem with the use of repeated stress paradigms, such as foot shock or restraint, is that they do not really reflect the chronic situation experienced by humans. Moreover, most of the challenges and threats human beings face in today's highly-demanding society are related to the way they interact with each other. Thus, it is probably not far out to state that social interactions are one of the most important sources of stress in human life. This may range from competition for social rank or quarrels in the family up to workplace bullying (Sgoifo and Meerlo, 2002). Complementary, Koolhaas et al. declared social stress as the probably most potent and naturalistic type of challenges (Koolhaas et al., 1997). Besides the social ones, psychological stressors are the most frequent and naturalistic types we are exposed to in daily life. Psychological stress can be caused by environmental

demands that exceed an individual's capacity to deal with, for instance loss of employment or victim of a natural disaster (Cohen et al., 2007). As insinuated by its name, psychosocial stress comprises the two most important aspects of stress in human life, the social and the psychological one.

Hence, repeated/chronic psychosocial stress paradigms are of clinical relevance and represent the most promising approach in animal research to unravel the molecular basis for stress-promoted pathologies since they more accurately reflect the human situation (Bartlang et al., 2012). One such ethologically relevant animal model is SD which has been suggested as a potential rodent animal model for social stress-induced depression (Kudryavtseva et al., 1991; Berton et al., 2006; Rygula et al., 2006). SD can be accomplished by forcing the experimental male animal (intruder) to intrude into the territory of an aggressive and unfamiliar male conspecific (resident), leading to subordination of the intruder (Kudryavtseva et al., 1991). SD can be observed in many species that interact with conspecifics and whose social life organization is mainly determined by aggressive relations, for instance the house mouse (Anderson and Hill, 1965). According to traditional definitions, repeated SD would represent a recurrent homotypic stressor. As mentioned above, repeated SD does not result in adaptation and, thus, rather represents a heterotypic stressor for the defeated animal due to the behavioral differences displayed by the varying dominant residents it is exposed to (Sgoifo et al., 2002). A standardized protocol for repeated SD stress in mice implies the physical separation of the intruder and resident by a perforated partition wall after the attack by the resident and the ensuing submission shown by the intruder (Berton et al., 2006; Golden et al., 2011; Lehmann and Herkenham, 2011). This physical separation seems to create an uncontrollable situation for both animals since the winner is unable to exclude

the defeated conspecific and the loser is unable to escape from the aggressor (Koolhaas et al., 2011).

The impact of the augmentative psychosocial stressor becomes clear by means of the persistent neurobiological and behavioral effects even after one single SD exposure. For example, Marini et al. found an increased CRH and GR mRNA expression in the rat hippocampus following a single exposure to SD (Marini et al., 2006). In another study, neurotrophin expression in brain cortical and subcortical areas was reduced in mice after an acute SD (Pizarro et al., 2004). Intriguingly, a single or double defeat episode can produce profound alterations in the daily rhythms of heart rate, body temperature, and locomotor activity (Tornatzky and Miczek, 1993; Meerlo et al., 1996b). When encountered on a repeated or chronic basis, SD induces a spectrum of behavioral, physiological, endocrine, and immunological changes. To name a few, repeatedly subordinated mice are characterized by a long-lasting aversion to social contact (Berton et al., 2006) as well as anxiety- (Kinsey et al., 2007) and depressive-like behavior (Lutter et al., 2008a). Moreover, these repeated stressful conditions have well-established consequences on rodent physiology, namely enlarged adrenal glands as well as altered HPA axis activity and body weight gain (Zelena et al., 1999). Further hallmarks of repeated/chronic SD are changes in immune responses (Gryazeva et al., 2001), core body temperature and home cage locomotor activity (Keeney et al., 2001). In addition, intermittent SD on 10 alternate days induces a significant, long-lasting reduction in the amplitude of the heart rate circadian rhythm.

1.3. INTERACTIONS BETWEEN THE CIRCADIAN AND THE STRESS SYSTEM

At first glance, the circadian and the stress system seem to represent two separate bodily control systems that are involved in adaptation to predictable and unpredictable stimuli, respectively. However, both systems are fundamental for survival, and thus, communicate with each other at various physiological and neuronal levels. Since the day/night changes are more general and happen continuously, the circadian system controls the stress system, whereas the latter adjusts the circadian rhythmicity of subsidiary peripheral clocks in response to different stressors (Nader et al., 2010).

This issue will be further discussed in the subsequent sections, focusing on the circadian GC rhythm and GC-induced changes in central and peripheral clocks.

1.3.1. The circadian release of glucocorticoids

As outlined in section 1.2.2, plasma concentrations of GC hormones follow a circadian rhythm under baseline conditions. Moreover, these circadian GC level changes were also verified in feces (Cavigelli et al., 2005) and in the hippocampus (Droste et al., 2008) in rats.

The daily fluctuation is usually locked to the environmental transition from dark-to-light or vice versa, and waking-up is preceded by a substantial surge in the release of these steroid hormones in all species. It is assumed that the increasing GC levels at awakening prepare the body for the upcoming activity phase, i.e. for a period of increased probability to encounter stressors or for increased energy expenditure during foraging (Kalsbeek et al., 2011; Dickmeis et al., 2013). Importantly, the daily

plasma GC rhythm is remarkably stable and persists when volunteers are subjected to constant bed rest or sleep deprivation for one to two nights (Kalsbeek et al., 2011). However, a flattening of the circadian GC rhythm following chronic stress has been observed in humans (Miller et al., 2007) and rodents (Sterlemann et al., 2008), suggesting a susceptibility of the circadian clock to certain environmental stimuli.

The circadian GC rhythm is not made up of a simple smooth change in hormone levels over 24 h, but rather the result of changes in the activity of an underlying ultradian ("less than a day") rhythm (Windle et al., 1998; Walker et al., 2012). These pulses occur with a relatively constant, roughly hourly, frequency (the rate of hormonal release), whereas the pulse amplitude (the amount of hormonal release) is variable. The active phase-related rise of GC secretion results from increases in the amplitude of the pulses, being maximal just before waking and declining thereafter to reach a trough early in the sleep phase (Buckingham, 2006; Kriegsfeld and Silver, 2006). Besides the circadian input, homeostatic and stress-related signals impact the amplitude of these secretory episodes.

Several studies indicate that the pulsatility of GC secretion appears to be important for the appropriate stress response (Windle et al., 1998; Lightman et al., 2008). In this regard it could be demonstrated that the time of stressor application determines the physiological response, depending on the phase of an endogenous basal pulse. While rats exposed to white noise for 10 min responded with an additional release of CORT when endogenous basal CORT levels increased just prior to stressor onset, no or only little CORT responses were detectable in animals in which basal CORT levels were falling at stressor initiation (Windle et al., 1998). This indicates that the basal GC pulsatility interacts dynamically with the ability of an organism to mount a stress response (Lightman et al., 2008). Termination of the

rising phase of the GC pulse by rapid feedback inhibition may be essential in the generation of such pulses. It is assumed that during the secretory phase of a pulse the fast rising GC causes a rapid feedback on ACTH release from the pituitary that results in the inhibitory phase of the pulse, which clears the GC according to its half time. This inhibitory phase is short lasting, thus allowing a new pulse to be initiated within a short time-frame (Lightman et al., 2008).

The daily GC rhythm has been of greatest importance in the history of the circadian timing system and is based on a multimodal regulation: (1) circadian changes in HPA axis activity, including changing levels of ACTH, and probably CRH, are under control of the SCN via its projections to the PVN (Dickmeis et al., 2013); (2) clock control over GC secretion operates via the sympathetic input to the adrenal gland, thereby modulating the sensitivity of the target organ to the incoming ACTH message (Ulrich-Lai et al., 2006a); and (3) the peripheral autonomous adrenal clock itself regulates genes involved in steroidogenesis as well as circadian ACTH sensitivity (Oster et al., 2006b).

It seems that the SCN control over GC secretion does not primarily operate through the HPA axis but rather via mechanism (2) and (3) (Dickmeis et al., 2013). However, each of these paths might dominate under different conditions: In constant darkness (DD), the adrenal clockwork appears to be essential for circadian CORT production in mice, while under normal light-dark (LD) conditions light information, probably transmitted via the splanchnic nerve, is capable to regulate daily changes in GC production even in adrenals lacking a functional clock (Son et al., 2008; Dickmeis et al., 2013).

In the following sections the three mechanisms are explained in detail.

1.3.1.1. Suprachiasmatic nucleus control over the hypothalamic-pituitary-adrenal axis

The above-mentioned rhythms of circulating GCs are paralleled by similar rhythms in ACTH, albeit less robust and of lower amplitude (Carnes et al., 1988; Carnes et al., 1989). Moreover, CRH synthesis seems to be under circadian control, since a diurnal variation in CRH mRNA has been shown in the PVN of rats (Watts et al., 2004; Girotti et al., 2009). However, it has to be noted that the ACTH release precedes the onset of CRH gene transcription substantially, implicating that these two processes might not necessarily be coupled to each other (Dickmeis, 2009).

Lesioning the SCN abolishes rhythms in GC and ACTH release (Moore and Eichler, 1972; Cascio et al., 1987), implying that the circadian aspect of the HPA axis is under control of the master circadian pacemaker. Indeed, the SCN conveys excitatory and inhibitory circadian pacemaker activity to the medioparvocellular PVN, where the CRH- and AVP-expressing neurons, projecting to the median eminence, are located (Sawchenko et al., 1984; Engeland and Arnhold, 2005; Dickmeis, 2009). The SCN targets the PVN either through direct connections or by innervating neighboring areas of the PVN, such as the sub-paraventricular zone (subPVZ) or the DMH, that contact the hypophysiotropic neurons as well (Dickmeis, 2009). These relay stations also provide a good explanation for the underlying mechanism of the 12 h reversal in plasma GC rhythms in nocturnal and diurnal species despite a similar timing of clock gene expression, neural activity, and transmitter release by the SCN. In both species AVP is released during the light phase. In the rat, AVP inhibits CRH-containing neurons in the PVN by contacting GABAergic interneurons in the subPVZ and DMH, thus suppressing CORT release during the light (inactive) phase. In contrast, in diurnal species AVP release during the light phase stimulates CRH-

containing neurons in the PVN since it acts on glutamatergic, instead of GABAergic, interneurons in the subPVZ and DMH, thereby promoting cortisol release during the light (active) phase (Kalsbeek et al., 2012). However, the circadian release pattern of AVP cannot account solely for the daily CORT rhythm in rodents. Despite the absence of an inhibitory AVP signal in the second half of the dark phase, basal CORT release is low, evoking sort of a mismatch situation between the daily CORT rhythm and the circadian AVP release (Kalsbeek et al., 1996). Thus, the existence of an additional stimulatory factor involved in the circadian control of the basal CORT release is proposed, probably involving neuromedin U (Kalsbeek et al., 2006).

1.3.1.2. The autonomic nervous system – an alternative pathway of circadian control

The robust CORT rhythm despite a modest rhythm in plasma ACTH suggests that a non-ACTH factor contributes to the circadian changes in adrenal activity (Ulrich-Lai et al., 2006a). In support, hypophysectomised rats still show rhythms in CORT secretion despite the absence of cycling ACTH levels (Meier, 1976). Considering the aforementioned close connection between the adrenal gland and the sympathetic branch of the ANS, an involvement of the SNS in this ACTH-independent connection has been proposed. Retrograde tracing experiments could confirm autonomic projections from the SCN to the adrenal gland via two relay stations. Pre-autonomic neurons in the PVN display the first relay station, which contact sympathetic pre-ganglionic neurons in the IML that, in turn, innervate the adrenal gland through the splanchnic nerve (Buijs et al., 1999). Notably, this pathway apparently does not overlap with the SCN connections to the PVN via the subPVZ and DMH outlined in section 1.3.1.1, indicating a separation of the autonomic from the HPA axis-targeted

route of SCN control at the level of the PVN (Dickmeis, 2009). Two lines of evidence support an involvement of the autonomic SCN-adrenal pathway in the diurnal control of GC secretion.

First, elaborate studies by Edwards (1987) and Engeland (1989) showed that electrical stimulation of the splanchnic nerve in different species increases the sensitivity of the adrenal cortex to ACTH (Edwards and Jones, 1987; Engeland and Gann, 1989). Vice versa, adrenal denervation abolished both the circadian GC rhythm (Jasper and Engeland, 1994) and the diurnal change in ACTH sensitivity (Ulrich-Lai et al., 2006a). Thus, it seems that the autonomic projections from the SCN via the PVN to the IML determine a time window during which the adrenal gland is most responsive to ACTH (Buijs et al., 1999; Kalsbeek et al., 2011). This neuronal SCN-adrenal gland connection represents an anatomical basis for multiple early studies that reported a circadian rhythm in the responsiveness of the HPA axis to stress (Zimmermann and Critchlow, 1967; Gibbs, 1970). Noteworthy, the type of stressor appears to be important in these responses: In rats, psychological stressors, such as novel environment or restraint elicit the largest response early during day (i.e. the inactive phase), whereas physical stressors like hypoglycemic shock do so at the onset of activity. One presumable reason for these differences might be that physical stress is relayed to the PVN mainly via the brain stem, while psychological stress information requires the interpretation from higher brain centers involving the limbic system. The SCN might differentially interfere with these signals from different brain areas by enhancing input from psychological stressors during day and inhibit physical stress input at the same time (Jacobson, 2005; Dickmeis, 2009).

Second, sympathetic innervation of the adrenal gland directly transmits photic information to the adrenal and leads to increased CORT release in mice,

independent of HPA axis activation (Ishida et al., 2005). Epinephrine release from the adrenal medulla by splanchnic innervation might be responsible for transmission of the photic signal to the adrenal cortex. Vice versa, adrenal denervation blocks the light-induced CORT release and abolishes photic induction of *Per1* in the adrenal cortex (Ishida et al., 2005; Son et al., 2011).

1.3.1.3. The adrenal peripheral clock

In addition to the rhythmic HPA axis activity and the neural connections described above, a third component contributes to the circadian control of GC secretion: The peripheral clock of the adrenal gland itself. By using adrenal slice cultures Oster et al. could show a stronger corticoid response following ACTH treatment in the evening (when the natural GC peak would occur) compared to the morning in wildtype (WT) mice. This effect was absent in adrenal cultures from *Per2/Cry1* double-mutant mice, which lack a functional molecular clock (Oster et al., 2006b). Transplantation studies from the same group revealed that an intact adrenal clock can sustain GC rhythmicity and ACTH sensitivity even in the absence of a functional SCN pacemaker (Oster et al., 2006b). Therefore, it is likely that the autonomous adrenal gland clock directs ACTH input into GC synthesis to a certain time of day (Kalsbeek et al., 2011).

Interestingly, differences regarding expression levels and phasing of cycling patterns of circadian clock gene mRNA in the adrenal gland were found between the adrenal medulla and the cortex (Dickmeis, 2009). While all clock genes, except for *Clock* and *Cry2*, exhibit a robust circadian rhythm in expression in the adrenal cortex (Oster et al., 2006b), the medulla merely expresses *Per1*, *Per3*, *Cry2*, and *Bmal1* rhythmically (Bittman et al., 2003; Torres-Farfan et al., 2006). Thus, the GC-producing layers of

the cortex are probably the main site of the intrinsic adrenal pacemaker. Besides the circadian expression of clock genes, many other genes oscillate in a diurnal fashion in the adrenal gland. Amongst these, the adrenal Mc2r itself and further downstream components exhibit circadian mRNA expression (Oster et al., 2006a). Moreover, genes associated with cholesterol biosynthesis and transport, such as the mitochondrial cholesterol transporter StAR, are also expressed rhythmically (Son et al., 2008; Girotti et al., 2009).

It is becoming increasingly evident that, apart from its role in gating ACTH sensitivity, the adrenal clock is critically involved in (re)synchronization processes of the circadian system following phase-shifts (Kalsbeek et al., 2011). For instance, adrenalectomized rats exhibit an accelerated re-entrainment rate to a shifted LD cycle (Sage et al., 2004). Moreover, re-entrainment of hormonal and locomotor rhythms were significantly accelerated in mice lacking an adrenal clock (Kiessling et al., 2010), further highlighting the adrenal gland in the capacity of regulators of behavioral adaptation to phase-shifts.

1.3.2. Stress affects the circadian clock

As outlined in section 1.2.4, circadian rhythms at the output level are strongly affected by acute and chronic SD. Furthermore, acute noise stress in rats flattens the circadian CORT rhythm and increases the frequency of ultradian pulses (Windle et al., 2001; Lightman et al., 2008). Several studies reported altered rhythms of sleep (Moreau et al., 1995), body temperature (Ushijima et al., 2006), locomotor activity (Gorka et al., 1996), and hormone secretion (Dubovicky et al., 2007; Maninger et al., 2010) following chronic mild, shaker, and restraint stress, respectively. These rhythm disturbances represent an imbalance between normally

precisely orchestrated physiological and behavioral processes and presumably render an individual susceptible to the development of pathological somatic (Knutsson, 2003) and mood changes (Meerlo et al., 1997). Alterations in the output of the circadian system may be attributable to the biological clock in the SCN or might arise from stress-induced changes in peripheral sub-oscillators. Arguments for either possibility will be brought forward in the sections below.

1.3.2.1. Stress effects on the master clock

In contrast to peripheral clocks, the central circadian pacemaker seems to be rather insensitive to GCs since neither MR nor GR expression could be detected in the SCN (Balsalobre et al., 2000), as aforementioned. Although social as well as non-social stressors alter the shape and amplitude of circadian output rhythms, this may not necessarily reflect changes in the central oscillator in the SCN. Early studies already indicated that acute SD does not perturb the central oscillator in the SCN, although its output is strongly masked (Meerlo et al., 1997; Meerlo and Daan, 1998). Masking describes a phenomenon, whereby a circadian response, such as locomotor activity is obscured, but not altered, by an exogenous factor. This enables an organism to respond immediately and in an appropriate way to environmental changes (Aschoff, 1960). In line with the reports for SD, a chronic stress protocol including forced swimming, restraint, and social stress did not affect the central oscillator but altered the amplitude of the activity rhythm in mice (Solberg et al., 1999). Furthermore, 10 days of repeated restraint stress did not affect the PER2 protein expression in the rat SCN, whereas the pattern of the daily locomotor activity was disrupted (Robison, 2005).

Although the above-mentioned studies demonstrate that the central circadian pacemaker is not affected by stress, other studies have suggested that in certain cases a stress signal may reach the SCN. For instance, water immersion and forced swimming for 10 min induced an increased release of AVP within the SCN (Engelmann et al., 1998). Moreover, exogenous GCs enhanced AVP mRNA expression in the SCN (Larsen et al., 1994), indicating that a GC signal can be sensed by the master clock, probably via intermediary GR-containing brain areas like the PVN, DMH or the raphe nucleus (Malek et al., 2007; Kiessling et al., 2010). In support, a study by Jiang et al. showed a reduction in amplitude of PER2 oscillation in the rat SCN following four weeks of chronic unpredictable stress (Jiang et al., 2011). Further, the same stress paradigm decreased CLOCK and BMAL1 protein expression in the SCN (Jiang et al., 2013). Similarly, repeated restraint stress over seven days decreased PER2 protein expression in the mouse SCN (Kinoshita et al., 2012), while acute exposure to predator scent stress resulted in an up-regulation of PER1 and PER2 protein in the SCN of male rats (Koresh et al., 2012). Together, these studies provide evidence that certain stressors are capable of perturbing core clock components in the SCN. Variations in these results may be assignable to the different animal models used, the type of stressor, as well as duration of stressor exposure.

While further investigation is clearly warranted to shed light on the mechanisms underlying the transmission of the GC signal to the central pacemaker, a direct effect of GCs on peripheral clocks is beyond doubt.

1.3.2.2. Glucocorticoid receptors – a checkpoint for the interaction between glucocorticoids and the circadian system at peripheral target tissues

In section 1.1.1.1, GCs were proposed to be attractive candidates for the entrainment of peripheral clocks on a molecular level. Such GC-mediated resetting of peripheral oscillators may be particularly important in acute stressful situations since organisms need to adjust the circadian rhythm-linked activity of their bodies to properly respond to stressors (Nader et al., 2010). For this reason, clock genes, such as *Per1*, and possibly *Per2*, possess a GRE in their promoter region (Balsalobre et al., 2000; So et al., 2009), implying that up-regulation of GCs by activation of the HPA axis might directly affect peripheral clocks in almost all organs and tissues. By binding to the GRE ligand-activated GRs/MRs can phase-shift the expression of several clock genes and clock-related genes, leading to the resetting of circadian rhythms (Nader et al., 2010). In support, the GC hormone analog dexamethasone was shown to induce the expression of *Per1* mRNA in cultured rat fibroblasts (Balsalobre et al., 2000) as well as *Bmal1* and *Clock* mRNA in neutrophils and lymphocytes (Nebzydoski et al., 2010). Likewise, mice exposed to acute restraint stress in order to raise GC levels exhibited elevated *Per1* mRNA expression in peripheral organs, such as the liver, heart, and kidney (Yamamoto et al., 2005). On the contrary, the transcription of *Rev-Erbα* and *Rorα* has been shown to be repressed by GCs (Torra et al., 2000). In any case, the GR-devoid SCN is proposed to reset peripheral clocks to their original phase following termination of the acute stressful situation (Balsalobre et al., 2000; Nader et al., 2010). In contrast, chronic stress might lead to a permanent desynchronization between the SCN and the clocks in peripheral tissues, rendering an organism more susceptible to the

development of diseases affecting the mood and the body (Albrecht, 2010). In line, chronic mild stress in BALB/c mice altered the rhythmic expression of core clock genes, such as *Clock*, *Bmal1*, *Per1*, and *Cry1* in the liver, but not the SCN, thereby feasibly contributing to metabolic disorders like insulin resistance (Takahashi et al., 2013). A recent study showed that the PER2 protein expression in the pituitary and adrenal gland was phase-advanced following chronic subordination stress, a stress model resulting in a complex syndrome consisting of autonomic, immune-endocrine, metabolic, and cognitive dysfunction (Razzoli et al., 2014).

In addition, direct interactions between clock factors and the GR at peripheral target tissues may occur. Indeed, CLOCK can acetylate the GR and thereby inhibit its transcriptional activity, possibly by reducing its ability to bind to the DNA (Nader et al., 2010). Furthermore, CRYs were also reported to prevent GR function via direct binding, resulting in a decreased transactivation potential on a GRE-controlled luciferase reporter gene (Lamia et al., 2011).

1.4. CONCLUDING REMARKS

In conclusion, the circadian system and the stress system are two highly conserved and interrelated regulatory networks aimed to ensure homeostasis. Although one might think that evolution has buffered the circadian system to unpredictable stressful stimuli, several studies applying different kinds of stressors demonstrated alterations at the level of the central circadian pacemaker and the peripheral clocks, as well as at the output level. However, stress-induced disturbances in peripheral rhythms in physiology and behavior do not necessarily reflect changes in the central clock but may rather be the result of shifted subsidiary peripheral clocks or due to masking effects. For instance, the shape of the body temperature rhythm can be modified by several exogenous and endogenous factors, independent of the clock, such as ambient temperature, activity and sleep or nesting material (Meerlo et al., 2002). In order to draw conclusions on the state of the central circadian pacemaker following stressor exposure further studies under constant, i.e. “free running”, conditions are required to measure intrinsic features of the SCN, such as period and phase.

On the other hand, the exact coordination of the multiple mechanisms involved in rhythmic GC activity and the question of how GC target cells sense and process this rhythmic information still remains to be fully elucidated.

In summary, further studies are required to understand the exact molecular interactions between these two systems and their interplay in the development of human pathology to resolve the outstanding issues.

1.5. AIM OF THE THESIS

The general aim of this PhD project was to investigate mutual interactions between psychosocial stress and the circadian clock. The overall objective is to gain insight into the time of day dependent effects of repeated stressor exposure on (1) behavioral, physiological/endocrine, and immunological/inflammatory and (2) peripheral and central clock parameters.

With respect to objective (1), it was intended to characterize the consequences of repeated SD during the dark/active and light/inactive phase on different behavioral parameters, such as home cage activity and social avoidance, physiological/endocrine parameters, such as body and adrenal weight, food intake, plasma ghrelin and leptin, plasma ACTH and CORT, adrenal *in vitro* ACTH sensitivity, as well as immunological/inflammatory parameters, such as cytokine secretion from mesenteric lymph node cells and severity of a subsequent pharmacologically-induced colitis. This issue is addressed in paper 1 (Bartlang et al., 2012) and in sections 3.5 and 3.6.

The second aim of this thesis was to characterize the effects of repeated SD during the dark/active and light/inactive phase on various rhythms *in vivo* (locomotor activity, body temperature) and *in vitro* (PER2 oscillations in the SCN and the peripheral adrenal gland). Part of this topic also includes the PER2-immunolabelling in the SCN. This issue is addressed in paper 2 (Bartlang et al., 2014) and paper 3 (Bartlang et al., submitted).

An important contribution to the present thesis is further the establishment of different techniques to enable the investigation of the circadian CORT rhythm: Assessment of the hippocampal CORT rhythm by *in vivo* microdialysis and assessment of *in vitro* CORT secretion by adrenal explants.

The performance of these techniques are described and discussed in detail in section 4.2. Furthermore, I initiated a collaboration with Prof. Dr. Henrik Oster from the University of Lübeck to assess the circadian CORT rhythm by non-invasive fecal CORT metabolite measures. This is an ongoing project and not part of the present thesis.

CHAPTER 2

MATERIAL AND METHODS

In the following, only the materials and methods are described which cannot be obtained from the materials and methods sections of the published articles (Bartlang et al., 2012, see full text version starting from page 152 and Bartlang et al., 2014, see full text version starting from page 165) and the submitted manuscript (see full text version starting from page 177).

2.1. MATERIAL

2.1.1. Antibodies for protein and immunohistochemical analysis

2.1.1.1. Primary antibodies

DENOTATION	COMPANY	SPECIES RAISED IN
MCR (H-300) sc-11412	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	rabbit polyclonal
AVP Receptor V3 (D-20) sc-18105	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	goat polyclonal
CRH-R1 (V-14) sc-12381	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	goat polyclonal
HMGCR (H-300) sc-33827	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	rabbit polyclonal
β-tubulin #2146	Cell Signaling Technology, GmbH, Frankfurt am Main, Germany	rabbit polyclonal
HSL #4107	Cell Signaling Technology, GmbH, Frankfurt am Main, Germany	rabbit polyclonal
Anti-Scavenging Receptor SR-B1 ab24603	Abcam, Cambridge, UK	rabbit polyclonal

DENOTATION	COMPANY	SPECIES RAISED IN
Anti-LDL Receptor ab30532	Abcam, Cambridge, UK	rabbit polyclonal

2.1.1.2. Secondary antibodies

DENOTATION	COMPANY	SPECIES RAISED IN
Anti goat (HRP-linked) sc-2020	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	donkey
Anti rabbit (HRP-linked) # 7074	Cell Signaling Technology, GmbH, Frankfurt am Main, Germany	goat

2.1.2. Protein extraction and western blotting

PRODUCT	COMPANY
Complete mini protease inhibitor	Roche Applied Science, Mannheim, Germany
Bicinchoninic Acid (BCA) Protein Assay Kit	Thermo Scientific, Rockford, USA
Enhanced Chemiluminescence Western Blotting Detection Reagent	GE Healthcare, Freiburg, Germany
PageRuler™ Prestained Protein Ladder Plus	Fermentas, Thermo Scientific, Rockford, USA
Re-Blot Plus Mild/Strong Solution	Millipore GmbH, Schwalbach, Germany
Nitrocellulose membrane, 0.45 µm	Bio-Rad, München, Germany

2.1.3. Neuroendocrine analysis

PRODUCT	COMPANY
Ghrelin ELISA Kit	LINCO Research, Missouri, USA
Leptin ELISA Kit	R&D Systems Ltd., Abingdon, UK
ACTH ELISA Kit	IBL International, Hamburg, Germany
CORT ELISA Kit	IBL International, Hamburg, Germany
EDTA tubes	Sarstedt, Nürnbrecht, Germany

2.1.4. In situ hybridization

PRODUCT	COMPANY
QIAquick Nucleotide Removal Kit	QIAGEN, Hilden, Germany
BioMax MR film	Kodak, Cedex, France
35S (sulfuric acid)	BIOTREND Chemikalien GmbH, Köln, Germany

2.1.5. Chemicals, enzymes, reagents and equipment

Chemicals, enzymes and reagents used in this thesis were obtained from Beckman Coulter (Krefeld, Germany), Biomol (Hamburg, Germany), Bio-Rad (München, Germany), BMG Labtech (Ortenberg, Germany), Braun (Melsungen, Germany), Carl Roth GmbH (Karlsruhe, Germany), Merck (Darmstadt, Germany), Sigma-Aldrich (Deisenhofen, Germany). Cell culture materials, general plastic material and other equipment were purchased from BD Biosciences (Heidelberg, Germany), Beckman Coulter (Krefeld, Germany), Eppendorf AG (Hamburg, Germany), GE Healthcare (Freiburg, Germany), Gibco® Life Technologies GmbH (Darmstadt, Germany), Leica Microsystems (Wetzlar, Germany), Sarstedt (Nürnbrecht, Germany), Thermo Scientific (Rockford, USA) and VWR GmbH (Darmstadt, Germany).

2.1.6. Software

SOFTWARE	PURPOSE	SOURCE
Endnote 9.0	Reference Management	Thomson
SPSS Statistics 21.0	Statistical analysis	IBM
Microsoft Office 2010	Text and data processing	Microsoft
GraphPad Prism 5	Graph processing	GraphPad Software
Leica QWin V3	Image analysis	Leica Microsystems
Leica FW 4000	Image analysis	Leica Microsystems

SOFTWARE	PURPOSE	SOURCE
NIH ImageJ 1.31	Image processing	http://rsb.info.nih.gov/ij/
Image Lab 4.1	Western Blot analysis	Bio-Rad Laboratories

2.2. METHODS

The first two paragraphs referring to mice and housing conditions and the SD paradigm are described in detail below since these sections are fundamental for the present thesis.

2.2.1. Animals

2.2.1.1. Mice and housing conditions

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19 – 22 g (experimental mice) were individually housed in standard polycarbonate mouse cages (16 x 22 x 14 cm) for one week before the start of the SD paradigm. CD1 male mice weighing 30 – 35 g were individually housed in polycarbonate observation cages (38 x 22 x 35 cm) and used as dominant animals (residents). All mice were kept under standard laboratory conditions (22 ± 2 °C, 60 ± 5 % humidity) and had free access to tap water and standard mouse diet.

Unless otherwise specified (mice used in paper 3; see section 3.3), all mice were exposed to a 12:12 h light-dark cycle (light phase: 200 ± 50 lux), whereas the time of lights-on is defined as *Zeitgeber* time (ZT)0 and lights-off as ZT12. All experimental protocols were approved by the Committee on Animal Health and Care of the local government and performed according to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their sufferings.

2.2.1.2. Social defeat paradigm

The SD paradigm was performed as described previously (Bartlang et al., 2012; Bartlang et al., 2014). Briefly, one week after delivery, experimental mice were weighed and assigned to a single-housed control (SHC), a social defeat light (SDL), and a social defeat dark (SDD) group in a weight-matched manner. SHC mice remained undisturbed in their home cage except for weighing and a weekly change of bedding. Based on previous studies, single housing is the adequate control group, as group housing itself was shown to be stressful for the mice (Singewald et al., 2009). SDL and SDD mice were exposed to SD daily for 2 h over 19 days (except for days 7 and 14), either at the beginning of the light phase (ZT1 – ZT3, SDL mice) or at the beginning of the dark phase (ZT13 – ZT15, SDD mice). During each SD session, the experimental mouse (intruder) was taken from its home cage and placed into the home cage (territory) of an aggressive and unfamiliar conspecific (resident) in order to induce a psychosocial stressful situation. After the first attack by the resident (see Fig. 6A) that resulted in submissive behavior from the intruder (see Fig. 6B), both mice were physically separated by a perforated partition wall (see Fig. 6C). This protected the intruder from physical injuries by the resident, but still allowed visual, olfactory, and auditory contact as described previously (Reber et al., 2006), thus generating an uncontrollable situation for the intruder. During the subsequent 2 h of sensory contact, food pellets, but not tap water, were available for all experimental mice including SHC animals. Following removal of the partition wall, direct social interaction between the resident and the intruder was allowed until the first attack by the resident that resulted in submissive behavior from the intruder again. Afterwards, the intruder was placed back into its home cage. To avoid habituation, each experimental mouse was exposed to a different resident

every day. SD was omitted on days 7 and 14 and home cage behavior of experimental mice was observed and scored at the beginning of the light phase (ZT0 – ZT3) and dark phase (ZT12 – ZT15) on days 0, 7, 14 and 20, respectively. The behavior was scored in 3 min time intervals, thereby distinguishing between locomotion, rearing, eating, drinking, grooming, digging, and climbing (active behavior) and inactive behavior. These 3 min scores were then summed up for the whole 180 min period per mouse and averaged per treatment group.

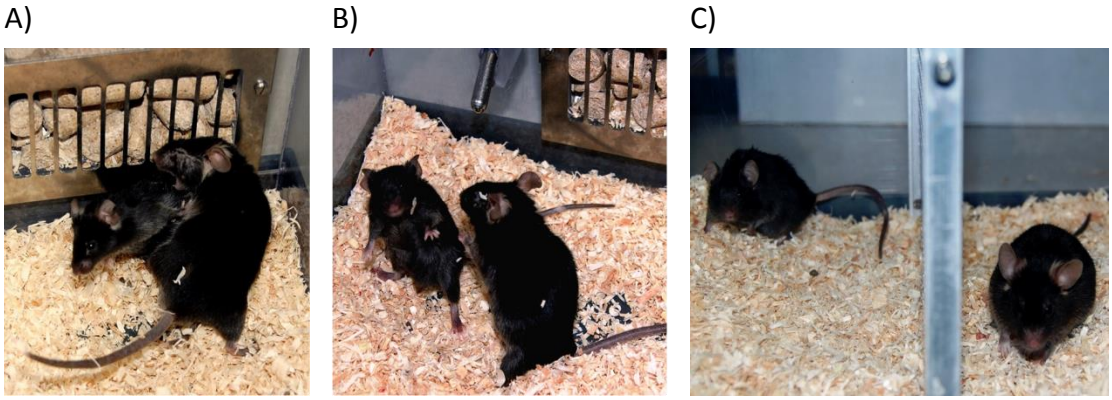


Figure 6: SD paradigm. The experimental mouse (intruder) was placed into the home cage of an aggressive and unfamiliar conspecific (resident). After the first attack by the resident (A, right mouse) that resulted in submissive behavior from the intruder (B, left mouse), both mice were physically separated by a perforated partition wall for 2 h (C). The pictures are mock scenes with male C57BL/6 mice used as resident and intruder.

2.2.1.3. Body weight development and food intake

To investigate whether the time of day of stressor exposure influences body weight development during the course of the 19 days of SD, SHC, SDL, and SDD mice were weighed daily in the afternoon between ZT7 and ZT8 from day 0 until day 20. In addition, another set of SHC, SDL, and SDD mice was weighed in the night between ZT19 and ZT20 from day 0 until day 3 (i.e. 3 days of SD exposure) to exclude that the time of weighing has an effect on body weight development.

Furthermore, to examine the daily food intake during the course of the 19 days of SD, the food was weighed daily between ZT7 and ZT8 until day 20, starting two days before the first stressor exposure. The food intake within 24 h was determined by subtracting the daily values from each other. To obtain more precise results, values for the basal (the two days before the first SD session), stress I (day 1 – day 6), stress II (day 8 – day 13), and stress III (day 15 – day 19) period of the SD paradigm were averaged.

2.2.1.4. Microdialysis

Implantation of microdialysis probes

On day 20 (i.e. one day after the last stressor exposure), experimental mice were anesthetized with isofluorane (Baxter GmbH, Unterschleißheim, Germany) and mounted on a stereotaxic frame. The self-constructed microdialysis probes (molecular cut-off: 18 kDa) were implanted stereotaxically within the right dorsal hippocampus by Rodrigue Maloumby (coordinates from bregma: 2.0 mm (lateral), 2.3 mm (posterior), 2.3 mm (ventral)) (Paxinos and Franklin, 1997; Paxinos and Watson, 1998). The probes were flushed and filled with sterile Ringer's solution (pH 7.4, Braun Melsungen AG, Melsungen, Germany). Two 5 cm long pieces of polyethylene tubing (PE 20, Karmann & Droll, Karlsfeld, Germany) filled with Ringer's solution were connected to the inflow and the outflow of the microdialysis probe and, together with two small stainless steel screws used for stabilization, fixed with dental cement (Kallocryl, Speiko-Dr. Speier GmbH, Münster, Germany). To avoid post-surgical infections, mice received 0.1 ml antibiotics (SC, Baytril® 2.5 % Bayer Vital GmbH, Leverkusen, Germany). After surgery, mice were individually

housed in experimental observation cages (38 x 22 x 35 cm). One day after surgery, mice were handled and familiarized with the experimental procedure.

Dialysate sampling and analysis

Two days after surgery (day 22), the microdialysis probe was connected via polyethylene tubing to a syringe mounted onto a microinfusion pump and perfused with sterile Ringer's solution (3.3 µl/min flow-rate, pH 7.4) starting at ZT0 to establish equilibrium between inside and outside of the microdialysis membrane. Two hours later (ZT2), microdialysis samples were collected in 60 min intervals under baseline conditions until ZT6. Then, mice were exposed to a swim stress for 5 min (water 21 °C). After the acute stressor exposure, mice were returned to their home cages and left undisturbed for the remainder of the experiment while sampling continued. After the swim stress procedure, dialysates were sampled between ZT6.5 and ZT14.5 in 60 min intervals again. Microdialysis samples were stored at -20 °C until being further processed for CORT quantification.

For CORT quantification, the dialysates were thawed on ice, lyophilized, and then dissolved in 15 µl phosphate buffered saline (PBS). Next, two dissolved samples were pooled each with (ZT2 – ZT4, ZT4 – ZT6, ZT6.5 – ZT8.5, ZT8.5 – ZT10.5, ZT10.5 – ZT12.5, ZT12.5 – ZT14.5) and analyzed undiluted using a commercially available ELISA kit for CORT (see 2.2.2.1).

2.2.2. Tissue extraction and preparation

2.2.2.1. Trunk blood collection and analysis of plasma ghrelin, leptin, corticosterone, and adrenocorticotropic hormone

SHC, SDL, and SDD mice were killed rapidly by decapitation either in the light phase (ghrelin and leptin: day 21, ZT5; CORT and ACTH: day 20, ZT1 – 3) or in the dark phase (ghrelin and leptin: day 20, ZT17; CORT and ACTH: day 20, ZT13 – 15). Therefore, each mouse was taken out from its home cage and was rapidly killed under CO_2 anesthesia within 3 min. Trunk blood was collected in EDTA-coated tubes (Sarstedt, Nürnbrecht, Germany) on ice and centrifuged at 4 °C (5000 rpm, 10 min). Plasma samples were stored at -20 °C until respective concentrations were assessed. Ghrelin, leptin, and ACTH plasma samples were analyzed using a commercially available ELISA kit for ghrelin (analytical sensitivity 0.02 ng/ml, intra-assay and inter-assay coefficients of variation ≤ 4.46 %, Linco Research, Missouri, USA), leptin (analytical sensitivity < 22 pg/ml, intra-assay and inter-assay coefficients of variation ≤ 7.6 %, R&D Systems Ltd., Abingdon, UK), and ACTH (analytical sensitivity 0.22 pg/ml, intra-assay and inter-assay coefficients of variation ≤ 7.1 %, IBL International, Hamburg, Germany). CORT plasma samples and lyophylized dialysates from the microdialysis experiment (see 2.2.1.4) were analyzed using a commercially available ELISA kit for CORT (analytical sensitivity < 0.564 ng/ml, intra-assay and inter-assay coefficients of variation ≤ 6.35 %, IBL International, Hamburg, Germany).

2.2.2.2. Removal of different brain regions

Following decapitation under CO_2 anesthesia, the eyes were removed from the head in order to prevent deformation and excitation of the optic nerve, which can damage the SCN. Then, the brain was taken out of the cranium and, thereby, the *dura mater* was removed to avoid any further damage of the brain. The optic nerves between the olfactory bulbs and the hemispheres were cut carefully as stretching the optic nerve can also lead to damage of the SCN and ruptured slices.

Histology for microdialysis

After decapitation, brains were removed, snap-frozen in methylbutane cooled on dry ice, and stored at -80 °C. Then, brains were cut into 40 µm coronal cryostat sections, and stained with cresyl violet for verification of probe placement. Only data of mice with correctly placed microdialysis probes were included in the analyses.

In situ hybridization (ISH)

After decapitation, brains were taken out, snap-frozen in methylbutane cooled on dry ice and stored at -80 °C. Then, a series of six 16 µm cryo-sections containing the respective brain areas (SCN, supraoptic nucleus (SON), PVN) were cut per mouse by Gabriele Schindler using a cryostat (at -22 °C). The slices were stored at -20 °C for subsequent ISH (see section 2.2.4).

2.2.2.3. Determination of body, pituitary, and adrenal weight

After anesthesia, mice were weighed immediately before decapitation to assess the effects of SD on body weight gain. Afterwards, the pituitary and the left and right adrenal gland of each animal were removed, pruned from fat and weighed separately. Subsequently, adrenal weights were summed per mouse (sum of respective left and right adrenal weight) and expressed as absolute adrenal weight (mg). Pituitaries were either frozen in liquid nitrogen (protein analysis) or embedded in protective freezing medium for immunohistochemistry (IHC) and stored at -80 °C until assayed. Left adrenal glands were stored in liquid nitrogen (protein analysis) and right adrenals were embedded in protective freezing medium (oil-red staining). The tissues were then stored at -80 °C until assayed.

2.2.3. Protein techniques

2.2.3.1. Preparation of total cell lysates

Generally, pituitary and adrenal gland tissue used for protein analysis was rapidly removed, immediately frozen in liquid nitrogen and stored at -80 °C until further processed. To avoid any protein degradation all subsequent analysis steps were carried out on ice. Pituitary and adrenal proteins were isolated using EDTA lysis buffer supplemented with complete mini protease inhibitor. Thereby, the buffer had to be prepared immediately before protein extraction. At first, tissue was homogenized with a Dounce homogenisator in an appropriate volume of EDTA lysis buffer (100 µl for pituitaries, 120 µl for adrenals). Samples were rotated at 4 °C for 1 h and, subsequently, cellular debris were pelleted by centrifugation (13000 rpm, 30

min, 4 °C; Eppendorf 5415R) and total protein concentration in the supernatant was determined by using a commercial kit (see 2.2.3.2).

EDTA lysis buffer	
0.05 mM	EDTA
250 mM	NaCl
50 mM	HEPES
0.5 %	Igepal (v/v)

2.2.3.2. Quantitative determination of protein concentrations

Protein concentrations in the samples of total cell lysates were determined by means of a commercial kit (BCA Protein Assay Kit, Thermo Scientific, Rockford, USA). The assay is based upon the colorimetric detection and quantification of total protein through chelation of the protein with cuprous. In alkaline medium the reduction of Cu^{+2} to Cu^{+1} by the peptide bonds of proteins occurs. As a second step, two BCA molecules form a complex with one Cu^{+} ion, resulting in a purple-colored reaction product with an absorption peak at 562 nm. Hence, the amount of the produced reaction complex is proportional to the amount of protein present in the sample. For quantification, 5 µl of the sample were mixed with 200 µl of the BCA working reagent (composed by 50 % of the alkaline reagent A, 48 % of reagent B that contains BCA, and 2 % of reagent C that contains cuprous sulfate). The mixture was incubated for 30 min at 37 °C and, after cooling to room temperature (RT), the absorbance was measured in an ELISA plate reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany). In order to calculate the protein concentration of each sample, the originated standard curve (0 – 2 µg/µl) was used.

2.2.3.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Equal amounts of protein extracts (40 µg for CRH-R1, 20 µg for all other measures) were mixed with denaturating buffer according to Laemmli (Laemmli, 1970) and boiled at 98 °C for 5 min in a thermoblock (Thermomixer Comfort, Eppendorf). After cooling, the proteins were separated by SDS-PAGE (10 % acrylamide resolving gel, 5 % acrylamide stacking gel; Bio-Rad system), according to their molecular weight. To determine the molecular weight of the proteins the PageRuler™ Prestained Protein Ladder Plus (Fermentas, Thermo Scientific, Rockford, USA) was used. The electrophoresis technique was performed with Mini-PROTEAN® Tetra Cell in electrophoresis buffer at 110 V.

4 x Resolving gel buffer (pH 8.8)	
1.5 M	Tris
10 %	SDS

4 x Stacking gel buffer (pH 6.8)	
0.5 M	Tris
10 %	SDS

Resolving gel (10 %)	
4 ml	30 % Acrylamide
3 ml	Resolving gel buffer
5 ml	H_2O dest
90 µl	APS
9 µl	TEMED

Stacking gel (5 %)	
670 µl	30 % Acrylamide
1 ml	Stacking gel buffer
2.3 ml	H_2O dest
30 µl	APS
5 µl	TEMED

4 x Loading Dye	
2.4 ml	1 M Tris (pH 6.8)
0.8 g	SDS
4 µl	Glycerol
1 ml	β-Mercaptoethanol
2.8 ml	H_2O bidest.
0.01 %	Bromphenol blue

Electrophoresis buffer	
25 mM	Tris
86 mM	Glycin
3.5 mM	SDS
Add 1 l H_2O bidest.	

2.2.3.4. Western blotting

Transfer on nitrocellulose membranes

In order to detect the proteins, they had to be transferred onto nitrocellulose membranes (Bio-Rad, München, Germany) by using Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, München, Germany). Therefore, a blotting "sandwich" consisting of two fiber pads, two whatman papers, the gel, one nitrocellulose membrane, two whatman papers and two fiber pads was assembled in a gel holder cassette with the membrane directed towards the anode. The electrophoretic transfer was carried out in a blotting chamber filled with transfer buffer at 100 V for 1 h. Before staining the fixed proteins on the membrane with specific antibodies, unspecific protein binding sites had to be blocked. For this purpose, the nitrocellulose membranes were incubated in blocking solution (see table 1) on an orbital shaker.

Transfer buffer (pH 8.3)	
25 mM	Tris
192 mM	Glycin
20 %	Methanol

Detection

After blocking of the membrane, the blocking solution was discarded and the membrane was incubated with a diluted solution of primary antibody under gentle agitation over night at 4 °C. The respective incubation time and dilution of the primary antibodies can be taken from table 1. After rinsing the membrane (3 x 15 min with Tris buffered saline/0.1 % Tween-20 (TBS-T) or PBS/0.1 % Tween-20 (PBS-T) to remove unbound primary antibody, the membrane was incubated with a secondary antibody that is linked to horseradish peroxidase (HRP) for 30 – 60 min at RT (see table 1). Subsequently, the membrane was washed again (3 x 15 min) with TBS-T/PBS-T. Enhanced Chemiluminescent Western Blotting detection reagent (ECL, GE Healthcare, Freiburg, Germany) was used for detecting immunoreactive bands. Therefore, membranes were incubated for 1 min with the ECL reagent, according to the manufacturer's instructions. Afterwards, bands were visualized by means of a Molecular Imager® ChemiDoc™ XRS+ system (Bio-Rad Laboratories, München, Germany). Image Lab™ Software (Bio-Rad Laboratories, München, Germany) was used for semiquantitative densitometric analysis and protein expression for each animal was normalized to β-tubulin expression and averaged per treatment group.

Stripping of membranes

In order to re-use the membrane for subsequent antibody probes, it had to be stripped (i.e. the antibody was removed efficiently) by using Re-Blot Plus Mild Antibody Stripping Solution (Millipore GmbH, Schwalbach, Germany). After verifying a successful stripping procedure, the membrane was blocked again (2 x 5 min) and, thereafter, incubated with another primary antibody. The stripping procedure was mainly used to detect the HSL and SR-B1 protein in the adrenal gland.

10 x TBS (pH 7.4)
500 ml Tris
1.5 M NaCl

TBS-T
0.1 % Tween in TBS

PBS (pH 7.4)	
140 mM	NaCl
3 mM	KCl
1.5 mM	KH_2PO_4
4mM	Na_2HPO_4

PBS-T
0.1 % Tween in PBS

PROTEIN OF INTEREST	BLOCKING	PRIMARY ANTIBODY	SECONDARY ANTIBODY	SIZE [kDA]
CRH-R1	5 % BSA/TBS-T 1 h at RT	1:200 in 5 % BSA/TBS-T o/n at 4 °C	Anti goat, 1:8500 in 3 % BSA/TBS-T 30 min at RT	~ 48
AVP-R1b	5 % MP/TBS-T 2 h at RT	1:400 in 5 % MP/TBS-T o/n at 4 °C	Anti goat, 1:1500 in TBS-T 30 min at RT	~ 48 - 50
β-tubulin	5 % MP/TBS-T 1 h at RT	1:1000 in 5 % MP/TBS-T o/n at 4 °C	Anti rabbit, 1:1000 in TBS-T 30 min at RT	~ 55
HSL	5 % BSA/TBS-T 1 h at RT	1:1000 in 5 % BSA/TBS-T o/n at 4 °C	Anti rabbit, 1:1500 in TBS-T 30 min at RT	~ 82
LDLR	5 % MP/TBS-T 1 h at RT	1:500 in 5 % MP/TBS-T o/n at 4 °C	Anti rabbit, 1:3000 in TBS-T 30 min at RT	~ 150
SR-B1	5 % MP/TBS-T 1 h at RT	1:1600 in 5 % MP/TBS-T o/n at 4 °C	Anti rabbit, 1:3000 in TBS-T 30 min at RT	~ 76
HMGCR	5 % MP/TBS-T 1 h at RT	1:500 in 5 % MP/TBS-T o/n at 4 °C	Anti rabbit, 1:3000 in TBS-T 30 min at RT	~ 76
Mc2r	5 % MP/TBS-T 1 h at RT	1:200 in 5 % MP/TBS-T o/n at 4 °C	Anti rabbit, 1:3000 in TBS-T 30 min at RT	~ 45

Table 1: Detection protocols for individual antibodies. Tris-buffered saline/0.1 % Tween-20 (TBS-T) supplemented with either bovine serum albumin (BSA) or milk powder (MP) was used to block the membranes. Incubation with the primary antibody took place at 4 °C overnight (o/n).

2.2.4. In situ hybridization of hypothalamic CRH and AVP mRNA

Assessment of hypothalamic CRH and AVP mRNA expression was performed as described in general previously (Reber et al., 2007; Peters et al., 2014). After decapitation, brains were rapidly removed, snap-frozen in methylbutane cooled on dry ice and stored at -80 °C. A series of six 16 µm cryo-cut sections of the hypothalamus including the SON, PVN, and the SCN was thaw-mounted onto slides at -22 °C and used to quantify hypothalamic CRH and AVP mRNA. Hybridization was performed together with Martina Fuchs by using specific 48-mer, 35S-labeled oligonucleotide probes for CRH (5'GGCCCGCGGCGCTCCAGAGACGGATCCCCTGCTCAGCAGGGCCCTGCA-3') and AVP (5'-GCAGAAGGCCCCGGCCGGCCCGTCCAGCTGCGTGGCGTTGCTCCGGTC-3') mRNA, respectively. Hybridized slices were exposed to BioMax MR film (Kodak, Cedex, France) under safe red light conditions for 28 days (CRH) or 3 days (AVP). CRH and AVP mRNA expression in the SON, PVN, and SCN were measured as optical density using NIH ImageJ 1.31 program (http://rsb.info.nih.gov/ij/). Bilateral measures were taken from two to four SON, PVN, and SCN sections for each brain, which were then pooled to provide individual means per mouse. For tissue background, the optical density of a non-hybridized region outside the PVN, SON, and SCN was measured.

2.2.5. Oil-red lipid staining in adrenal tissue

In order to determine the effects of repeated SD on the availability of cortical cholesterol esters, adrenal cryo-sections (5 µm) were stained with oil-red as previously described (Ramirez-Zacarias et al., 1992; Füchsl et al., 2013b). Briefly, six cryo-sections per adrenal gland were fixed in 4 % paraformaldehyde (PFA) for 72 h. Afterwards, sections were washed in distilled water, rinsed in 60 % isopropyl alcohol for 5 min and then stained in a freshly filtered oil-red solution (Certistain Oilred O, Merck, Darmstadt, Germany) for 15 min. The sections were then washed with 60 % isopropyl alcohol followed by distilled water. Sections were mounted with glycerine jelly and covered with a glass cover slip. The entire area of all lipid droplets [mm^2] as well as the cortex area [mm^2] containing these lipid droplets (= relative cortical lipid per area) were measured in digitized images using Leica FW 4000 Software (Leica Microsystems, Wetzlar, Germany). The adrenal medulla and the glomerulosa layer of the cortex were excluded from the measurement of the adrenal stained cortex areas, thus allowing specific quantification of the lipid droplets in adrenal areas involved in CORT synthesis, the fasciculata and reticularis layer of the cortex (Rosol et al., 2001) (see Fig. 7). The results for six adrenal sections per mouse were pooled (individually for left and right adrenals) to provide individual means.

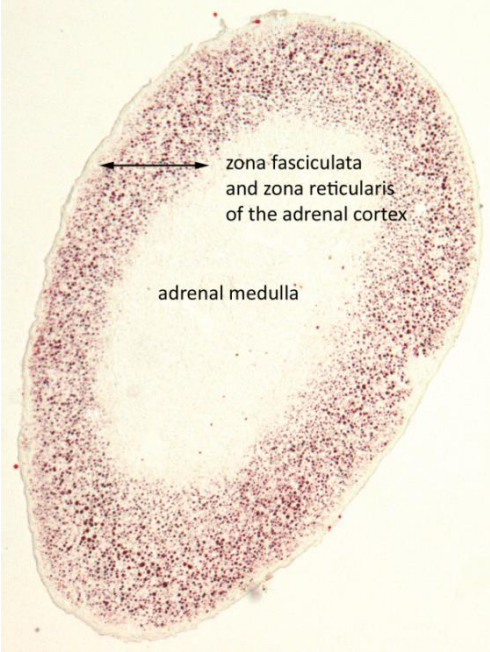


Figure 7: Adrenal cortical lipids stained by oil-red (5 x optical zoom). Six adrenal slices per animal were stained and lipid droplets content was assessed using a semi-quantitative measurement of the ratio between the stained area [mm^2] and the adrenal cortex area [mm^2].

2.2.6. Statistics

For statistical comparisons, the software package SPSS (version 21.0) was used. Data were first tested for normal distribution applying a one-sample Kolmogorov-Smirnov test. Since all data presented in this thesis were normally distributed, they were subsequently tested for significant differences by using either two-way analysis of variance (ANOVA) for parameters depending on two factors (e.g. factor SD paradigm and factor time of day of tissue collection) or ANOVA for repeated measures (rm ANOVA) for parameters measured more times (e.g. body weight, food intake, hippocampal CORT rhythm), followed by Fisher's least significant difference (LSD) *post hoc* analysis if appropriate. Due to the application on different gels, Student's t-test was applied for analysis of CRH-R1, AVP-R1b, Mc2r, SR-B1, LDLR, HSL, and HMGCR protein expression. Data are presented as mean + SEM and considered as significantly different with $p \leq 0.05$.

CHAPTER 3

RESULTS

In the following, I will shortly describe the main findings of my publications included in this thesis as well as of one new manuscript, which has been submitted to Chronobiology International. The full text versions of the articles and the manuscript can be read starting from page 152. Additionally, I will present data which are so far not part of a manuscript (see 3.5 and 3.6) and describe the established techniques performed during this PhD project (see 3.4).

3.1. TIME MATTERS: PATHOLOGICAL EFFECTS OF REPEATED PSYCHOSOCIAL STRESS DURING THE ACTIVE, BUT NOT INACTIVE, PHASE OF MALE MICE (PAPER 1)

In this study we were interested whether and to what extent a clinically relevant chronic/intermittent psychosocial stressor affects selected behavioral, neuroendocrine/physiological, and immunological/inflammatory parameters depending on the time of day of stressor exposure. Therefore, male mice were exposed to repeated SD (2h/day; 19 days) either during the light (SDL mice) or dark (SDD mice) phase, and behavioral (home cage activity, social preference, and anxiety-related behavior), neuroendocrine and physiological (basal plasma ACTH and CORT concentrations as well as adrenal *in vitro* responsiveness to ACTH), and immunological (interferon (IFN)-γ secretion from mesenteric lymph node cells and the severity of dextran sulfate sodium (DSS)-induced colitis) parameters were assessed at different times of the day. We found that the effects of repeated SD exposure on these parameters strongly depend on the time of day of stressor exposure, thus highlighting the importance of the circadian clock in this context. While physiological factors, such as absolute adrenal gland weight and adrenal ACTH responsiveness, were more affected by SD during the light/inactive phase

(SDL mice), behavioral and immunological parameters like active home cage behavior, social preference, IFN-γ secretion from mesenteric lymph node cells, and severity of DSS-induced colitis were more affected by SD during the dark/active phase (SDD mice). The prolonged increased adrenal gland weight and the decreased adrenal ACTH responsiveness seen in SDL mice are likely to represent beneficial adaptations to the predictable stressor occurring every day in the morning. In contrast, decreased interest in an unfamiliar conspecific, loss of general activity, flattened or shifted diurnal CORT rhythmicity, and a shift towards a more pro-inflammatory body milieu, occurring particularly in SDD mice, represent well-accepted hallmarks of affective disorders, such as depression. Overall, we could demonstrate that equally severe stressors have a more pronounced negative outcome when applied during the dark/active phase. Moreover, our data also suggest that the physiological changes seen in mice stressed during the rest period (SDL mice) might represent beneficial adaptations rather than maladaptive consequences.

(For details see results sections of Bartlang et al., 2012)

3.2. REPEATED PSYCHOSOCIAL STRESS AT NIGHT, BUT NOT DAY, AFFECTS THE CENTRAL MOLECULAR CLOCK (PAPER 2)

Based on previous studies showing that chronic stress affects PER2 protein expression in the SCN (Jiang et al., 2011; Kinoshita et al., 2012) and the PER2 protein rhythm in the adrenal gland (Razzoli et al., 2014), we aimed to investigate the effects of repeated SD on the circadian clock in transgenic PERIOD2::LUCIFERASE (PER2::LUC) and WT mice. Further, since we demonstrated in Paper 1 that the outcome of repeated SD is more negative when applied during the dark/active phase as compared with the light/inactive phase of male C57BL/6 mice, we exposed the animals in this study to repeated SD (2h/day) either in the early light phase (SDL mice) or early dark phase (SDD mice) across 19 days. We found higher PER2::LUC rhythm amplitude and increased PER2 protein expression in the posterior part of the SCN of SDD mice compared with SDL and SHC mice. In contrast, PER2::LUC rhythms and PER2 protein expression in the SCN of SDL mice were not affected. Furthermore, *Per2* and *Cry1* mRNA expression in the adrenal gland was reduced in mice stressed during the dark/active phase (SDD mice). However, mice stressed during the light/inactive phase (SDL mice) exhibited a 2 h phase-advance of the PER2::LUC rhythm in the adrenal gland compared to SHC mice. Furthermore, plasma levels of BDNF and BDNF mRNA in the SCN were elevated in the SDL group. Taken together, we could show that the SCN molecular rhythmicity is affected by repeated SDD, but not SDL, while the peripheral adrenal clock is mainly influenced by SDL. The observed increase in BDNF in the SDL group may act protective against

the negative consequences of repeated psychosocial stress. (For details see results sections of Bartlang et al., 2014)

3.3. REPEATED PSYCHOSOCIAL STRESS AT NIGHT, BUT NOT DAY, DELAYS THE ENTRAINED ACTIVITY RHYTHM OF MALE MICE (PAPER 3, SUBMITTED)

Based on our findings in Paper 2, demonstrating that the molecular rhythmicity in the SCN was affected by repeated SDD, but not SDL, we applied *in vivo* biotelemetry for measuring stress-induced changes in two output rhythms of the circadian clock, i.e. activity and core body temperature. In order to unravel whether potential differences between SDL and SDD mice are due to alterations of the endogenous clock, we exposed WT and *Per1/Per2* double-mutant mice, which lack a functional clock, to the predictable stressor (2h/day) either in the early light phase (SDL mice) or in the early dark phase (SDD mice). Overall, more pronounced stress effects could be seen in the activity rhythm as compared to the body temperature rhythm. Throughout the 19 days of SD procedure, SDL and SDD mice were similarly affected and no differences between WT and *Per1/Per2* mutant mice were detectable, suggesting that the acute alterations upon SD exposure are a direct response to the social stressor. In contrast, considerable differences in the activity between SDL and SDD mice were found in the entrained 14-day post-stress period. The majority of WT SDD mice showed a lack of the activity peak at ZT13 under LD post-stress conditions, whereas the remaining SDD mice exhibited a delayed activity peak compared to SHC and SDL mice at that time. This effect was particularly seen in WT, but not *Per1/Per2* mutant, SDD mice, implementing that the delay/absent peak in WT SDD mice results from an altered circadian clock. In coincidence with the findings from Paper 2, we could show that SDD has long-lasting consequences for

the functional output of the biological clock, that might be linked to the more negative outcome of SD exposure during the dark versus the light phase.

3.4. ESTABLISHED TECHNIQUES

3.4.1. Assessment of the corticosterone rhythm in the hippocampus by *in vivo* microdialysis

Statistical analysis of the hippocampal CORT rhythm across 24 h did not reveal any significant effect of the time of day of sampling (Fig. 8).

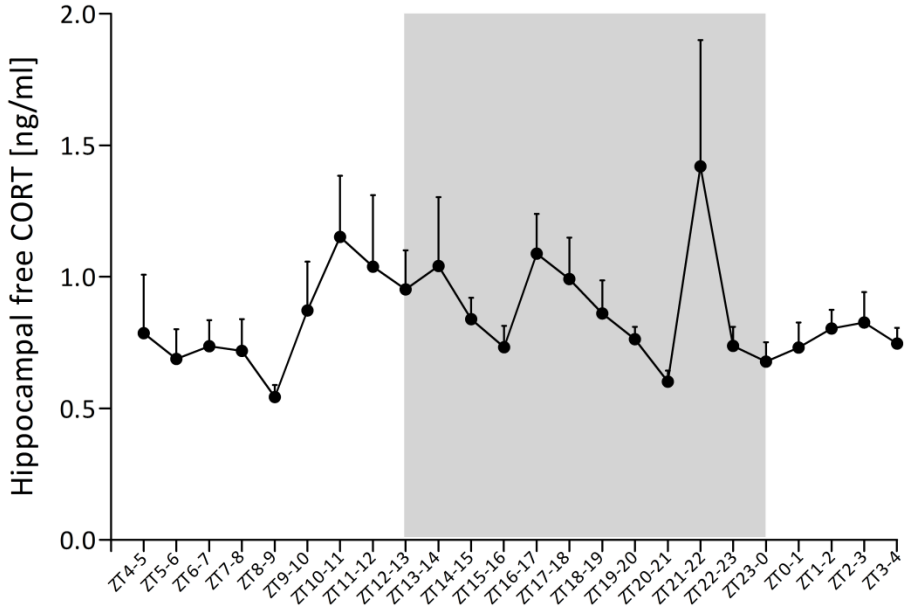


Figure 8: Establishment of the assessment of the 24 h hippocampal CORT rhythm by *in vivo* microdialysis technique. Two days after implantation of the microdialysis probe within the right dorsal hippocampus, microdialysis samples of C57BL/6N mice (n = 4) were collected in 60 min intervals around the clock. The grey bar indicates the dark phase (starting at ZT12). Data represent the mean + SEM.

3.4.2. Assessment of *in vitro* corticosterone secretion by adrenal explants

Pre-experiment 1: Adrenal *in vitro* CORT secretion was found to be dependent on both the time of day of stimulation and the medium used (saline or ACTH) ($F_{7,42}$ = 8.34; $p \leq 0.001$) (Fig. 9). *Post hoc* analysis revealed a significant increase in CORT secretion after *in vitro* ACTH stimulation at ZT8 – 9, ZT9 – 10, ZT11 – 12, and ZT12 – 13 ($p \leq 0.05$).

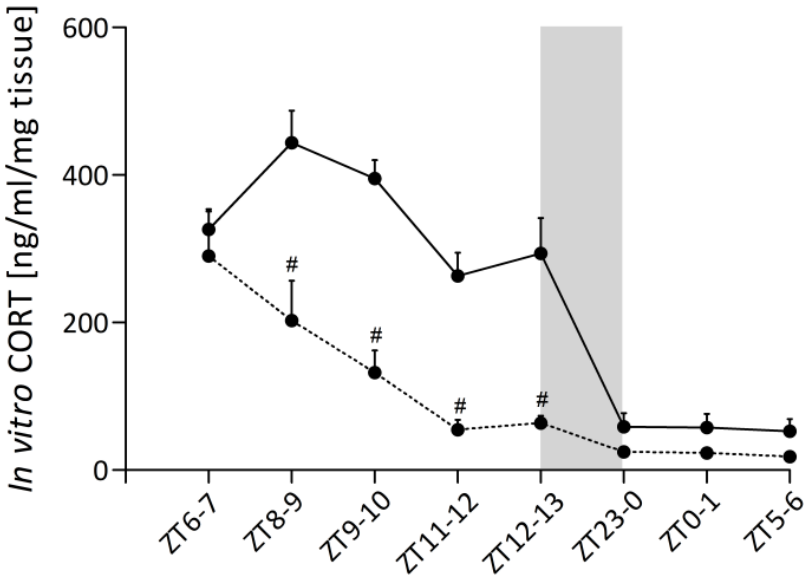


Figure 9: Establishment of the assessment of adrenal CORT secretion across 24 h *in vitro* – pre-experiment 1. Following decapitation at ZT1, left adrenal glands (n = 4) were removed and pruned from fat tissue. Subsequently, adrenals were cut into two halves, each containing cortical and medullary tissues. The halves were then weighed and pre-incubated in 200 µl DMEM/F-12 for 4 h before any further treatment. Culture medium was then replaced and each half of one adrenal was incubated hourly with either medium containing saline (basal; dashed line) or medium containing ACTH (100 nM; solid line) during a time period of 24 h. CORT concentrations in the supernatants (ng/ml) were quantified and expressed per mg of adrenal tissue (ng/ml per mg). The grey bar indicates the dark phase (ZT12 – ZT0). Data represent the mean + SEM. # represent $p \leq 0.05$ vs ACTH-induced CORT secretion.

Pre-experiment 2: Statistical analysis of *in vitro* CORT secretion by left adrenal explants showed a significant effect of both the time of day of stimulation and the medium used (saline or ACTH) ($F_{6,60}$ = 79.36; p ≤ 0.001) (Fig. 10A). When right adrenal explants were stimulated, CORT secretion was also dependent on both the time of day of stimulation and the medium used (saline or ACTH) ($F_{6,60}$ = 15.11; p ≤ 0.001) (Fig. 10B). *Post hoc* analyses revealed a significant increase in CORT secretion after *in vitro* ACTH stimulation at ZT11 – 12, ZT12 – 13, ZT18 – 19, ZT0 – 1, and ZT6 – 7 (p ≤ 0.05) for left and right adrenal gland explants, respectively.

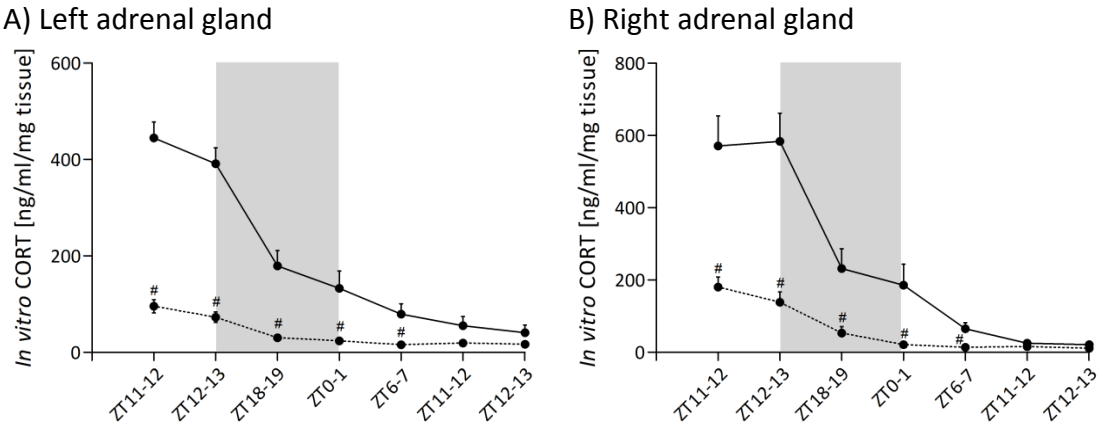


Figure 10: Establishment of the assessment of adrenal CORT secretion across 24h *in vitro* – pre-experiment 2. Following decapitation at ZT1, left (A) and right (B) adrenal glands (n = 6/body side) were removed and pruned from fat tissue. Subsequently, each left and right adrenal gland was cut into two halves, each containing cortical and medullary tissues. The halves were then weighed and pre-incubated in 200 µl DMEM/F-12 for 4 h before any further treatment. Culture medium was then replaced and left adrenal halves were incubated hourly with either medium containing saline (basal; dashed line) or medium containing ACTH (100 nM; solid line) during a time period of 24 h. Right adrenal halves were incubated with either medium containing saline (basal; dashed line) or medium containing ACTH (100 nM; solid line) for 1 h at time points shown in the graphs above. For all other time points across the 24 h right adrenal halves were incubated with medium for 1 h. Afterwards, CORT concentrations in the supernatants (ng/ml) were quantified and expressed per mg of adrenal tissue (ng/ml per mg). The grey bar indicates the dark phase (ZT12 – ZT0). Data represent the mean + SEM. # represent p ≤ 0.05 vs ACTH-induced CORT secretion.

3.5. EFFECTS OF REPEATED SOCIAL DEFEAT ON DIFFERENT METABOLIC PARAMETERS

3.5.1. Basal plasma ghrelin and leptin levels

Basal plasma ghrelin levels were found to be dependent on the time of day of trunk blood collection ($F_{1,39}$ = 61.80; $p \leq 0.001$) (Fig. 11A). *Post hoc* tests showed that all three treatment groups (SHC, SDL, SDD) had significantly higher ghrelin levels ($p \leq 0.01$) during the light phase as compared to the dark phase.

Basal plasma leptin levels were also dependent on the time of day of trunk blood collection ($F_{1,39}$ = 28.77; $p \leq 0.001$) (Fig. 11B). *Post hoc* analysis revealed that SHC ($p \leq 0.01$) and SDL ($p \leq 0.001$), but not SDD, mice showed lower leptin levels during the light phase as compared to the dark phase.

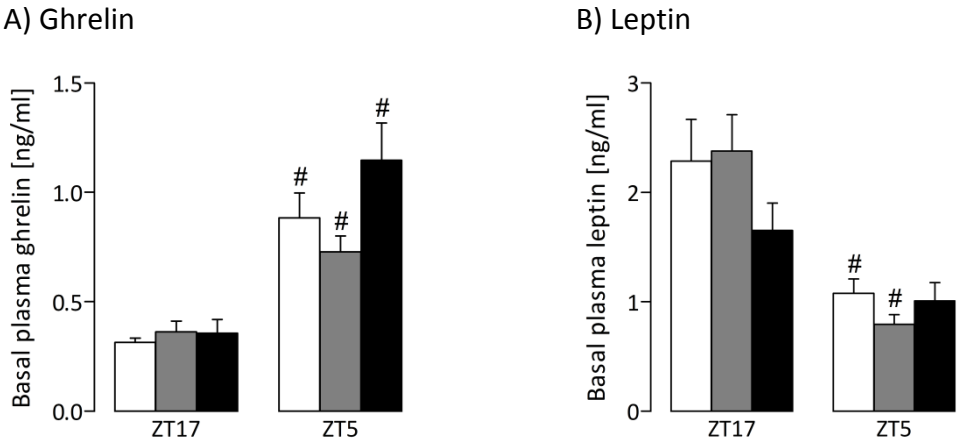


Figure 11: Effects of 19 days of SDL and SDD on basal ghrelin (A) and leptin (B) concentrations in trunk blood measured in the dark phase (ZT17) and light phase (ZT5). Following decapitation under brief inhalation anesthesia on day 20 in the dark phase (ZT17, peak of circulating leptin and trough of circulating ghrelin (Bodosi et al., 2004)), basal ghrelin [ng/ml] and leptin [ng/ml] concentrations were determined in the trunk blood of SHC (white bars; n = 8), SDL (grey bars; n = 8), and SDD (black bars; n = 8) mice. Another set of SHC (white bars; n = 7), SDL (grey bars; n = 7), and SDD (black bars; n = 7) mice was killed on day 21 in the light phase (ZT5, peak of circulating ghrelin and trough of circulating leptin (Bodosi et al., 2004)) and trunk blood was collected for determination of plasma ghrelin [ng/ml] and leptin [ng/ml]. Data represent the mean + SEM. # represent $p \leq 0.05$ vs respective group at ZT17.

3.5.2. Body weight development

Body weight development assessed at ZT7 – ZT8 during the course of the 19 days of SD was found to be dependent on both the SD paradigm employed and the day of observation ($F_{40,780} = 3.86$; $p \leq 0.001$) (Fig. 12). *Post hoc* tests showed that, on days where SD took place, SDL mice exhibited lower body weight as compared to SHC and SDD mice ($p \leq 0.05$). In general, all three treatment groups (SHC, SDL, SDD) gained body weight across the 19 days of SD ($p \leq 0.05$).

To exclude that the time of day of body weight assessment exerts influence, a separate set of SHC, SDL, and SDD mice was weighed in the night between ZT19 and ZT20 from day 0 (before the start of the SD paradigm) until day 3 (i.e. after 3 SD

sessions) (inserted graph Fig. 12). It could be shown that body weight development at night was not dependent on the SD paradigm employed.

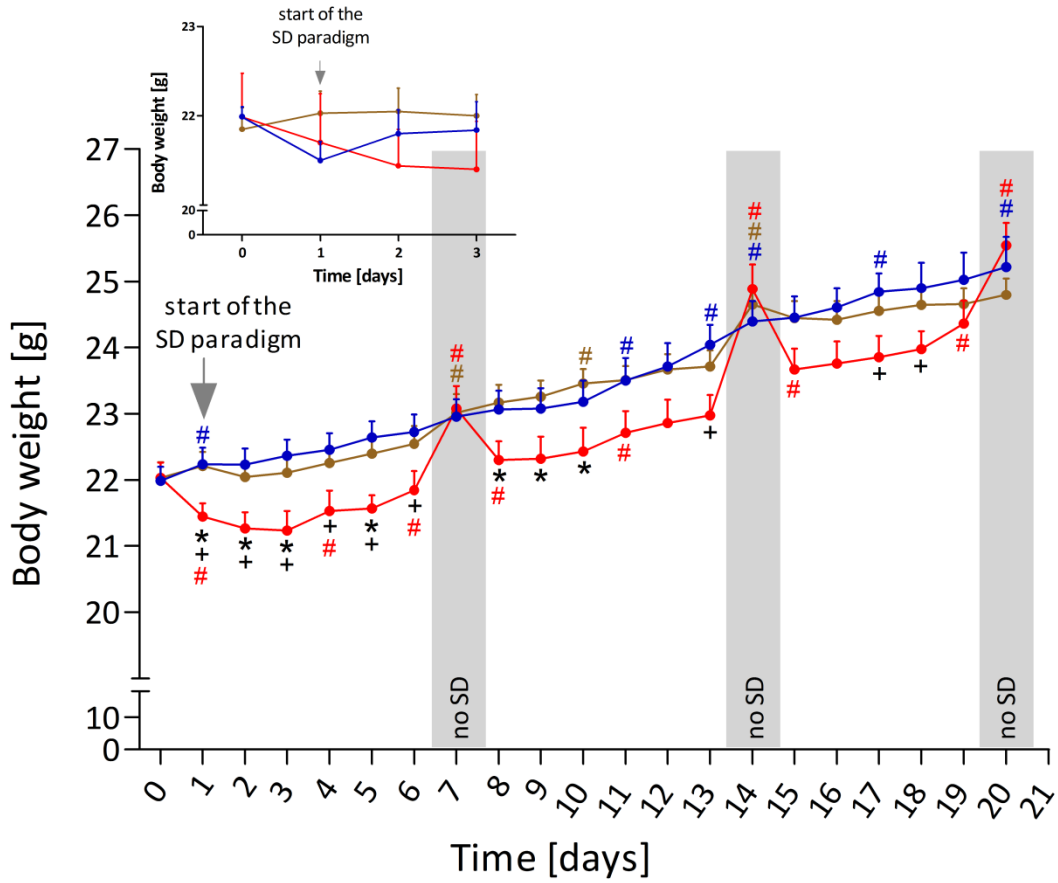


Figure 12: Effects of 19 days of SDL and SDD on body weight development during the course of the 19 days of SD. SHC (brown lines; n = 14), SDL (red lines; n = 14), and SDD (blue lines; n = 14) mice were weighed daily from day 0 (before the start of the SD paradigm) until day 20 (one day after the last SD) between ZT7 and ZT8. The inserted graph shows the body weight development of SHC (brown lines; n = 15), SDL (red lines; n = 7), and SDD (blue lines; n = 8) mice between ZT19 and ZT20 on the first three days of the SD paradigm. Data represent the mean + SEM. * represent $p \leq 0.05$ vs respective SHC mice; + represent $p \leq 0.05$ vs respective SDD mice; # represent $p \leq 0.05$ vs respective group on the day before.

3.5.3. Food intake

Daily food intake during the course of the 19 days of SD was dependent on both the SD paradigm employed and the day of observation ($F_{12,234}$ = 4.21; p ≤ 0.001) (Fig. 13). *Post hoc* tests showed that, especially on stress-free days (day 7, 14, and 20), SDD mice ate significantly less (p ≤ 0.05) compared with SHC and SDL mice. All three treatment groups (SHC, SDL, SDD) exhibited a significant fluctuation in food intake with higher food consumption on stress-free days as compared to periods of SD exposure (p ≤ 0.05), as revealed by *post hoc* analysis.

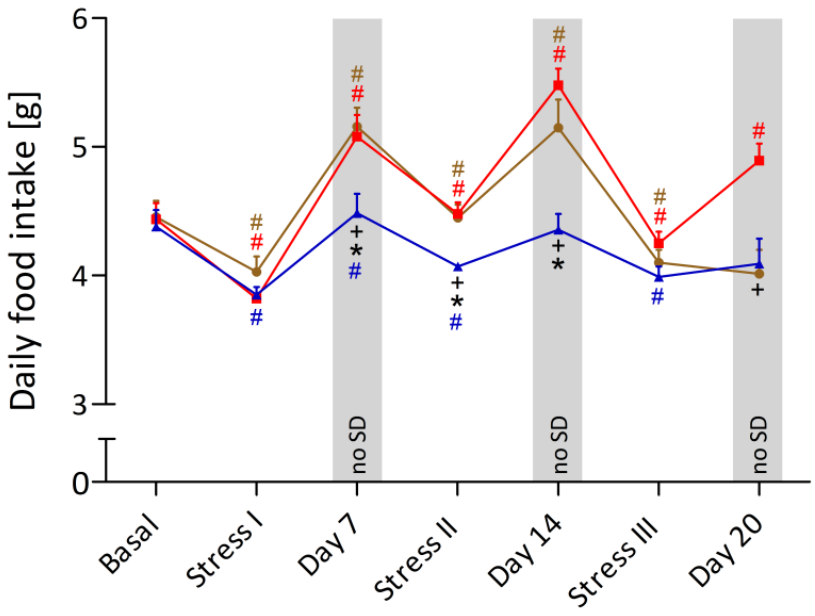


Figure 13: Effects of 19 days of SDL and SDD on daily food intake. The food from SHC (brown lines; n = 14), SDL (red lines; n = 14), and SDD (blue lines; n = 14) mice was weighed daily between ZT7 and ZT8 and the difference within 24 h was calculated by subtracting the daily values from each other. For clarification, the mean of the values assessed on basal days (two days before the start of the SD paradigm) and on days when SD exposure took place (day 1 – 6 = Stress I; day 8 – 13 = Stress II; day 15 – 19 = Stress III) was calculated for every mouse, respectively, and averaged per treatment group. Data represent the mean + SEM. * represent p ≤ 0.05 vs respective SHC mice; + represent p ≤ 0.05 vs respective SDL mice; # represent p ≤ 0.05 vs respective group at the time point before.

3.6. EFFECTS OF REPEATED SOCIAL DEFEAT ON HYPOTHALAMIC-PITUITARY-ADRENAL AXIS ACTIVITY

3.6.1. Hypothalamus

3.6.1.1. CRH mRNA expression in the suprachiasmatic nucleus, paraventricular nucleus, and supraoptic nucleus

CRH mRNA expression in the hypothalamic SCN, PVN, and SON was neither dependent on the SD paradigm employed nor on the time of day of tissue collection (Fig. 14).

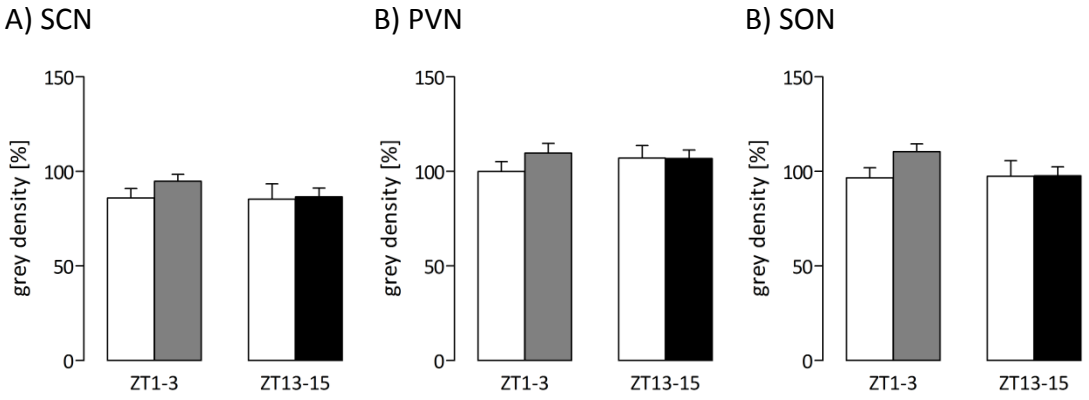


Figure 14: Effects of 19 days of SDL and SDD on CRH mRNA expression within the SCN (A), PVN (B), and SON (C). Following decapitation under brief inhalation anesthesia on day 20 in the light phase (ZT1 – 3, time of previous SDL exposure), brains were removed from SHC (white bars; n = 8) and SDL (grey bars; n = 9) mice and snap-frozen in methylbutane. On day 20 in the dark phase (ZT13 – 15, time of previous SDD exposure), brains were removed from another set of SHC (white bars; n = 8) and SDD (black bars; n = 9) mice and snap-frozen in methylbutane. Afterwards, a series of 16 µm cryo-cut sections of the hypothalamus including the SCN, PVN, and SON was thaw-mounted onto slides at -20 °C and CRH mRNA expression was quantified by ISH. Data represent the mean + SEM.

3.6.1.2. AVP mRNA expression in the suprachiasmatic nucleus, paraventricular nucleus, and supraoptic nucleus

AVP mRNA expression in the hypothalamic SCN, PVN, and SON was neither dependent on the SD paradigm employed nor on the time of day of tissue collection (Fig. 15).

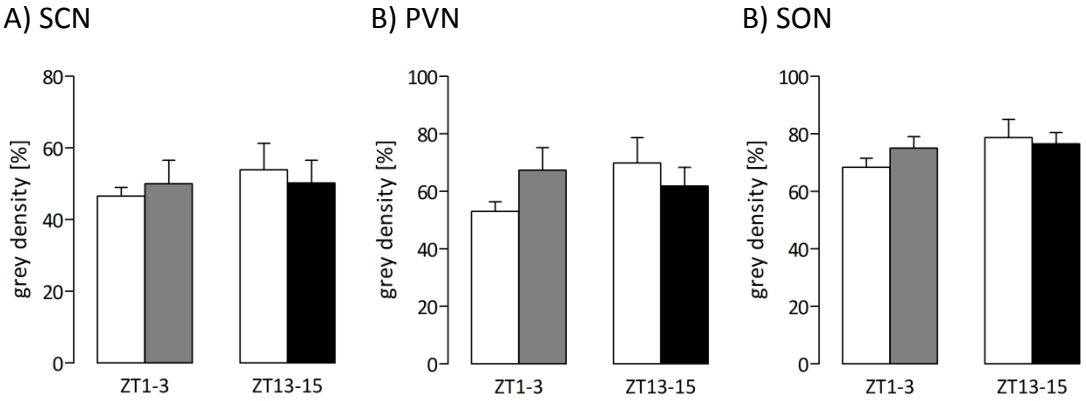


Figure 15: Effects of 19 days of SDL and SDD on AVP mRNA expression within the SCN (A), PVN (B), and SON (C). Following decapitation under brief inhalation anesthesia on day 20 in the light phase (ZT1 – 3, time of previous SDL exposure), brains were removed from SHC (white bars; n = 8) and SDL (grey bars; n = 9) mice and snap-frozen in methylbutane. On day 20 in the dark phase (ZT13 – 15, time of previous SDD exposure), brains were removed from another set of SHC (white bars; n = 8) and SDD (black bars; n = 9) mice and snap-frozen in methylbutane. Afterwards, a series of 16 µm cryo-cut sections of the hypothalamus including the SCN, PVN, and SON was thaw-mounted onto slides at -20 °C and AVP mRNA expression was quantified by ISH. Data represent the mean + SEM.

3.6.2. Pituitary

3.6.2.1. Pituitary weight and plasma adrenocorticotropic hormone

Absolute pituitary weight was not dependent on the SD paradigm employed or on the time of day of tissue collection (Fig. 16A).

Plasma ACTH was found to be dependent on both the SD paradigm employed and the time of day of tissue collection ($F_{1,28} = 4.48$; $p \leq 0.05$) (Fig. 16B). *Post hoc* analysis revealed that SHC mice exhibited significantly higher plasma ACTH levels at ZT13 – 15 compared to ZT1 – 3 ($p \leq 0.001$), while SDD mice showed lower plasma ACTH ($p \leq 0.05$) compared to the SHC group at ZT13 – 15.

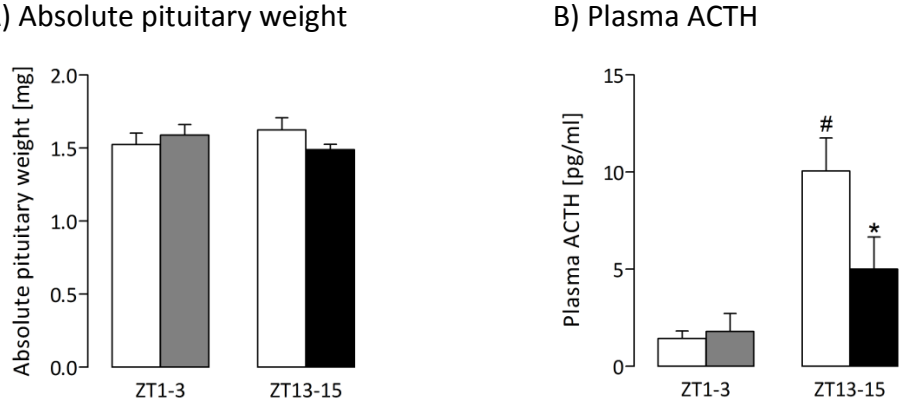


Figure 16: Effects of 19 days of SDL and SDD on absolute pituitary weight (A) and plasma ACTH (B). Following decapitation under brief inhalation anesthesia on day 20 in the light phase (ZT1 – 3, time of previous SDL exposure), trunk blood from SHC (white bars; n = 8) and SDL (grey bars; n = 9) mice was collected and pituitaries were removed and weighed. On day 20 in the dark phase (ZT13 – 15, time of previous SDD exposure), another set of SHC (white bars; n = 6 – 8) and SDD (black bars; n = 9) mice was decapitated under brief inhalation anesthesia, trunk blood was collected, and pituitaries were removed and weighed. Afterwards, plasma ACTH was quantified by ELISA. Data represent the mean + SEM. * represent $p \leq 0.05$ vs respective SHC mice (decapitated at ZT13 – 15); # represent $p \leq 0.05$ vs respective SHC group decapitated at ZT1 – 3.

3.6.2.2. CRH-R1 and AVP-R1b protein expression

Statistical analysis employing Student's t-test showed that relative pituitary CRH-R1 protein expression was not different between SDL and respective SHC mice decapitated at ZT1 – 3 (Fig. 17A). Following SDD, CRH-R1 protein expression in the pituitary was significantly lower compared with respective SHC mice decapitated at ZT13 – 15 ($p \leq 0.01$), as revealed by t-test analysis.

Applying Student's t-test, a significant decrease in relative pituitary AVP-R1b protein expression could be detected for SDL ($p \leq 0.001$) and SDD mice ($p \leq 0.01$), respectively (Fig. 17B).

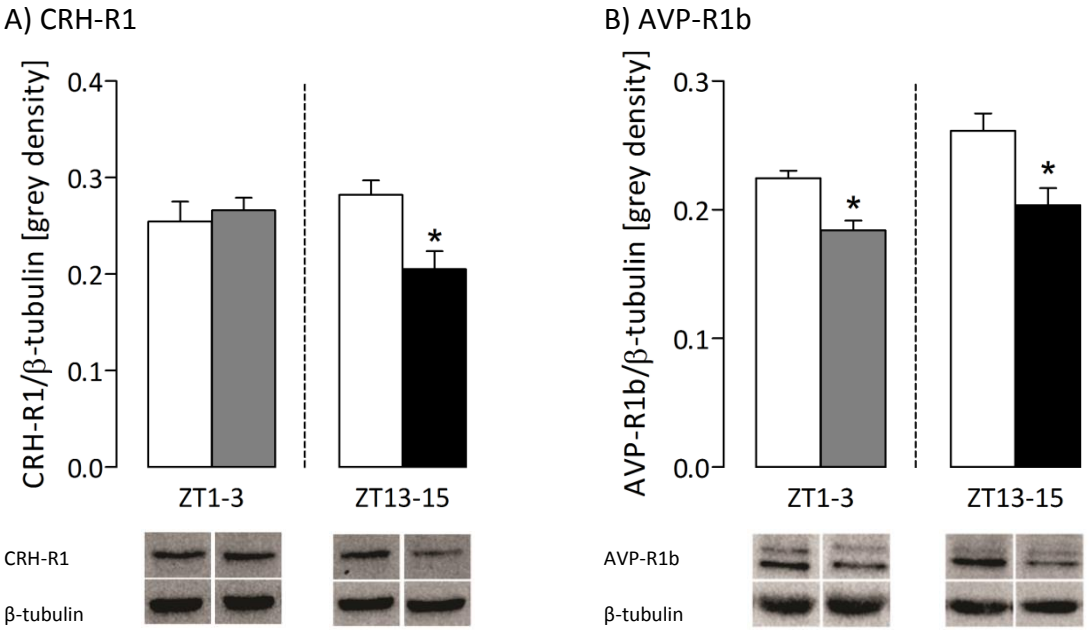


Figure 17: Effects of 19 days of SDL and SDD on relative CRH-R1 (A) and AVP-R1b (B) protein expression in the pituitary. Following decapitation under brief inhalation anesthesia on day 20 in the light phase (ZT1 – 3, time of previous SDL exposure), pituitaries were removed from SHC (white bars; n = 8) and SDL (grey bars, n = 7 – 8) mice. On day 20 in the dark phase (ZT13 – 15, time of previous SDD exposure), pituitaries were removed from another set of SHC (white bars, n = 8) and SDD (black bars, n = 8) mice. Subsequently, protein was extracted from the pituitaries for determination of relative CRH-R1 and AVP-R1b protein expression [grey density], all normalized to the loading control β-tubulin. Data represent the mean + SEM. * represent $p \leq 0.05$ vs respective SHC mice. Representative images of bands detected for CRH-R1 (~ 48 kDa; A) and AVP-R1b (~ 48 – 50 kDa; B) and respective loading control β-tubulin (~ 55 kDa; A/B) are shown for SHC+SDL and SHC+SDD mice.

3.6.3. Adrenal glands

3.6.3.1. Adrenal gland weight and plasma corticosterone

Absolute adrenal gland weight was dependent on the SD paradigm employed ($F_{1,30}$ = 37.69; $p \leq 0.001$) (Fig. 18A). *Post hoc* tests showed that both SDL and SDD mice had a higher absolute adrenal gland weight ($p \leq 0.001$) compared to their respective SHC groups.

Plasma CORT was found to be dependent on both the SD paradigm employed and the time of day of tissue collection ($F_{1,29}$ = 5.26; $p \leq 0.05$) (Fig. 18B). *Post hoc* analysis revealed higher plasma CORT levels of SHC mice at ZT13 – 15 compared to SHC mice at ZT1 – 3 ($p \leq 0.001$). Furthermore, SDD mice showed lower plasma CORT concentrations compared with respective SHC mice ($p \leq 0.01$), but still higher levels than SDL mice ($p \leq 0.001$).

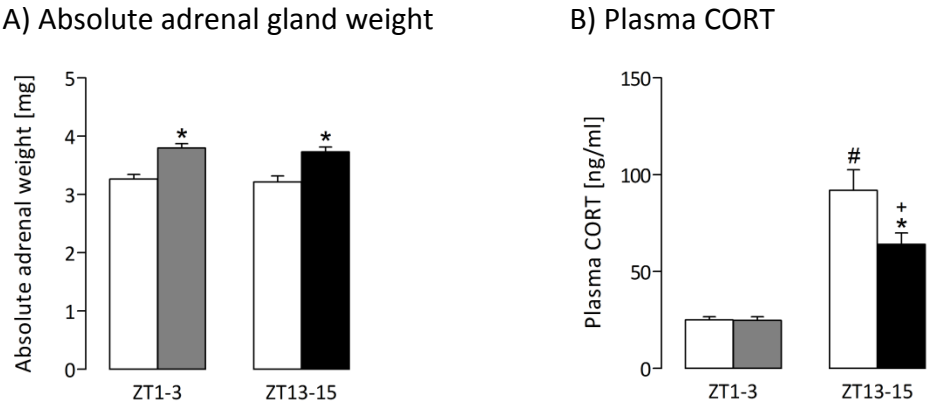


Figure 18: Effects of 19 days of SDL and SDD on absolute adrenal gland weight (A) and plasma CORT (B). Following decapitation under brief inhalation anesthesia on day 20 in the light phase (ZT1 – 3, time of previous SDL exposure), trunk blood from SHC (white bars; n = 8) and SDL (grey bars; n = 9) mice was collected for quantification of basal plasma CORT and adrenal glands were removed, pruned from fat and weighed. On day 20 in the dark phase (ZT13 – 15, time of previous SDD exposure), a separate cohort of SHC (white bars; n = 8) and SDD (black bars; n = 8 – 9) mice was decapitated under brief inhalation anesthesia, trunk blood was collected for plasma CORT quantification, and adrenal glands were removed and weighed. Data represent the mean + SEM. * represent $p \leq 0.05$ vs respective SHC mice; + represent $p \leq 0.05$ vs respective SDL mice; # represent $p \leq 0.05$ vs respective SHC group decapitated at ZT1 – 3.

3.6.3.2. Mc2r and SR-B1 protein expression

Relative adrenal Mc2r protein expression was neither different between SDL and SHC mice, nor between SDD and respective SHC animals (Fig. 19A).

Relative adrenal SR-B1 protein expression was not affected by SDL. In contrast, t-test analysis revealed that SDD mice exhibited a decreased SR-B1 protein expression compared with respective SHC mice killed between ZT13 – 15 ($p \leq 0.01$) (Fig. 19B).

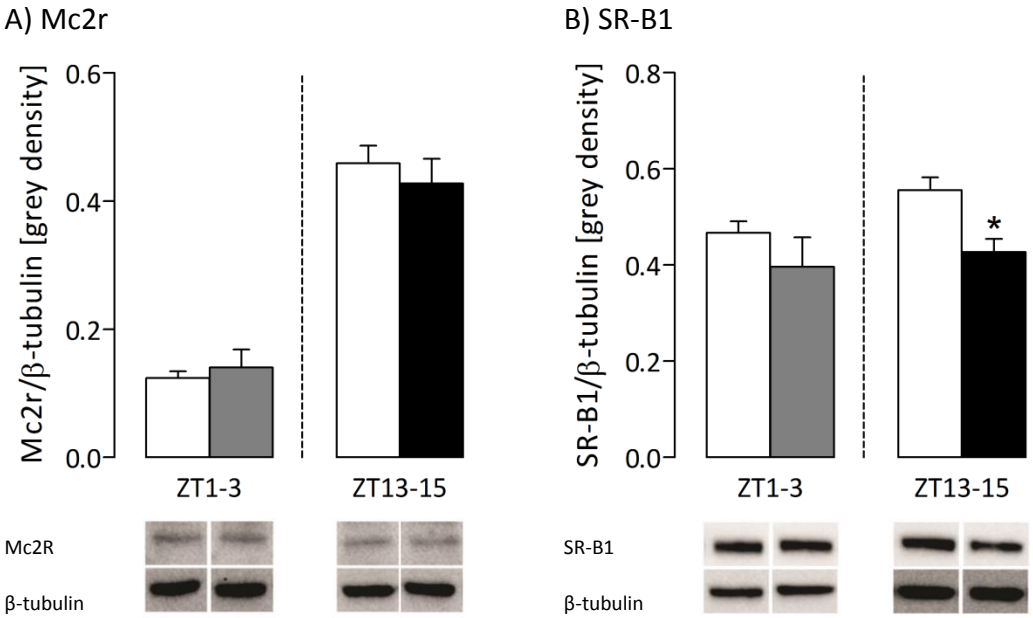


Figure 19: Effects of 19 days of SDL and SDD on relative Mc2r (A) and SR-B1 (B) protein expression in the left adrenal gland. Following decapitation under brief inhalation anesthesia on day 20 in the light phase (ZT1 – 3, time of previous SDL exposure), left adrenal glands were removed from SHC (white bars; n = 8) and SDL (grey bars, n = 9) mice. On day 20 in the dark phase (ZT13 – 15, time of previous SDD exposure), left adrenal glands were removed from another set of SHC (white bars, n = 8) and SDD (black bars, n = 9) mice. Subsequently, protein was extracted from the adrenal glands for determination of relative Mc2r and SR-B1 protein expression [grey density], all normalized to the loading control β-tubulin. Data represent the mean + SEM. * represent $p \leq 0.05$ vs respective SHC mice. Representative images of bands detected for Mc2r (~45 kDa; A) and SR-B1 (~ 76 kDa; B) and respective loading control β-tubulin (~ 55 kDa; A/B) are shown for SHC+SDL and SHC+SDD mice.

3.6.3.3. LDLR and HSL protein expression

Statistical analysis employing Student's t-test showed that LDLR protein expression was not affected by SDL and SDD (Fig. 20A).

Relative HSL protein expression was significantly reduced following SDL ($p \leq 0.05$), but not SDD, as revealed by t-test analysis (Fig. 20B).

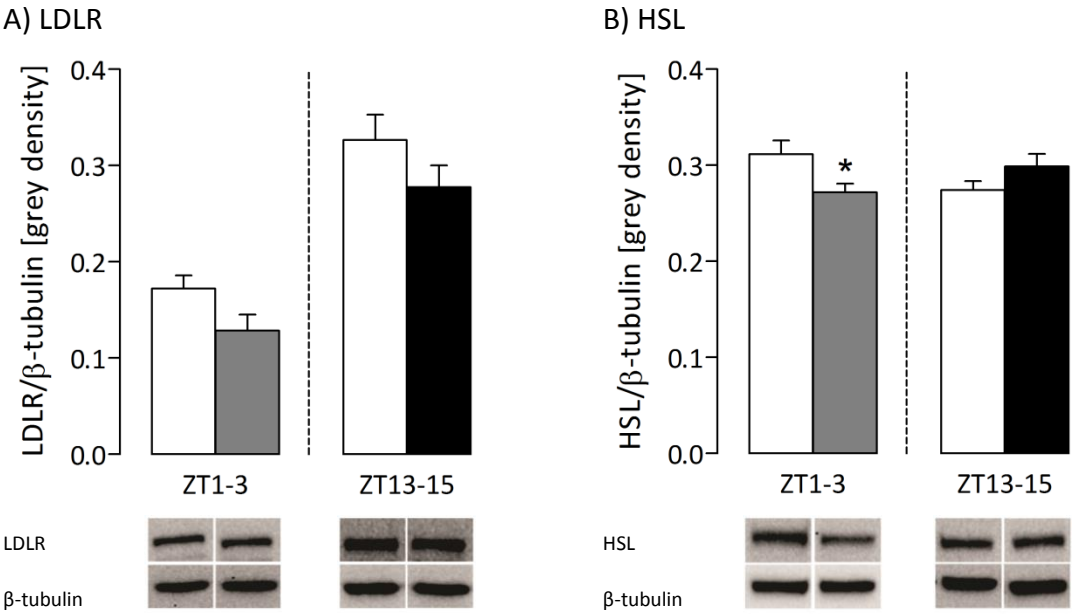


Figure 20: Effects of 19 days of SDL and SDD on relative LDLR (A) and HSL (B) protein expression in the left adrenal gland. Following decapitation under brief inhalation anesthesia on day 20 in the light phase (ZT1 – 3, time of previous SDL exposure), left adrenal glands were removed from SHC (white bars; n = 8) and SDL (grey bars, n = 9) mice. On day 20 in the dark phase (ZT13 – 15, time of previous SDD exposure), left adrenal glands were removed from another set of SHC (white bars, n = 8) and SDD (black bars, n = 9) mice. Subsequently, protein was extracted from the adrenal glands for determination of relative LDLR and HSL protein expression [grey density], all normalized to the loading control β-tubulin. Data represent the mean + SEM. * represent $p \leq 0.05$ vs respective SHC mice. Representative images of bands detected for LDLR (~150 kDa; A) and HSL (~ 82 kDa; B) and respective loading control β-tubulin (~ 55 kDa; A/B) are shown for SHC+SDL and SHC+SDD mice.

3.6.3.4. HMGCR protein expression

T-test analysis showed that SDL mice exhibited significantly lower HMGCR protein expression than respective SHC mice ($p \leq 0.01$) (Fig. 21).

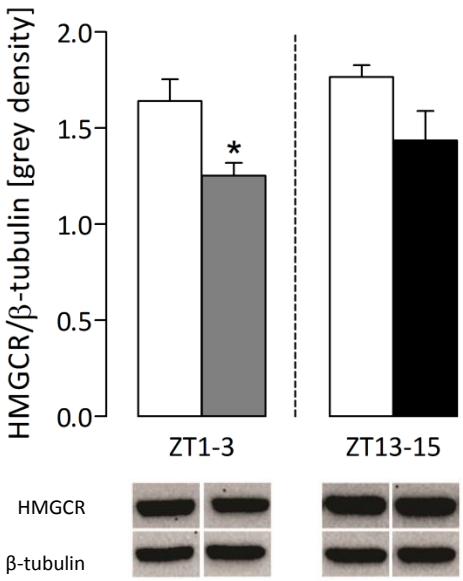


Figure 21: Effects of 19 days of SDL and SDD on relative HMGCR protein expression in the left adrenal gland. Following decapitation under brief inhalation anesthesia on day 20 in the light phase (ZT1 – 3, time of previous SDL exposure), left adrenal glands were removed from SHC (white bars; n = 8) and SDL (grey bars, n = 9) mice. On day 20 in the dark phase (ZT13 – 15, time of previous SDD exposure), left adrenal glands were removed from another set of SHC (white bars, n = 8) and SDD (black bars, n = 9) mice. Subsequently, protein was extracted from the adrenal glands for determination of relative HMGCR protein expression [grey density], normalized to the loading control β-tubulin. Data represent the mean + SEM. * represent $p \leq 0.05$ vs respective SHC mice. Representative images of bands detected for HMGCR (~76 kDa) and respective loading control β-tubulin (~ 55 kDa) are shown for SHC+SDL and SHC+SDD mice.

3.6.3.5. Adrenal cortical lipids

Adrenal cholesterol stores were neither dependent on the SD paradigm employed nor on the time of day of tissue collection (Fig. 22).

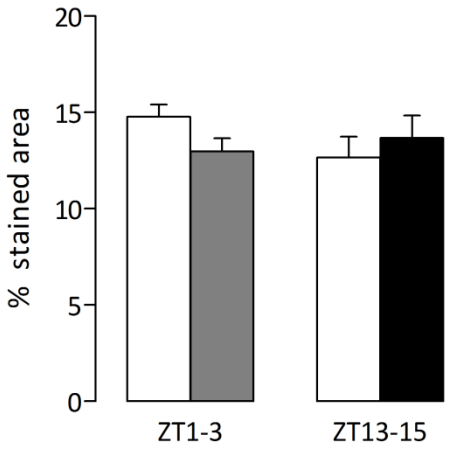


Figure 22: Effects of 19 days of SDL and SDD on cortical lipid droplets in the right adrenal gland. Following decapitation under brief inhalation anesthesia on day 20 in the light phase (ZT1 – 3, time of previous SDL exposure), right adrenal glands were removed from SHC (white bars; n = 7) and SDL (grey bars, n = 9) mice. On day 20 in the dark phase (ZT13 – 15, time of previous SDD exposure), right adrenal glands were removed from another set of SHC (white bars, n = 8) and SDD (black bars, n = 9) mice. Subsequently, right adrenals were cut on a cryostat and one series of cryo-sections was stained with Oil-red for lipid vesicles quantification. Per animal, six different sections were analyzed and averaged for the percentage of stained lipid droplets in relation to the total cortex area. Data represent the mean + SEM.

3.6.4. Hippocampal free corticosterone rhythm

The CORT rhythm within the hippocampus was found to be dependent on the time of day ($F_{5,100}$ = 12.68; p ≤ 0.001) (Fig. 23). *Post hoc* tests showed that 5 minutes of forced swim stress at ZT6 induced a rise in hippocampal free CORT (p ≤ 0.05) in SDL and SDD mice between ZT6.5 and ZT8.5 in the light phase. Towards the end of the light phase (ZT10.5 – ZT12.5), SHC and SDL mice showed a significant increase in free hippocampal CORT concentrations (p ≤ 0.05) compared with levels measured two hours before.

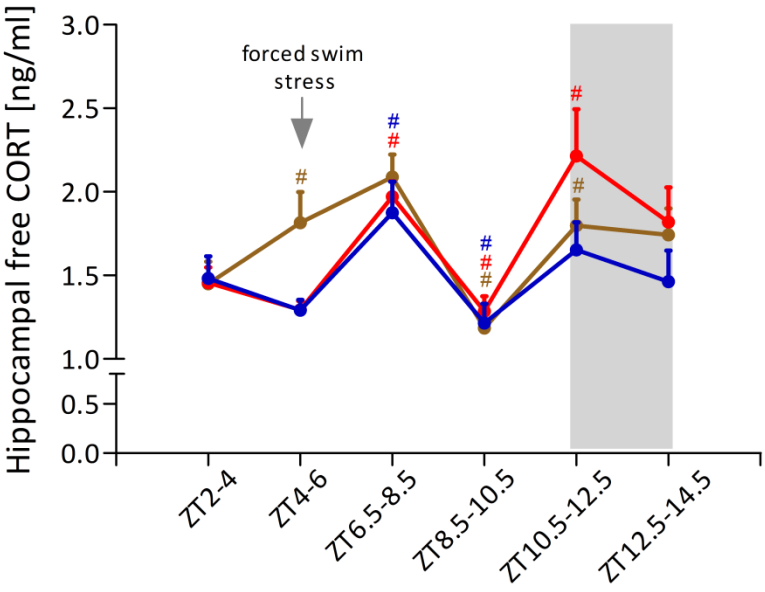


Figure 23: Effects of 19 days of SDL and SDD on hippocampal free CORT levels. SHC, SDL, and SDD mice were implanted stereotaxically with a microdialysis probe within the right dorsal hippocampus on day 20. On day 22, microdialysis samples of SHC (brown lines; n = 8), SDL (red lines, n = 7), and SDD (blue lines, n = 8) mice were collected in 60 min intervals between ZT2 and ZT14.5. At ZT6, mice were exposed to a forced swim stress for 5 min and, afterwards, sampling was continued in 60 min intervals from ZT6.5 until ZT14.5. The grey bar indicates the dark phase (starting at ZT12). Data represent the mean + SEM. # represent p ≤ 0.05 vs previous sample of the respective group.

CHAPTER 4

GENERAL DISCUSSION

4.1. DISCUSSION OF THE PRESENT RESULTS

In this thesis, data are presented which describe the effects of repeated SD on various (1) behavioral, physiological/endocrine and immunological/inflammatory and (2) peripheral and central clock parameters. Parts of these data have been published or are currently submitted for publication and have, therefore, been already discussed in detail (see full text papers and manuscript starting from page 152). Nonetheless, I will briefly discuss some of these data here in the context of unpublished results and the current literature again, as some recent publications have not been taken into account in the previous discussion sections yet.

4.1.1. Metabolism and physiology

Body weight

One of the most profound alterations resulting from social subordination is a reduction in body weight. In line, several rodent studies have reported decreased body weights and/or body weight gain during and following acute and repeated/chronic SD (Meerlo et al., 1996a; Koolhaas et al., 1997; Buwalda et al., 2005; Czeh et al., 2007; Iio et al., 2014). In our work of 2012 (Bartlang et al., 2012), we reported that delta body weight between day 0 and day 20 was not affected by 19 days of SDL and SDD. Since these data do not allow any conclusions to be drawn on body weight development during the actual SD procedure, we aimed to refine our previous findings by a daily body weight assessment. In line with the aforementioned literature, body weight measured between ZT7 and ZT8 (i.e. 4 – 5 h after termination of SDL and 16 – 17 h after termination of SDD) was significantly reduced in SDL, but not SDD, mice following one single stressor exposure. Given

that body weight measured between ZT19 and ZT20 was not different between the three groups, our findings indicate that body weight is particularly affected by SD at the beginning of the light phase, but not dark phase. In support, Rybkin et al. (1997) reported a greater body weight loss in rats restrained acutely at the beginning of the light cycle compared to the dark cycle (Rybkin et al., 1997). Furthermore, in another study body weight was not affected by acute restraint stress during the dark phase (Gorka and Adamik, 1993).

The decreased body weight and body weight gain in SDL mice compared to SHC and SDD mice (assessed daily between ZT7 to ZT8) was evident throughout the whole psychosocial stress procedure. However, this effect was only transient, since body weight was comparable between all three treatment groups on stress-free days (day 7, 14, and 20), indicating a fast recovery of SDL mice following cessation of stressor exposure. In line, dynamic body weight in response to subordination stress has been observed in the visible burrow system (VBS), a rodent model of chronic social stress, in which mixed gender colonies are housed together for two weeks and male rats/mice rapidly develop a dominance hierarchy (Arakawa et al., 2007; Tamashiro et al., 2007). Subordinate male rats within these VBS groups showed a rapid and sustained body weight loss throughout the 14 days (Blanchard et al., 1993; Blanchard et al., 1995; Tamashiro et al., 2004). When removed from their VBS colonies and allowed to recover for 21 days in individual cages, subordinate animals regained body weight.

Food intake

Possible mechanisms for a decreased body weight during repeated SD include reduced food intake (Iio et al., 2012) and stress-induced enhancement of metabolic activity (Johren et al., 1991; Kramer et al., 1999). Thus, daily food intake was determined between ZT7 and ZT8. Interestingly, all three treatment groups showed a fluctuating food consumption with higher intake on stress-free days (day 7, 14, and 20) compared with days of SD exposure. However, this was significantly less pronounced in SDD compared with both SHC and SDL mice. Given that food intake has been measured between ZT7 and ZT8, it is likely that the attenuated increase in food intake of SDD mice on stress-free days is due to the fact that they are expecting the next SD to happen in the beginning of the dark phase on these days as well. The observation that SHC mice also displayed variable food consumption, namely an increase on stress-free days for SDL and SDD mice, suggests that these mice show empathic behavior with their conspecifics exposed to SD in the same animal room. In support, emotional empathy in response to observing conspecific distress has been reported for several species, such as rats (Ben-Ami Bartal et al., 2011), mice (Langford et al., 2006), and pigeons (Watanabe and Ono, 1986).

As food intake was comparable between the three groups on days of SD exposure, reduced body weight/body weight gain of SDL mice on these days cannot be ascribed to diminished food consumption. As mentioned above, differences in metabolic activity might also be due to alterations in HPA axis activity. In line, mean body temperature was significantly elevated in WT SDL mice during the first half of the light phase (ZT0 to ZT6) throughout the 19 days of SD exposure, whereas SDD did not result in increased body temperature compared to SHC mice at any time of the day during this period (Bartlang et al., submitted). Akin to body temperature,

the more persistent increase in adrenal mass following 19 days of SDL compared to SDD (Bartlang et al., 2012) further indicates a hypermetabolic state in SDL mice, given that GCs are critically involved in the regulation of energy supply by increasing catabolic metabolism. Though future studies assessing heart rate and respiration measures are needed to provide additional insight, the present data support the idea of a higher metabolic rate in SDL mice that might be causative for the body weight loss during the SD paradigm.

Ghrelin and Leptin

Body weight and food intake are centrally regulated by the hypothalamus that contains numerous neuronal systems, which play important roles in the regulation of energy homeostasis (Woods et al., 1998). Amongst the hypothalamic centers associated with the regulation of energy balance are the PVN, DMH, and the ARC. Of those, the ARC is of particular interest as it integrates signals not only from the brain stem, but also the periphery, given its feature of a circumventricular organ (Cone et al., 2001; Cowley et al., 2003). Hypothalamic ARC circuits are directly responsive to a number of circulating hunger and satiety signals, such as the anorexigenic hormone leptin and the orexigenic hormone ghrelin (Minor et al., 2009). The peripheral information about the current state of energy availability is integrated by first-order neurons within the ARC, which translate and convey this message to second-order neuron centers of the DMH and the PVN (Schwartz and Porte, 2005). Under basal conditions, leptin and ghrelin display distinct diurnal rhythms with their peak values occurring at opposite times of the day (in the rat plasma leptin peaks at ZT17, whereas plasma ghrelin peaks at ZT5) (Bodosi et al., 2004). While the adipocyte-derived leptin serves as satiety signal, resulting in

decreased food intake (Elmquist et al., 1998), ghrelin, synthesized predominantly by specialized gastrointestinal endocrine cells, stimulates food intake and increases body weight (Tschop et al., 2000; van der Lely et al., 2004). Besides its well-known rise during periods of energy insufficiency, several recent studies have demonstrated stress-associated elevations of ghrelin. For instance, increased plasma and serum concentrations of ghrelin have been observed in response to repeated/chronic SD that might persist for some time beyond the duration of the stress protocol (Lutter et al., 2008b; Patterson et al., 2013). In contrast, we did not observe any difference in plasma ghrelin between the three treatment groups. However, although our data do not indicate any effect of repeated SD on ghrelin levels, we cannot exclude that concentrations measured at other time points than at ZT5 (peak) and ZT17 (trough) were different between the groups.

In accordance with the disturbed plasma CORT rhythm in SDD mice (Bartlang et al., 2012), a lack of diurnal differences in plasma leptin concentrations was observed in SDD mice, indicating an absent or shifted leptin rhythmicity. Given the presence of GRs within fat cells (Feldman and Loose, 1977) and the well-established influence of GCs on adipocytes (Campbell et al., 2011; Lee et al., 2013), a disturbed plasma CORT rhythm might affect the release of leptin by adipose tissue, thereby influencing the plasma leptin rhythm. Although gross (i.e. twice) analyses of plasma CORT and leptin concentrations may not be sufficient to examine the impact of SD on the output of the circadian clock, these data suggest that SDD disrupts peripheral hormonal rhythms. In contrast to repeated SDD, leptin levels on day 20 (ZT17) and day 21 (ZT5) were not altered following 19 days of SDL. In line, the reduction in body weight of SDL mice was restricted to days of stressor exposure, while body weight was similar to SHC mice on stress-free days (day 7, 14, and 20). In order to

shed light on the underlying cause of body weight reduction of SDL mice, the measurement of hunger and satiety hormones on days of SD exposure would be of certain interest.

Considering the reciprocal relationship between the SCN and the ARC (Yi et al., 2006) and the aforementioned neuronal connections between the ARC and the PVN as well as the SCN and the PVN, alterations in plasma CORT and leptin levels allude to changes in the central circadian pacemaker. This assumption is supported by our finding showing higher PER2::LUC rhythm amplitude and increased PER2 protein expression in the SCN of SDD mice (Bartlang et al., 2014). Moreover, the functional output of the biological clock was considerably affected by repeated SDD. This was reflected by an absent or phase-delayed activity peak at ZT13 in WT SDD mice in the entrained 14-day post-stress period under LD conditions (Bartlang et al., submitted). Since this effect was absent in *Per1/Per2* double-mutant mice, which lack a functional clock, SDD-induced alterations in the circadian output are related to changes in the central circadian clock. Considering the entrainment of peripheral oscillators, such as the adrenal gland and fat cells, by the central clock in the SCN, the observed changes in CORT and leptin rhythmicity might emanate from stress-induced alterations in the central pacemaker. Taking into account that CORT and leptin constitute systemic feedback signals to brain areas (the PVN and the ARC) that are connected to the SCN in a multimodal fashion, it is also possible that changes in the SCN result from alterations in peripheral rhythms. Although either mechanism is possible, the second option is more plausible given the robustness and the resilience of the central circadian pacemaker. Thus, it is reasonable to deduce that repeated SDD results in a disruption of the HPA axis and, consequently, of the plasma CORT rhythm. Given the aforementioned influence of GCs on

adipocytes, the disturbed plasma CORT rhythm might affect leptin levels and, thus, lead to a disturbed rhythm in plasma leptin and probably further peripheral hormonal rhythms. CORT and leptin, in turn, provide feedback to the PVN and ARC via their respective receptors. Based on the multimodal connection between the SCN, the ARC, and the PVN, the increased PER2::LUC rhythm amplitude and PER2 protein expression in the central circadian pacemaker might be counteractive measures to the flattening of peripheral output rhythms.

4.1.2. Hypothalamic-pituitary-adrenal axis

It is important to note that HPA axis measures on day 20 have been performed at the times of previous SDL (ZT1 to ZT3) and SDD (ZT13 to ZT15) exposure, respectively, in order to reveal possible adaptational effects in response to the repeated predictable stressor exposure.

Hypothalamus

When released together from the parvocellular PVN into portal capillaries, AVP potentiates the ACTH-releasing activity of CRH (Gillies et al., 1982). In addition, AVP from the magnocellular SON (Wotjak et al., 2002) and PVN (Wotjak et al., 1996) might contribute to HPA axis regulation by fine-tuning ACTH secretion (Landgraf, 2005). Several findings have pointed towards a central role of the two major HPA axis mediators, CRH and AVP, in the neurobiology of depression. For instance, an elevated number of CRH-expressing neurons and increased CRH mRNA in the PVN have been reported for depressed patients (Raadsheer et al., 1994; Raadsheer et al., 1995). On the other hand, a higher number of AVP-expressing neurons in the PVN (Purba et al., 1996) and enhanced AVP mRNA production in the SON (Meynen et al., 2006) are commonly associated with MDD. Furthermore, an increased number of AVP-expressing neurons and a decreased amount of AVP mRNA in the SCN have been observed in depressed subjects (Zhou et al., 2001).

Likewise, a variety of repeated/chronic stress paradigms, such as restraint, foot shock, and social subordination cause a reliable increase in CRH mRNA expression within the parvocellular PVN (Imaki et al., 1991; Sawchenko et al., 1993; Makino et al., 1995; Albeck et al., 1997). Akin to human studies, repeated/chronic stress in rats generally increases AVP mRNA in the PVN (Aguilera, 1994; Makino et al., 1995; Ma

and Lightman, 1998). Moreover, accumulating evidence suggests an influence of acute stressor exposure, such as swim stress and foot shock, on the release of AVP within the SCN (Engelmann et al., 1998) and AVP mRNA levels in the central circadian pacemaker (Handa et al., 2007).

Given the decreased interest in an unfamiliar conspecific, the loss of general activity, the shift towards a more pro-inflammatory body milieu, the rhythm disturbances in plasma CORT (Bartlang et al., 2012) and leptin, and the decreased food intake, all representing well-accepted hallmarks of depression, displayed by SDD mice, we aimed to assess CRH and AVP mRNA in different hypothalamic brain regions. However, in contrast to the existing literature mentioned above, CRH mRNA was not affected in any hypothalamic brain region assessed. It is important to note that, whereas CRH serves as the principal stimulus of ACTH secretion in acute stressful situations, a switch from CRH to AVP-driven ACTH release occurs in response to repeated/chronic stressor exposure (Scott and Dinan, 1998; Makino et al., 2002). This is manifested by enhanced AVP synthesis in CRH-producing cells within the parvocellular PVN following repeated/chronic stress, such as restraint (De Goeij et al., 1992; Aguilera, 1994) and foot shock (Sawchenko et al., 1993). Thus, CRH mRNA must not necessarily be altered after repeated SD. In line, no change in CRH mRNA in the PVN has been observed upon chronic social stress in the VBS (Choi et al., 2006) and repeated exposure of rats to cold stress (Hatalski et al., 1998). Decreased CRH mRNA in the PVN resulting from chronic inflammatory stress (Harbuz et al., 1992) further demonstrates that stress-induced effects on CRH transcripts are stressor specific. Of note, only one study (Reghunandanan and Reghunandanan, 2006) described the existence of CRH mRNA in the SCN, whereas, at least to my knowledge, no similar published reports are present so far.

With respect to AVP mRNA levels in the PVN, SON, and SCN, no differences between SHC, SDL, and SDD mice were observed. In line, others have also shown no effects of chronic stress on AVP mRNA levels in these hypothalamic brain regions (Herman et al., 1989; Herman et al., 1995; Choi et al., 2006). The observed lack of difference in AVP mRNA in the SCN could be explained by the fact that GCs influence the expression of AVP only during a precise time window in the diurnal cycle (i.e. in the afternoon), coinciding with the time where entrainment of the circadian pacemaker with non-photic cues is possible. In this regard, chronic administration of exogenous GCs significantly enhanced AVP mRNA expression only at ZT5, while rhythmic expression of AVP mRNA was unaffected at ZT11, ZT17, and ZT23 (Larsen et al., 1994). Given the unchanged plasma CORT levels of SDL mice decapitated between ZT1 to ZT3 and the decapitation of SDD mice between ZT13 to ZT15 (i.e. at a time where GCs are ineffective), no influence on AVP mRNA in the SCN of SDL and SDD mice is deemed likely.

Importantly, while a plethora of studies has demonstrated that the AVP mRNA content of the SON and PVN is increased upon chronic osmotic stress (Burbach et al., 1984; Sherman et al., 1986; Zingg et al., 1986; Lightman and Young, 1987), duration and frequency of certain stressors, such as repeated restraint, are critical factors for the stress-induced alterations of AVP mRNA levels (Ma et al., 1997; Ma and Lightman, 1998). Thus, altered AVP mRNA upon repeated stressor exposure is not a constant finding.

Taken together, since no differences in CRH and AVP mRNA could be detected in the hypothalamic PVN, SON, and SCN, it is tempting to speculate that the depressive-like symptoms displayed by SDD mice do not result from alterations in the CRH and AVP system within these three hypothalamic nuclei. However, it has to

be mentioned that SDL and SDD mice were decapitated on day 20 at a time when they would expect the next stressor exposure (ZT1 – ZT3 for SDL mice, ZT13 – ZT15 for SDD mice), thus it might be that changes in CRH and AVP mRNA would be apparent at different times of the diurnal cycle, such as at their peak expression. Yet, while peak expression of CRH mRNA in the rat PVN has been observed in the afternoon (Kwak et al., 1993), an absent diurnal AVP mRNA rhythmicity in the SON and PVN has been reported (Uhl and Reppert, 1986).

Pituitary gland

Increased pituitary gland weight is often used as hallmark sign of repeated/chronic stress, as shown for chronic subordinate colony housing (Füchsl et al., 2013a) and chronic immobilization (Kapitonova et al., 2010). Following SDL and SDD, no alterations in pituitary weight could be detected, which is consistent with the study of Slattery et al., showing that SD/overcrowding had no effect on absolute and relative pituitary weight (Slattery et al., 2011).

Surprisingly, and in contrast to unaffected pituitary weight, plasma ACTH was decreased in SDD mice on day 20 between ZT13 – ZT15 (corresponding to the time of previous SDD exposure), while circulating ACTH levels were not altered in SDL mice between ZT1 to ZT3 (corresponding to the time of previous SDL exposure). As outlined in the introduction, the absence or presence of an HPA axis response at a certain time of day is specific for the type of stimulus encountered by an organism (Dunn et al., 1972; Engeland et al., 1977; Kant et al., 1986). Decreased plasma ACTH levels between ZT13 to ZT15, following 19 days of SDD, suggest an adjustment of HPA axis activity to repeated predictable SDD. Whereas adaptation to repeated psychosocial stress has been demonstrated in humans (Gerra et al., 2001; Wust et

al., 2005), SD in rodents generally does not produce habituation upon repeated exposure (Tornatzky and Miczek, 1993). However, taking into account the threefold higher plasma ACTH concentrations 25 min following SDD on day 4 (Bartlang et al., 2012), it is reasonable to deduce that 19 days of SDD result in an alleviated HPA axis response to the predictable stressor at the level of the pituitary. Of note, although adaptation is considered a beneficial self-adjusting ability, plasma ACTH and concentrations below control levels might indicate an "over-adjustment", thus representing a failure in self-adjusting abilities. The depressive-like symptoms exhibited by SDD mice (Bartlang et al., 2012) are in favor of a decreased HPA axis activity to be maladaptive. On the other hand, it is important to note that circulating ACTH levels have been assessed on day 20 at the anticipated time of SDD exposure. This limits the generalizability of the results and deserves further study, since plasma ACTH levels have not been assessed directly after the last SD on day 19.

ACTH secretion is regulated in a multifactorial way, involving the stimulatory input of hypothalamic CRH and AVP as well as the inhibitory effect of GCs (Antoni, 1986). Consistent with the decreased concentrations of plasma ACTH, CRH-R1 and AVP-R1b protein expression was reduced in the pituitaries of SDD mice on day 20 between ZT13 to ZT15. As CRH and AVP are considered important ACTH secretagogues, decreased ACTH secretion by the pituitary gland of SDD mice might be attributed to the reduced CRH-R1 and AVP-R1b protein expression in the pituitary. Although CRH receptor numbers are not considered a major determinant of corticotrope responsiveness, the severely blunted ACTH responses in CRH and CRH-R1 knockout mice indicate that CRH, and at least a certain amount of CRH-R1, are required for the responsiveness of the pituitary gland to most stressors

(Turnbull and Rivier, 1997; Venihaki and Majzoub, 1999; Aguilera et al., 2004). Given that decreased plasma ACTH levels and down-regulated CRH-R1 and AVP-R1b were accompanied by reduced plasma CORT concentrations in SDD mice at the same time, it is reasonable to speculate that predictable exposure to SD during the active phase increases sensitivity of the negative feedback system of the HPA axis. This might be accomplished by an enhanced number and/or GC sensitivity of GRs and MRs at different levels of the HPA axis, resulting in lower CORT concentrations to be sufficient for enhanced negative feedback inhibition. This hypothesis needs further experimental evaluations, for instance determination of GR/MR mRNA and protein expression as well as functionality at different levels of the HPA axis. With respect to GR functionality, a dexamethasone suppression test would offer valuable clues to determine the feedback response at the level of the pituitary gland (Cole et al., 2000). In healthy subjects, administration of the synthetic GC dexamethasone initiates engagement of negative HPA axis feedback inhibition, thus reducing circulating CORT levels (Butler et al., 2013). Hence, if GC sensitivity is indeed increased in SDD mice, prolonged GC suppression would be expected.

In conclusion, changes at the central component of the HPA axis, the pituitary gland, occur mainly following repeated SDD. Given the more negative outcome of SDD vs SDL, it is reasonable to deduce that the changes at the pituitary level, namely a reduction in plasma ACTH as well as CRH-R1 and AVP-R1b protein expression at activity onset, represent a failure in HPA axis adjustment.

Adrenal gland

Besides pituitary gland weight, repeated/chronic stress commonly increases adrenal gland weight. In line, absolute adrenal weight on day 20 was increased in SDL mice at ZT1 – ZT3 and SDD mice between ZT13 to ZT15, respectively. Stress-induced changes in adrenal growth may either be due to cellular hyperplasia (increase in the number of cells) or hypertrophy (increased cell size). Given that cellular hypertrophy and hyperplasia in the zona fasciculata of the adrenal gland have been associated with increased adrenal responsiveness to ACTH (Ulrich-Lai et al., 2006b), it would be interesting to determine the causative factor for adrenal enlargement in SDL mice given the attenuated adrenal responsiveness to ACTH *in vitro* following 19 days of SDL (Bartlang et al., 2012).

With respect to plasma CORT levels, decreased concentrations between ZT13 – ZT15 on day 20 could be observed in SDD mice, while circulating CORT was not affected by SDL. In support, several studies revealed hypocorticism as a consequence of repeated/chronic stressor exposure (Sanchez et al., 1998; Malkesman et al., 2006; Reber et al., 2006; Adzic et al., 2009). Causative factors for hypocorticism involve (1) reduced biosynthesis or release of the respective releasing hormones on various HPA axis levels (i.e. CRH/AVP from the hypothalamus, ACTH from the pituitary gland, and GCs from the adrenal gland) with a concomitant reduced stimulation of the respective target receptors, (2) hypersecretion of one secretagogue with subsequent down-regulation of the respective target receptors, and (3) enhanced sensitivity to GC feedback (Heim et al., 2000; Raison and Miller, 2003; Fries et al., 2005).

Consistent with the reduced plasma CORT levels on day 20 between ZT13 to ZT15, plasma ACTH concentrations were decreased in SDD mice at that time period, as

aforementioned. Hence, a reduction in circulating CORT might result from decreased ACTH release from the pituitary gland, followed by reduced Mc2r stimulation. Despite unchanged Mc2r protein expression, decreased stimulation of the adrenal target receptor for ACTH is conceivable. Upon ligand binding, the Mc2r undergoes conformational changes that stimulate adenylyl cyclase, resulting in elevated intracellular cAMP and subsequent activation of protein kinase A (Xing et al., 2010). Thus, assessment of *in vitro* cAMP and protein kinase A responses to ACTH would be helpful to investigate the status of Mc2r stimulation.

Furthermore, hypocorticism might also arise from reduced CORT biosynthesis. As stated in the introduction, CORT is synthesized from cholesterol by multiple delivery pathways, including the endocytic and selective uptake of cholesteryl esters via LDLR and SR-B1, respectively (Kraemer, 2007). In line with the decreased plasma CORT levels, SR-B1 protein expression was reduced in SDD mice, implicating that the selective uptake of LDL and high density lipoprotein cholesteryl esters into the cell might at least partly account for the HPA axis breakdown and rhythm disturbances following repeated predictable stressor exposure in the dark phase. In contrast, LDLR protein expression in the adrenal gland was not affected. Evidence for a different modulation of adrenal LDLR and SR-B1 expression comes from the study of Füchsl et al., where chronically stressed mice exhibited an increase in adrenal SR-B1 expression without changes in LDLR (Füchsl et al., 2013b).

Cholesterol esters that enter the cell via SR-B1 are further modified by the intracellular neutral lipase HSL. Despite a reduced SR-B1 protein expression, no difference in HSL protein expression between SHC and SDD mice has been found. In contrast, a decrease in HSL could be observed in SDL mice. Despite normal basal CORT levels, the knockdown of the gene encoding for HSL was shown to result in a

prominent reduction of ACTH-induced CORT secretion from isolated adrenal cells (Kraemer and Shen, 2002). Intriguingly, we recently showed that responsiveness of adrenal explants to ACTH *in vitro* was severely blunted in SDL mice compared with SHC and SDD mice (Bartlang et al., 2012). Though further work is required to provide additional insight, these data suggest that a reduction in HSL accounts for the reduced adrenal ACTH sensitivity in SDL mice during the light phase, which is deemed an adaptational process to the predictable stressor.

Along with the reduced HSL protein expression, adrenal HMGCR protein expression was lower in SDL mice compared to SHC animals. This enzyme catalyzes the limiting step in cholesterol synthesis. Thus, the reduction in *de novo* adrenal cholesterol synthesis might be essential to maintain normal basal morning CORT levels despite increased adrenal weight. Commonly, this enzyme is suppressed by cholesterol derived from the internalization and degradation of LDL via the LDLR (Faust et al., 1982). Therefore, assessment of LDL levels would further clarify this issue.

The storage and supply of cholesterol and fatty acids constitute the primary functions of lipid droplets. Importantly, adrenal lipid droplet physiology is strongly affected by ACTH. For instance, depletion of adrenal lipid droplets has been shown following long-term ACTH administration (Mazzocchi et al., 1986). Similarly, exposure of rats to 60 minutes of high temperature, resulting in increased plasma ACTH levels, depleted adrenal lipid droplets (Koko et al., 2004). Furthermore, rats subjected to chronic noise stress exhibited reduced lipid droplets in the zona fasciculata of the adrenal gland (Oliveira et al., 2009). However, oil-red staining in adrenal cryo-sections revealed no differences in the amount of cortical lipid droplets between SHC, SDL, and SDD mice. In support, mice exposed to chronic psychosocial stress had a comparable relative amount of adrenal lipids to

unstressed controls (Füchsl et al., 2013b). This implicates that the effects of repeated/chronic stress on cholesterol esters in the adrenal cortex depend on the species and the stress paradigm employed.

Taken together, it is reasonable to speculate that normal CORT levels, despite enlarged adrenal glands, in SDL mice might be mediated by a decreased HSL expression, which could explain the observed ACTH insensitivity. In contrast, the hypocorticism displayed by SDD mice might arise from the reduced selective uptake of LDL and high density lipoprotein cholesteryl esters into the cell via the SR-B1. As aforementioned, an enhanced sensitivity to GC feedback is also conceivable. Importantly, as plasma hypocorticism (total CORT) does not necessarily mean that also the biological active fraction of free CORT is reduced, assessment of free CORT or CBG levels are of particular interest. Though further studies investigating CBG levels are still warranted, the microdialysis data demonstrate reduced free hippocampal CORT levels in SDD mice between ZT10 – ZT12.5, supporting the idea that plasma hypocorticism (total CORT) in these animals is indeed paralleled by a decrease in free CORT. Hence, a combination of different routes might account for the decreased plasma CORT levels in SDD mice.

Considering the increased HPA axis activity at lights-off in normal healthy mice, decreased CORT levels in SDD vs SHC mice at that time of the day might be interpreted as maladaptive consequence of repeated SDD. In support, a hypoactive HPA axis activity is frequently observed in stress-related disorders, such as chronic fatigue syndrome, burn out, and post-traumatic stress disorder, where an initial stage of HPA axis hyperactivity eventually evolves into hypoactivity (Fries et al., 2005; Miller et al., 2007). Unfortunately, no CORT measures following acute SD are available so far. In order to shed light on the underlying cause and onset of

hypocorticism, as well as a possible relation between HPA axis activity and depressive-like symptoms in SDD mice, it would be of particular interest to measure the 24 h CORT rhythm after an acute SD and at the end of the SD paradigm. This issue has been addressed together with Prof. Dr. Henrik Oster from the University of Lübeck by non-invasive fecal CORT metabolite measures, and is currently under investigation.

4.1.3. Final conclusions

It was already proposed in the 60's and 70's of the last century that, besides its role in controlling the secretion of hormones under basal conditions, the circadian clock modulates the responsiveness of hormonal systems, such as the HPA axis, to environmental stimuli (Zimmermann and Critchlow, 1967; Gibbs, 1970; Dunn et al., 1972). At present, it is generally accepted that exposure to psychological stressors, such as novel environment, elicits a greater HPA axis activation in the early morning compared to the early evening in rats (Gallant, 1979; Torrellas et al., 1981; Kant et al., 1986). In contrast, physical stressors cause the largest stress response at the onset of activity (Dickmeis, 2009). Although the potential interaction of the time of day of stressor exposure and the stress response is commonly acknowledged nowadays, only few studies have addressed this issue so far. This PhD project aimed to refine previous findings concerning the mutual interactions between psychosocial stress and the circadian clock in C57BL/6 mice. In collaboration with several helpful co-workers, I have tried to tap the full range of available techniques in order to shed some light on this issue. I will draw my final conclusions of this project in the following.

In the present thesis it is shown that, besides the well-established physical and psychological stressors, psychosocial ones also modulate the responsiveness of the stress system, depending on the time of day of stressor exposure; a phenomenon that, at least to my knowledge, has never been described before. Given that psychosocial stressors more closely resemble the human conditions, these findings are of clinical relevance. Exposure to 19 days of SDD results in a more negative outcome compared to repeated SDL exposure. Moreover, different levels of the mammalian circadian clock, namely the central pacemaker in the SCN, the

subordinated adrenal clock, and various peripheral output rhythms, are affected by repeated psychosocial stressor exposure, depending on the time of day of stressor presentation. These differences concerning the circadian system might be the prerequisites, or consequences, of either adaptive or maladaptive behavioral, physiological, and immunological alterations following repeated SDL and SDD, respectively. It remains to be elucidated whether the alterations in the SCN precede the peripheral changes or are rather a result of disturbed peripheral output rhythms.

One possible mechanism underlying the diurnal differences in stress effects might be that the time of stressor presentation for SDD mice coincidences with the time at which the clock responds very sensitive to light with phase delays, whereas it does barely respond during the day (the time at which SDL mice were exposed to the stressor). Thus, the time window at the beginning of the night is especially important for the resetting processes of the circadian clock, required for the appropriate timing of physiological and behavioral processes. Consequently, repeated stressor exposure during the early dark phase would lead to circadian misalignment. The depressive-like behavior, the hormonal rhythm disturbances, as well as the increased IFN-γ secretion and severity of DSS-induced colitis, all representing well-accepted hallmark signs of shift-work disorders, are in favor of this possibility.

Although the mechanism underlying the differences in the stress response between SDL and SDD mice could not be revealed within the scope of the present thesis, the findings provide deeper insight into the interaction between the circadian and the stress system and provide a basis for further investigation in future studies.

4.2. ESTABLISHED TECHNIQUES – PERFORMANCE AND ANALYSIS

In the course of my dissertation I attempted to establish different techniques in order to measure the diurnal CORT rhythm (*in vivo* and *in vitro*), which I will discuss in the following.

4.2.1. Assessment of the corticosterone rhythm in the hippocampus by *in vivo* microdialysis

Given that free CORT levels in the rat brain also show a circadian and ultradian rhythm (Droste et al., 2008; Droste et al., 2009), I aimed to establish the *in vivo* microdialysis technique used in the studies mentioned above in mice because of three reasons. First, I wanted to assess whether the time of day of stressor exposure impacts the basal circadian CORT rhythm. Second, I tried to show that the stress response (i.e. CORT release) to a novel, heterotypic stressor can be measured in the same conscious and freely behaving animals. Third, solely the free (i.e. biologically active) fraction of CORT is determined by microdialysis in the brain (Linthorst et al., 1994; Droste et al., 2008). This is due to the fact that the brain is devoid of CBGs that mediate the GC transport in the plasma by sequestrating GCs, thus inhibiting their activity on target cells.

The protocol I used for *in vivo* microdialysis in mice was adapted from the aforementioned studies of Droste et al. (2008, 2009), in which microdialysis was performed in the hippocampus of female and male rats. Since the hippocampus plays a pivotal role in HPA axis regulation, this brain site was also selected as target region in my experiment. The protocol I used is described in detail in section 2.2.1.4.

Due to the absence of CBGs in the brain, I expected very low CORT concentrations in the microdialysates as only 10 % of total CORT is free in the plasma. Given that the more sensitive radioimmunoassay was applied for measuring 10 min or 30 min samples in the study of Droste et al., 60 min sample intervals were chosen and dialysates were lyophilized in the present thesis to ensure that sufficient CORT concentrations were obtained that are detectable by ELISA. Importantly, the direct measurement of free CORT levels in the brain has the advantage over measurement of plasma hormone levels that it provides a better reflection of CORT available for binding to its respective receptors in the brain (Reul et al., 2000).

As shown in Fig. 8, hippocampal free CORT concentrations were increased prior to the onset of the dark (active) phase. Although statistical comparisons did not reveal any significant effect, probably due to the small group size (n = 4), these findings indicate the reliability of the established mouse protocol for assessing the circadian CORT rhythm in the hippocampus. Moreover, given that daytime levels of total plasma CORT are at around 20 ng/ml (Zhang et al., 2009; Costa-Nunes et al., 2014), the obtained CORT concentrations between 0.5 – 2.0 ng/ml (corresponding to 10 % of total plasma CORT) were in the expected range.

Since the obtained results were at the limit of detection, I decided to pool two consecutive 1 h-samples for measuring the CORT rhythm in the hippocampus in the ensuing experiment following 19 days of either SDL or SDD (see 3.6.4). Furthermore, in this experiment I sampled dialysates between ZT2 to ZT14.5, as I was primarily interested in the CORT levels at the time points at which the former stressor exposure took place (SDL: ZT1 – ZT3; SDD: ZT13 – ZT15). Additionally, mice were exposed to a novel heterotypic stressor, i.e. forced swim for 5 min (a combined physical and psychological stressor), and hippocampal CORT levels were assessed to

determine whether repeated SDL and SDD affects the subsequent CORT response to an acute heterotypic stressor differently. Although no difference in the forced swim-induced CORT response within the hippocampus could be seen between SDL and SDD mice, the increase in free hippocampal CORT concentrations at the end of the light phase/beginning of the dark phase was absent in SDD mice only, suggesting disturbances in the diurnal CORT rhythm in these mice (see Fig. 23).

4.2.2. Assessment of *in vitro* corticosterone secretion by adrenal explants

Based on the fact that stimulation of adrenal explants *in vitro* allows assessment of both basal as well as ACTH-induced CORT production/secretion, I aimed to establish the adrenal *in vitro* stimulation across 24 h to reveal possible diurnal differences in the basal and/or ACTH-induced CORT response of adrenal explants between SHC, SDL, and SDD mice. Importantly, the adrenal CORT secretion during *in vitro* stimulation does not reflect the potential of the adrenal to secret CORT at the moment of killing, but at the actual stimulation period (Torres-Farfan et al., 2011; Yoder et al., 2014).

The protocol I used was adapted from the one established in our laboratory before, in which adrenal stimulation was performed over 6 h (Uschold-Schmidt et al., 2012) (for details see materials and methods section of (Bartlang et al., 2012)). In the first pre-experiment, only left adrenal glands were cut into two halves, each containing cortical and medullary tissues. Then, the halves were weighed and pre-incubated in 200 µl DMEM/F-12 for 4 h before any further treatment. Culture medium was then replaced and each half was incubated with medium either containing saline (basal) or saline plus ACTH (100 nM) for 1 h (in contrast to 6 h in the study of (Uschold-

Schmidt et al., 2012)) during a time period of 24 h, starting at ZT6. Following the hourly incubation, supernatants were removed and medium containing saline or ACTH was added again for 1 h. To get a first impression on the feasibility of this technique, only selected time points (ZT6 – 7, ZT8 – 9, ZT9 – 10, ZT11 – 12, ZT12 – 13, ZT 23 – 0, ZT0 – ZT1, ZT5 – 6) were chosen for the analysis by ELISA. As shown in Fig. 9, although basal CORT concentrations continuously declined, an ACTH-induced increase in CORT secretion could be seen until ZT12 – 13, demonstrating the principle validity of this technique. However, the gradual reduction in basal and ACTH-induced CORT concentrations (except for the moderate increase at ZT12) and the complete lack of adrenal responsiveness to ACTH between ZT23 to ZT6 (i.e. the last three time points) suggest a breakdown of adrenal functionality *in vitro*. Therefore, an additional pre-experiment was performed, in which incubation period was extended. Furthermore, to rule out that hourly treatment of adrenal explants is responsible for the steady decline in CORT secretion, left and right adrenal explants were used for *in vitro* stimulation in the follow-up experiment. Thereby, left adrenal glands were treated exactly as in the experiment before, i.e. each half was incubated with medium either containing saline (basal) or saline plus ACTH (100 nM) for 1 h, respectively, across 24 h, starting at ZT8. In contrast, right adrenal glands were incubated with medium (with the medium changed hourly) across 24 h, starting at ZT8, and saline/saline plus ACTH was applied at selected time points (ZT11 – 12, ZT12 – 13, ZT18 – 19, ZT0 – 1, ZT6 – 7, ZT11 – 12, ZT12 – 13). As shown in Fig. 10, CORT concentrations from left and right adrenal glands do not differ markedly from each other, indicating that hourly administration of saline/ACTH is not responsible for the continuous decline. Furthermore, the lack of increase in basal and ACTH-induced CORT secretion at the last two time points (ZT11 – 12 and ZT12 – 13; beginning of the dark phase) suggests a breakdown of adrenal

functionality *in vitro* following several hours in culture. Thus, adrenal *in vitro* stimulation is not suitable for determination of the 24 h CORT rhythm, at least in my hands and with the conditions employed by me.

REFERENCES

Abboud FM (2010) The Walter B. Cannon Memorial Award Lecture, 2009. Physiology in perspective: The wisdom of the body. In search of autonomic balance: the good, the bad, and the ugly. Am J Physiol Regul Integr Comp Physiol 298:R1449-1467.

Abercrombie ED, Keller RW, Jr., Zigmond MJ (1988) Characterization of hippocampal norepinephrine release as measured by microdialysis perfusion: pharmacological and behavioral studies. Neuroscience 27:897-904.

Abrahamson EE, Moore RY (2001) Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. Brain Res 916:172-191.

Adzic M, Djordjevic J, Djordjevic A, Niciforovic A, Demonacos C, Radojcic M, Krstic-Demonacos M (2009) Acute or chronic stress induce cell compartment-specific phosphorylation of glucocorticoid receptor and alter its transcriptional activity in Wistar rat brain. J Endocrinol 202:87-97.

Aguilar-Roblero R, Morin LP, Moore RY (1994) Morphological correlates of circadian rhythm restoration induced by transplantation of the suprachiasmatic nucleus in hamsters. Exp Neurol 130:250-260.

Aguilera G (1994) Regulation of pituitary ACTH secretion during chronic stress. Front Neuroendocrinol 15:321-350.

Aguilera G (2011) HPA axis responsiveness to stress: implications for healthy aging. Exp Gerontol 46:90-95.

Aguilera G, Nikodemova M, Wynn PC, Catt KJ (2004) Corticotropin releasing hormone receptors: two decades later. Peptides 25:319-329.

Albeck DS, McKittrick CR, Blanchard DC, Blanchard RJ, Nikulina J, McEwen BS, Sakai RR (1997) Chronic social stress alters levels of corticotropin-releasing factor and arginine vasopressin mRNA in rat brain. J Neurosci 17:4895-4903.

Albrecht U (2010) Circadian clocks in mood-related behaviors. Ann Med 42:241-251.

Albrecht U (2012) Timing to perfection: the biology of central and peripheral circadian clocks. Neuron 74:246-260.

Anderson PK, Hill JL (1965) Mus musculus: Experimental Induction of Territory Formation. Science 148:1753-1755.

Antoni FA (1986) Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. Endocr Rev 7:351-378.

Arakawa H, Blanchard DC, Blanchard RJ (2007) Colony formation of C57BL/6J mice in visible burrow system: identification of eusocial behaviors in a background strain for genetic animal models of autism. Behav Brain Res 176:27-39.

Armario A, Castellanos JM, Balasch J (1984) Adaptation of anterior pituitary hormones to chronic noise stress in male rats. Behav Neural Biol 41:71-76.

Armario A, Valles A, Dal-Zotto S, Marquez C, Belda X (2004) A single exposure to severe stressors causes long-term desensitisation of the physiological response to the homotypic stressor. Stress 7:157-172.

Aschoff J (1960) Exogenous and endogenous components in circadian rhythms. Cold Spring Harb Symp Quant Biol 25:11-28.

Asher G, Schibler U (2011) Crosstalk between components of circadian and metabolic cycles in mammals. Cell Metab 13:125-137.

Balsalobre A (2002) Clock genes in mammalian peripheral tissues. Cell Tissue Res 309:193-199.

Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schutz G, Schibler U (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 289:2344-2347.

Barclay JL, Tsang AH, Oster H (2012) Interaction of central and peripheral clocks in physiological regulation. Prog Brain Res 199:163-181.

Bartlang MS, Savelyev SA, Johansson AS, Reber SO, Helfrich-Förster C, Lundkvist GB (2014) Repeated psychosocial stress at night, but not day, affects the central molecular clock. Chronobiol Int 31:996-1007.

Bartlang MS, Neumann ID, Slattery DA, Uschold-Schmidt N, Kraus D, Helfrich-Förster C, Reber SO (2012) Time matters: pathological effects of repeated psychosocial stress during the active, but not inactive, phase of male mice. J Endocrinol 215:425-437.

Bartolomucci A, Palanza P, Sacerdote P, Panerai AE, Sgoifo A, Dantzer R, Parmigiani S (2005) Social factors and individual vulnerability to chronic stress exposure. Neurosci Biobehav Rev 29:67-81.

Ben-Ami Bartal I, Decety J, Mason P (2011) Empathy and pro-social behavior in rats. Science 334:1427-1430.

Benedetti F, Dallaspezia S, Fulgosi MC, Lorenzi C, Serretti A, Barbini B, Colombo C, Smeraldi E (2007) Actimetric evidence that CLOCK 3111 T/C SNP influences sleep and activity patterns in patients affected by bipolar depression. Am J Med Genet B Neuropsychiatr Genet 144B:631-635.

Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, Nestler EJ (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science 311:864-868.

Bhatnagar S, Meaney MJ (1995) Hypothalamic-pituitary-adrenal function in chronic intermittently cold-stressed neonatally handled and non handled rats. J Neuroendocrinol 7:97-108.

Bittman EL, Doherty L, Huang L, Paroskie A (2003) Period gene expression in mouse endocrine tissues. Am J Physiol Regul Integr Comp Physiol 285:R561-569.

Blanchard DC, Sakai RR, McEwen B, Weiss SM, Blanchard RJ (1993) Subordination stress: behavioral, brain, and neuroendocrine correlates. Behav Brain Res 58:113-121.

Blanchard DC, Spencer RL, Weiss SM, Blanchard RJ, McEwen B, Sakai RR (1995) Visible burrow system as a model of chronic social stress: behavioral and neuroendocrine correlates. Psychoneuroendocrinology 20:117-134.

Bodosi B, Gardi J, Hajdu I, Szentirmai E, Obal F, Jr., Krueger JM (2004) Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. Am J Physiol Regul Integr Comp Physiol 287:R1071-1079.

Bremner JD, Krystal JH, Southwick SM, Charney DS (1996) Noradrenergic mechanisms in stress and anxiety: I. Preclinical studies. Synapse 23:28-38.

Britton KT, Segal DS, Kuczenski R, Hauger R (1992) Dissociation between in vivo hippocampal norepinephrine response and behavioral/neuroendocrine responses to noise stress in rats. Brain Res 574:125-130.

Buckingham JC (2006) Glucocorticoids: exemplars of multi-tasking. Br J Pharmacol 147 Suppl 1:S258-268.

Buijs RM, Wortel J, Van Heerikhuize JJ, Feenstra MG, Ter Horst GJ, Romijn HJ, Kalsbeek A (1999) Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. Eur J Neurosci 11:1535-1544.

Burbach JP, De Hoop MJ, Schmale H, Richter D, De Kloet ER, Ten Haaf JA, De Wied D (1984) Differential responses to osmotic stress of vasopressin-neurophysin mRNA in hypothalamic nuclei. Neuroendocrinology 39:582-584.

Butler TR, Ariwodola OJ, Weiner JL (2013) The impact of social isolation on HPA axis function, anxiety-like behaviors, and ethanol drinking. Front Integr Neurosci 7:102.

Buwalda B, Kole MH, Veenema AH, Huininga M, de Boer SF, Korte SM, Koolhaas JM (2005) Long-term effects of social stress on brain and behavior: a focus on hippocampal functioning. Neurosci Biobehav Rev 29:83-97.

Caldelas I, Poirel VJ, Sicard B, Pevet P, Challet E (2003) Circadian profile and photic regulation of clock genes in the suprachiasmatic nucleus of a diurnal mammal Arvicanthis ansorgei. Neuroscience 116:583-591.

Campbell JE, Peckett AJ, D'Souza A M, Hawke TJ, Riddell MC (2011) Adipogenic and lipolytic effects of chronic glucocorticoid exposure. Am J Physiol Cell Physiol 300:C198-209.

Cannon WB (1929) Organization for physiological homeostasis. Physiol Rev 9:399-431.

Carnes M, Lent S, Feyzi J, Hazel D (1989) Plasma adrenocorticotropic hormone in the rat demonstrates three different rhythms within 24 h. Neuroendocrinology 50:17-25.

Carnes M, Kalin NH, Lent SJ, Barksdale CM, Brownfield MS (1988) Pulsatile ACTH secretion: variation with time of day and relationship to cortisol. Peptides 9:325-331.

Cascio CS, Shinsako J, Dallman MF (1987) The suprachiasmatic nuclei stimulate evening ACTH secretion in the rat. Brain Res 423:173-178.

Cavigelli SA, Monfort SL, Whitney TK, Mechref YS, Novotny M, McClintock MK (2005) Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. J Endocrinol 184:153-163.

Cho S, Yujnovsky I, Doi M, Sassone-Corsi P (2005) Transplantation of mouse embryo fibroblasts: an approach to study the physiological pathways linking the suprachiasmatic nucleus and peripheral clocks. Methods Enzymol 393:469-478.

Choi DC, Nguyen MM, Tamashiro KL, Ma LY, Sakai RR, Herman JP (2006) Chronic social stress in the visible burrow system modulates stress-related gene expression in the bed nucleus of the stria terminalis. Physiol Behav 89:301-310.

Chrousos GP (1998) Stressors, stress, and neuroendocrine integration of the adaptive response. The 1997 Hans Selye Memorial Lecture. Ann N Y Acad Sci 851:311-335.

Chrousos GP (2009) Stress and disorders of the stress system. Nat Rev Endocrinol 5:374-381.

Cintra A, Bhatnagar M, Chadi G, Tinner B, Lindberg J, Gustafsson JA, Agnati LF, Fuxe K (1994) Glial and neuronal glucocorticoid receptor immunoreactive cell populations in developing, adult, and aging brain. Ann N Y Acad Sci 746:42-61; discussion 61-43.

Cohen S, Janicki-Deverts D, Miller GE (2007) Psychological stress and disease. Jama 298:1685-1687.

Cole MA, Kim PJ, Kalman BA, Spencer RL (2000) Dexamethasone suppression of corticosteroid secretion: evaluation of the site of action by receptor measures and functional studies. Psychoneuroendocrinology 25:151-167.

Cole RJ, Loving RT, Kripke DF (1990) Psychiatric aspects of shiftwork. Occup Med 5:301-314.

Cone RD, Cowley MA, Butler AA, Fan W, Marks DL, Low MJ (2001) The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. Int J Obes Relat Metab Disord 25 Suppl 5:S63-67.

Costa-Nunes J, Zubareva O, Araujo-Correia M, Valenca A, Schroeter CA, Pawluski JL, Vignisse J, Steinbusch H, Hermes D, Phillipines M, Steinbusch HM, Strekalova T (2014) Altered emotionality, hippocampus-dependent performance and expression of NMDA receptor subunit mRNAs in chronically stressed mice. Stress 17:108-116.

Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL (2003) The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37:649-661.

Czeh B, Muller-Keuker JI, Rygula R, Abumaria N, Hiemke C, Domenici E, Fuchs E (2007) Chronic social stress inhibits cell proliferation in the adult medial prefrontal cortex: hemispheric asymmetry and reversal by fluoxetine treatment. Neuropsychopharmacology 32:1490-1503.

Danilenko KV, Putilov AA, Russkikh GS, Duffy LK, Ebbesson SO (1994) Diurnal and seasonal variations of melatonin and serotonin in women with seasonal affective disorder. Arctic Med Res 53:137-145.

De Goeij DC, Dijkstra H, Tilders FJ (1992) Chronic psychosocial stress enhances vasopressin, but not corticotropin-releasing factor, in the external zone of the median eminence of male rats: relationship to subordinate status. Endocrinology 131:847-853.

de Kloet ER, Joels M, Holsboer F (2005) Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6:463-475.

Dhabhar FS (2000) Acute stress enhances while chronic stress suppresses skin immunity. The role of stress hormones and leukocyte trafficking. Ann N Y Acad Sci 917:876-893.

Dibner C, Schibler U, Albrecht U (2010) The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annu Rev Physiol 72:517-549.

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

Dickmeis T, Weger BD, Weger M (2013) The circadian clock and glucocorticoids--interactions across many time scales. Mol Cell Endocrinol 380:2-15.

Droste SK, de Groote L, Lightman SL, Reul JM, Linthorst AC (2009) The ultradian and circadian rhythms of free corticosterone in the brain are not affected by gender: an in vivo microdialysis study in Wistar rats. J Neuroendocrinol 21:132-140.

Droste SK, de Groote L, Atkinson HC, Lightman SL, Reul JM, Linthorst AC (2008) Corticosterone levels in the brain show a distinct ultradian rhythm but a delayed response to forced swim stress. Endocrinology 149:3244-3253.

Dubovicky M, Mach M, Key M, Morris M, Paton S, Lucot JB (2007) Diurnal behavioral and endocrine effects of chronic shaker stress in mice. Neuro Endocrinol Lett 28:846-853.

Duhart JM, Leone MJ, Paladino N, Evans JA, Castanon-Cervantes O, Davidson AJ, Golombek DA (2013) Suprachiasmatic astrocytes modulate the circadian clock in response to TNF-alpha. J Immunol 191:4656-4664.

Dunlap JC (1999) Molecular bases for circadian clocks. Cell 96:271-290.

Dunn J, Scheving L, Millet P (1972) Circadian variation in stress-evoked increases in plasma corticosterone. Am J Physiol 223:402-406.

Edwards AV, Jones CT (1987) The effect of splanchnic nerve stimulation on adrenocortical activity in conscious calves. J Physiol 382:385-396.

Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES (2000) The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. Pharmacol Rev 52:595-638.

Elmquist JK, Maratos-Flier E, Saper CB, Flier JS (1998) Unraveling the central nervous system pathways underlying responses to leptin. Nat Neurosci 1:445-450.

Engeland WC, Gann DS (1989) Splanchnic nerve stimulation modulates steroid secretion in hypophysectomized dogs. Neuroendocrinology 50:124-131.

Engeland WC, Arnhold MM (2005) Neural circuitry in the regulation of adrenal corticosterone rhythmicity. Endocrine 28:325-332.

Engeland WC, Shinsako J, Winget CM, Vernikos-Danellis J, Dallman MF (1977) Circadian patterns of stress-induced ACTH secretion are modified by corticosterone responses. Endocrinology 100:138-147.

Engelmann M, Ebner K, Landgraf R, Wotjak CT (1998) Swim stress triggers the release of vasopressin within the suprachiasmatic nucleus of male rats. Brain Res 792:343-347.

Eskin A (1979) Identification and physiology of circadian pacemakers. Introduction. Fed Proc 38:2570-2572.

Everds NE, Snyder PW, Bailey KL, Bolon B, Creasy DM, Foley GL, Rosol TJ, Sellers T (2013) Interpreting stress responses during routine toxicity studies: a review of the biology, impact, and assessment. Toxicol Pathol 41:560-614.

Faust JR, Luskey KL, Chin DJ, Goldstein JL, Brown MS (1982) Regulation of synthesis and degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase by low density lipoprotein and 25-hydroxycholesterol in UT-1 cells. Proc Natl Acad Sci U S A 79:5205-5209.

Feldman D, Loose D (1977) Glucocorticoid receptors in adipose tissue. Endocrinology 100:398-405.

Feldman S, Weidenfeld J (1999) Glucocorticoid receptor antagonists in the hippocampus modify the negative feedback following neural stimuli. Brain Res 821:33-37.

Ferguson AV, Latchford KJ, Samson WK (2008) The paraventricular nucleus of the hypothalamus - a potential target for integrative treatment of autonomic dysfunction. Expert Opin Ther Targets 12:717-727.

Field MD, Maywood ES, O'Brien JA, Weaver DR, Reppert SM, Hastings MH (2000) Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanisms. Neuron 25:437-447.

Filipski E, Li XM, Levi F (2006) Disruption of circadian coordination and malignant growth. Cancer Causes Control 17:509-514.

Foster RG, Hankins MW (2007) Circadian vision. Curr Biol 17:R746-751.

Foster RG, Provencio I, Hudson D, Fiske S, De Grip W, Menaker M (1991) Circadian photoreception in the retinally degenerate mouse (rd/rd). J Comp Physiol A 169:39-50.

Fries E, Hesse J, Hellhammer J, Hellhammer DH (2005) A new view on hypocortisolism. Psychoneuroendocrinology 30:1010-1016.

Füchsl AM, Langgartner D, Reber SO (2013a) Mechanisms underlying the increased plasma ACTH levels in chronic psychosocially stressed male mice. PLoS One 8:e84161.

Füchsl AM, Uschold-Schmidt N, Reber SO (2013b) Chronic psychosocial stress in male mice causes an up-regulation of scavenger receptor class B type 1 protein in the adrenal glands. Stress 16:461-468.

Fukuhara C, Dirden JC, Tosini G (2000) Circadian expression of period 1, period 2, and arylalkylamine N-acetyltransferase mRNA in the rat pineal gland under different light conditions. Neurosci Lett 286:167-170.

Gallant S (1979) Serum levels of corticosterone and 18-hydroxy-11-deoxycorticosterone in the female rat at the high and low points of the circadian rhythm. Steroids 33:183-195.

Gardill BR, Vogl MR, Lin HY, Hammond GL, Muller YA (2012) Corticosteroid-binding globulin: structure-function implications from species differences. PLoS One 7:e52759.

Gatt JM, Nemeroff CB, Dobson-Stone C, Paul RH, Bryant RA, Schofield PR, Gordon E, Kemp AH, Williams LM (2009) Interactions between BDNF Val66Met polymorphism and early life stress predict brain and arousal pathways to syndromal depression and anxiety. Mol Psychiatry 14:681-695.

Gerra G, Zaimovic A, Mascetti GG, Gardini S, Zambelli U, Timpano M, Raggi MA, Brambilla F (2001) Neuroendocrine responses to experimentally-induced psychological stress in healthy humans. Psychoneuroendocrinology 26:91-107.

Gibbs FP (1970) Circadian variation of ether-induced corticosterone secretion in the rat. Am J Physiol 219:288-292.

Gillespie CF, Phifer J, Bradley B, Ressler KJ (2009) Risk and resilience: genetic and environmental influences on development of the stress response. Depress Anxiety 26:984-992.

Gillies GE, Linton EA, Lowry PJ (1982) Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. Nature 299:355-357.

Girotti M, Weinberg MS, Spencer RL (2009) Diurnal expression of functional and clock-related genes throughout the rat HPA axis: system-wide shifts in response to a restricted feeding schedule. Am J Physiol Endocrinol Metab 296:E888-897.

Golden SA, Covington HE, 3rd, Berton O, Russo SJ (2011) A standardized protocol for repeated social defeat stress in mice. Nat Protoc 6:1183-1191.

Golombek DA, Rosenstein RE (2010) Physiology of circadian entrainment. Physiol Rev 90:1063-1102.

Gorka Z, Adamik P (1993) The effect of reserpine and stress on feeding behaviour in the light and dark phases of the diurnal cycle in rats. J Pharm Pharmacol 45:137-138.

Gorka Z, Moryl E, Papp M (1996) Effect of chronic mild stress on circadian rhythms in the locomotor activity in rats. Pharmacol Biochem Behav 54:229-234.

Gorrigan RJ, Guasti L, King P, Clark AJ, Chan LF (2011) Localisation of the melanocortin-2-receptor and its accessory proteins in the developing and adult adrenal gland. J Mol Endocrinol 46:227-232.

Grandin LD, Alloy LB, Abramson LY (2006) The social zeitgeber theory, circadian rhythms, and mood disorders: review and evaluation. Clin Psychol Rev 26:679-694.

Grissom N, Iyer V, Vining C, Bhatnagar S (2007) The physical context of previous stress exposure modifies hypothalamic-pituitary-adrenal responses to a subsequent homotypic stress. Horm Behav 51:95-103.

Gryazeva NI, Shurlygina AV, Verbitskaya LV, Mel'nikova EV, Kudryavtseva NN, Trufakin VA (2001) Changes in various measures of immune status in mice subject to chronic social conflict. Neurosci Behav Physiol 31:75-81.

Guldner FH (1983) Numbers of neurons and astroglial cells in the suprachiasmatic nucleus of male and female rats. Exp Brain Res 50:373-376.

Guo H, Brewer JM, Champhekar A, Harris RB, Bittman EL (2005) Differential control of peripheral circadian rhythms by suprachiasmatic-dependent neural signals. Proc Natl Acad Sci U S A 102:3111-3116.

Hall JC (1990) Genetics of circadian rhythms. Annu Rev Genet 24:659-697.

Handa RJ, Zoeller RT, McGivern RF (2007) Changes in vasoactive intestinal peptide and arginine vasopressin expression in the suprachiasmatic nucleus of the rat brain following footshock stress. Neurosci Lett 425:99-104.

Harbuz MS, Rees RG, Eckland D, Jessop DS, Brewerton D, Lightman SL (1992) Paradoxical responses of hypothalamic corticotropin-releasing factor (CRF) messenger ribonucleic acid (mRNA) and CRF-41 peptide and adenohypophysial proopiomelanocortin mRNA during chronic inflammatory stress. Endocrinology 130:1394-1400.

Hastings MH, Maywood ES, O'Neill JS (2008) Cellular circadian pacemaking and the role of cytosolic rhythms. Curr Biol 18:R805-R815.

Hastings MH, Ebling FJ, Grosse J, Herbert J, Maywood ES, Mikkelsen JD, Sumova A (1995) Immediate-early genes and the neural bases of photic and non-photic entrainment. Ciba Found Symp 183:175-189; discussion 190-177.

Hatalski CG, Guirguis C, Baram TZ (1998) Corticotropin releasing factor mRNA expression in the hypothalamic paraventricular nucleus and the central nucleus of the amygdala

is modulated by repeated acute stress in the immature rat. J Neuroendocrinol 10:663-669.

Heim C, Ehlert U, Hellhammer DH (2000) The potential role of hypocortisolism in the pathophysiology of stress-related bodily disorders. Psychoneuroendocrinology 25:1-35.

Helfrich-Förster C (2004) The circadian clock in the brain: a structural and functional comparison between mammals and insects. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 190:601-613.

Herman JP, Adams D, Prewitt C (1995) Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. Neuroendocrinology 61:180-190.

Herman JP, Schafer KH, Sladek CD, Day R, Young EA, Akil H, Watson SJ (1989) Chronic electroconvulsive shock treatment elicits up-regulation of CRF and AVP mRNA in select populations of neuroendocrine neurons. Brain Res 501:235-246.

Holgert H, Dagerlind A, Hokfelt T (1998) Immunohistochemical characterization of the peptidergic innervation of the rat adrenal gland. Horm Metab Res 30:315-322.

Iio W, Tokutake Y, Matsukawa N, Tsukahara T, Chohnan S, Toyoda A (2012) Anorexic behavior and elevation of hypothalamic malonyl-CoA in socially defeated rats. Biochem Biophys Res Commun 421:301-304.

Iio W, Takagi H, Ogawa Y, Tsukahara T, Chohnan S, Toyoda A (2014) Effects of chronic social defeat stress on peripheral leptin and its hypothalamic actions. BMC Neurosci 15:72.

Imaki T, Nahan JL, Rivier C, Sawchenko PE, Vale W (1991) Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. J Neurosci 11:585-599.

Ishida A, Mutoh T, Ueyama T, Bando H, Masubuchi S, Nakahara D, Tsujimoto G, Okamura H (2005) Light activates the adrenal gland: timing of gene expression and glucocorticoid release. Cell Metab 2:297-307.

Jacobson L (2005) Hypothalamic-pituitary-adrenocortical axis regulation. Endocrinol Metab Clin North Am 34:271-292, vii.

Jasper MS, Engeland WC (1994) Splanchnic neural activity modulates ultradian and circadian rhythms in adrenocortical secretion in awake rats. Neuroendocrinology 59:97-109.

Jiang WG, Li SX, Zhou SJ, Sun Y, Shi J, Lu L (2011) Chronic unpredictable stress induces a reversible change of PER2 rhythm in the suprachiasmatic nucleus. Brain Res 1399:25-32.

Jiang WG, Li SX, Liu JF, Sun Y, Zhou SJ, Zhu WL, Shi J, Lu L (2013) Hippocampal CLOCK protein participates in the persistence of depressive-like behavior induced by chronic unpredictable stress. Psychopharmacology (Berl) 227:79-92.

Johnson RF, Moore RY, Morin LP (1989) Lateral geniculate lesions alter circadian activity rhythms in the hamster. Brain Res Bull 22:411-422.

Johren O, Topp H, Sander G, Schoch G, Fuchs E (1991) Social stress in tree shrews increases the whole-body RNA degradation rates. Naturwissenschaften 78:36-38.

Jones BE (2005) From waking to sleeping: neuronal and chemical substrates. Trends Pharmacol Sci 26:578-586.

Kalsbeek A, van Heerikhuize JJ, Wortel J, Buijs RM (1996) A diurnal rhythm of stimulatory input to the hypothalamo-pituitary-adrenal system as revealed by timed intrahypothalamic administration of the vasopressin V1 antagonist. J Neurosci 16:5555-5565.

Kalsbeek A, Fliers E, Hofman MA, Swaab DF, Buijs RM (2012) Vasopressin and the output of the hypothalamic biological clock. J Neuroendocrinol 22:362-372.

Kalsbeek A, van der Spek R, Lei J, Endert E, Buijs RM, Fliers E (2011) Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. Mol Cell Endocrinol 349:20-29.

Kalsbeek A, Palm IF, La Fleur SE, Scheer FA, Perreau-Lenz S, Ruiter M, Kreier F, Cailotto C, Buijs RM (2006) SCN outputs and the hypothalamic balance of life. J Biol Rhythms 21:458-469.

Kant GJ, Mougey EH, Meyerhoff JL (1986) Diurnal variation in neuroendocrine response to stress in rats: plasma ACTH, beta-endorphin, beta-LPH, corticosterone, prolactin and pituitary cyclic AMP responses. Neuroendocrinology 43:383-390.

Kapitonova MY, Kuznetsov SL, Khlebnikov VV, Zagrebin VL, Morozova Z, Degtyar YV (2010) Immunohistochemical characteristics of the hypophysis in normal conditions and chronic stress. Neurosci Behav Physiol 40:97-102.

Karatsoreos IN, Bhagat SM, Bowles NP, Weil ZM, Pfaff DW, McEwen BS (2010) Endocrine and physiological changes in response to chronic corticosterone: a potential model of the metabolic syndrome in mouse. Endocrinology 151:2117-2127.

Keeney A, Jessop DS, Harbuz MS, Marsden CA, Hogg S, Blackburn-Munro RE (2006) Differential effects of acute and chronic social defeat stress on hypothalamic-pituitary-adrenal axis function and hippocampal serotonin release in mice. J Neuroendocrinol 18:330-338.

Keeney AJ, Hogg S, Marsden CA (2001) Alterations in core body temperature, locomotor activity, and corticosterone following acute and repeated social defeat of male NMRI mice. Physiol Behav 74:177-184.

Keller-Wood ME, Dallman MF (1984) Corticosteroid inhibition of ACTH secretion. Endocr Rev 5:1-24.

Kerr DS, Campbell LW, Applegate MD, Brodish A, Landfield PW (1991) Chronic stress-induced acceleration of electrophysiologic and morphometric biomarkers of hippocampal aging. J Neurosci 11:1316-1324.

Kiessling S, Eichele G, Oster H (2010) Adrenal glucocorticoids have a key role in circadian resynchronization in a mouse model of jet lag. J Clin Invest 120:2600-2609.

Kinoshita C, Miyazaki K, Ishida N (2012) Chronic stress affects PERIOD2 expression through glycogen synthase kinase-3beta phosphorylation in the central clock. Neuroreport 23:98-102.

Kinsey SG, Bailey MT, Sheridan JF, Padgett DA, Avitsur R (2007) Repeated social defeat causes increased anxiety-like behavior and alters splenocyte function in C57BL/6 and CD-1 mice. Brain Behav Immun 21:458-466.

Kishi T, Kitajima T, Ikeda M, Yamanouchi Y, Kinoshita Y, Kawashima K, Okochi T, Ozaki N, Iwata N (2008) Association analysis of nuclear receptor Rev-erb alpha gene (NR1D1) with mood disorders in the Japanese population. Neurosci Res 62:211-215.

Knutsson A (2003) Health disorders of shift workers. Occup Med (Lond) 53:103-108.

Koch BC, Nagtegaal JE, Kerkhof GA, ter Wee PM (2009) Circadian sleep-wake rhythm disturbances in end-stage renal disease. Nat Rev Nephrol 5:407-416.

Koko V, Djordjeviae J, Cvijiae G, Davidoviae V (2004) Effect of acute heat stress on rat adrenal glands: a morphological and stereological study. J Exp Biol 207:4225-4230.

Koolhaas JM, De Boer SF, De Rutter AJ, Meerlo P, Sgoifo A (1997) Social stress in rats and mice. Acta Physiol Scand Suppl 640:69-72.

Koolhaas JM, Bartolomucci A, Buwalda B, de Boer SF, Flugge G, Korte SM, Meerlo P, Murison R, Olivier B, Palanza P, Richter-Levin G, Sgoifo A, Steimer T, Stiedl O, van Dijk G, Wohr M, Fuchs E (2011) Stress revisited: a critical evaluation of the stress concept. Neurosci Biobehav Rev 35:1291-1301.

Koresh O, Kozlovsky N, Kaplan Z, Zohar J, Matar MA, Cohen H (2012) The long-term abnormalities in circadian expression of Period 1 and Period 2 genes in response to stress is normalized by agomelatine administered immediately after exposure. Eur Neuropsychopharmacol 22:205-221.

Kraemer FB (2007) Adrenal cholesterol utilization. Mol Cell Endocrinol 265-266:42-45.

Kraemer FB, Shen WJ (2002) Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis. J Lipid Res 43:1585-1594.

Kramer M, Hiemke C, Fuchs E (1999) Chronic psychosocial stress and antidepressant treatment in tree shrews: time-dependent behavioral and endocrine effects. Neurosci Biobehav Rev 23:937-947.

Kriegsfeld LJ, Silver R (2006) The regulation of neuroendocrine function: Timing is everything. Horm Behav 49:557-574.

Kudryavtseva NN, Bakshtanovskaya IV, Koryakina LA (1991) Social model of depression in mice of C57BL/6J strain. Pharmacol Biochem Behav 38:315-320.

Kwak SP, Morano MI, Young EA, Watson SJ, Akil H (1993) Diurnal CRH mRNA rhythm in the hypothalamus: decreased expression in the evening is not dependent on endogenous glucocorticoids. Neuroendocrinology 57:96-105.

Kwon I, Choe HK, Son GH, Kim K (2011) Mammalian molecular clocks. Exp Neurobiol 20:18-28.

Kyrou I, Tsigos C (2009) Stress hormones: physiological stress and regulation of metabolism. Curr Opin Pharmacol 9:787-793.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lamia KA, Papp SJ, Yu RT, Barish GD, Uhlenhaut NH, Jonker JW, Downes M, Evans RM (2011) Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. Nature 480:552-556.

Landgraf R (2005) Neuropeptides in anxiety modulation. Handb Exp Pharmacol:335-369.

Langford DJ, Crager SE, Shehzad Z, Smith SB, Sotocinal SG, Levenstadt JS, Chanda ML, Levitin DJ, Mogil JS (2006) Social modulation of pain as evidence for empathy in mice. Science 312:1967-1970.

Larsen PJ, Vrang N, Moller M, Jessop DS, Lightman SL, Chowdrey HS, Mikkelsen JD (1994) The diurnal expression of genes encoding vasopressin and vasoactive intestinal peptide within the rat suprachiasmatic nucleus is influenced by circulating glucocorticoids. Brain Res Mol Brain Res 27:342-346.

Lavebratt C, Sjoholm LK, Partonen T, Schalling M, Forsell Y (2010) PER2 variantion is associated with depression vulnerability. Am J Med Genet B Neuropsychiatr Genet 153B:570-581.

Lee MJ, Pramyothin P, Karastergiou K, Fried SK (2013) Deconstructing the roles of glucocorticoids in adipose tissue biology and the development of central obesity. Biochim Biophys Acta 1842:473-481.

Lehmann ML, Herkenham M (2011) Environmental enrichment confers stress resiliency to social defeat through an infralimbic cortex-dependent neuroanatomical pathway. J Neurosci 31:6159-6173.

Lewy AJ, Sack RL, Miller LS, Hoban TM (1987) Antidepressant and circadian phase-shifting effects of light. Science 235:352-354.

Li JD, Hu WP, Zhou QY (2012) The circadian output signals from the suprachiasmatic nuclei. Prog Brain Res 199:119-127.

Lightman SL (2008) The neuroendocrinology of stress: a never ending story. J Neuroendocrinol 20:880-884.

Lightman SL, Young WS, 3rd (1987) Vasopressin, oxytocin, dynorphin, enkephalin and corticotrophin-releasing factor mRNA stimulation in the rat. J Physiol 394:23-39.

Lightman SL, Wiles CC, Atkinson HC, Henley DE, Russell GM, Leendertz JA, McKenna MA, Spiga F, Wood SA, Conway-Campbell BL (2008) The significance of glucocorticoid pulsatility. Eur J Pharmacol 583:255-262.

Linthorst AC, Flachskamm C, Holsboer F, Reul JM (1994) Local administration of recombinant human interleukin-1 beta in the rat hippocampus increases serotonergic neurotransmission, hypothalamic-pituitary-adrenocortical axis activity, and body temperature. Endocrinology 135:520-532.

Lundkvist GB (2014) personal communication.

Lutter M, Krishnan V, Russo SJ, Jung S, McClung CA, Nestler EJ (2008a) Orexin signaling mediates the antidepressant-like effect of calorie restriction. J Neurosci 28:3071-3075.

Lutter M, Sakata I, Osborne-Lawrence S, Rovinsky SA, Anderson JG, Jung S, Birnbaum S, Yanagisawa M, Elmquist JK, Nestler EJ, Zigman JM (2008b) The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. Nat Neurosci 11:752-753.

Ma XM, Lightman SL (1998) The arginine vasopressin and corticotrophin-releasing hormone gene transcription responses to varied frequencies of repeated stress in rats. J Physiol 510 (Pt 2):605-614.

Ma XM, Levy A, Lightman SL (1997) Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: a study of both AVP and corticotropin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA. Endocrinology 138:4351-4357.

Makino S, Smith MA, Gold PW (1995) Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. Endocrinology 136:3299-3309.

Makino S, Hashimoto K, Gold PW (2002) Multiple feedback mechanisms activating corticotropin-releasing hormone system in the brain during stress. Pharmacol Biochem Behav 73:147-158.

Malek ZS, Sage D, Pevet P, Raison S (2007) Daily rhythm of tryptophan hydroxylase-2 messenger ribonucleic acid within raphe neurons is induced by corticoid daily surge and modulated by enhanced locomotor activity. Endocrinology 148:5165-5172.

Malkesman O, Maayan R, Weizman A, Weller A (2006) Aggressive behavior and HPA axis hormones after social isolation in adult rats of two different genetic animal models for depression. Behav Brain Res 175:408-414.

Manenschijn L, Schaap L, van Schoor NM, van der Pas S, Peeters GM, Lips P, Koper JW, van Rossum EF (2013) High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease. J Clin Endocrinol Metab 98:2078-2083.

Maninger N, Capitanio JP, Mason WA, Ruys JD, Mendoza SP (2010) Acute and chronic stress increase DHEAS concentrations in rhesus monkeys. Psychoneuroendocrinology 35:1055-1062.

Marchant EG, Watson NV, Mistlberger RE (1997) Both neuropeptide Y and serotonin are necessary for entrainment of circadian rhythms in mice by daily treadmill running schedules. J Neurosci 17:7974-7987.

Marini F, Pozzato C, Andreetta V, Jansson B, Arban R, Domenici E, Carboni L (2006) Single exposure to social defeat increases corticotropin-releasing factor and glucocorticoid receptor mRNA expression in rat hippocampus. Brain Res 1067:25-35.

Mazzocchi G, Malendowicz LK, Rebuffat P, Robba C, Gottardo G, Nussdorfer GG (1986) Short- and long-term effects of ACTH on the adrenal zona glomerulosa of the rat. A coupled stereological and enzymological study. Cell Tissue Res 243:303-310.

Mazzoccoli G, Palmieri O, Corritore G, Latiano T, Bossa F, Scimeca D, Biscaglia G, Valvano MR, D'Inca R, Cucchiara S, Stronati L, Annese V, Andriulli A, Latiano A (2012) Association study of a polymorphism in clock gene PERIOD3 and risk of inflammatory bowel disease. Chronobiol Int 29:994-1003.

McClung CA (2007) Circadian genes, rhythms and the biology of mood disorders. Pharmacol Ther 114:222-232.

McEwen BS (2000) Allostasis and allostatic load: implications for neuropsychopharmacology. Neuropsychopharmacology 22:108-124.

Meerlo P, Daan S (1998) Aggressive and sexual social stimuli do not phase shift the circadian temperature rhythm in rats. Chronobiol Int 15:231-240.

Meerlo P, Sgoifo A, Turek FW (2002) The effects of social defeat and other stressors on the expression of circadian rhythms. Stress 5:15-22.

Meerlo P, van den Hoofdakker RH, Koolhaas JM, Daan S (1997) Stress-induced changes in circadian rhythms of body temperature and activity in rats are not caused by pacemaker changes. J Biol Rhythms 12:80-92.

Meerlo P, Overkamp GJ, Daan S, Van Den Hoofdakker RH, Koolhaas JM (1996a) Changes in Behaviour and Body Weight Following a Single or Double Social Defeat in Rats. Stress 1:21-32.

Meerlo P, De Boer SF, Koolhaas JM, Daan S, Van den Hoofdakker RH (1996b) Changes in daily rhythms of body temperature and activity after a single social defeat in rats. Physiol Behav 59:735-739.

Meier AH (1976) Daily variation in concentration of plasma corticosteroid in hypophysectomized rats. Endocrinology 98:1475-1479.

Meyer-Bernstein EL, Morin LP (1996) Differential serotonergic innervation of the suprachiasmatic nucleus and the intergeniculate leaflet and its role in circadian rhythm modulation. J Neurosci 16:2097-2111.

Meyer-Bernstein EL, Jetton AE, Matsumoto SI, Markuns JF, Lehman MN, Bittman EL (1999) Effects of suprachiasmatic transplants on circadian rhythms of neuroendocrine function in golden hamsters. Endocrinology 140:207-218.

Meynen G, Unmehopa UA, van Heerikhuize JJ, Hofman MA, Swaab DF, Hoogendijk WJ (2006) Increased arginine vasopressin mRNA expression in the human hypothalamus in depression: A preliminary report. Biol Psychiatry 60:892-895.

Miller AH, Spencer RL, Pulera M, Kang S, McEwen BS, Stein M (1992) Adrenal steroid receptor activation in rat brain and pituitary following dexamethasone: implications for the dexamethasone suppression test. Biol Psychiatry 32:850-869.

Miller GE, Chen E, Zhou ES (2007) If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans. Psychol Bull 133:25-45.

Minor RK, Chang JW, de Cabo R (2009) Hungry for life: How the arcuate nucleus and neuropeptide Y may play a critical role in mediating the benefits of calorie restriction. Mol Cell Endocrinol 299:79-88.

Molinoff PB (1984) Alpha- and beta-adrenergic receptor subtypes properties, distribution and regulation. Drugs 28 Suppl 2:1-15.

Monk TH, Buysse DJ, Frank E, Kupfer DJ, Dettling J, Ritenour AM (1994) Nocturnal and circadian body temperatures of depressed outpatients during symptomatic and recovered states. Psychiatry Res 51:297-311.

Moore RY (1996) Entrainment pathways and the functional organization of the circadian system. Prog Brain Res 111:103-119.

Moore RY, Lenn NJ (1972) A retinohypothalamic projection in the rat. J Comp Neurol 146:1-14.

Moore RY, Eichler VB (1972) Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res 42:201-206.

Moore RY, Speh JC (1993) GABA is the principal neurotransmitter of the circadian system. Neurosci Lett 150:112-116.

Moore RY, Speh JC, Card JP (1995) The retinohypothalamic tract originates from a distinct subset of retinal ganglion cells. J Comp Neurol 352:351-366.

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

Moreau JL, Scherschlicht R, Jenck F, Martin JR (1995) Chronic mild stress-induced anhedonia model of depression; sleep abnormalities and curative effects of electroshock treatment. Behav Pharmacol 6:682-687.

Morin LP (2007) SCN organization reconsidered. J Biol Rhythms 22:3-13.

Morin LP, Allen CN (2006) The circadian visual system, 2005. Brain Res Rev 51:1-60.

Mrosovsky N, Edelstein K, Hastings MH, Maywood ES (2001) Cycle of period gene expression in a diurnal mammal (Spermophilus tridecemlineatus): implications for nonphotic phase shifting. J Biol Rhythms 16:471-478.

Murray G, Michalak EE, Levitt AJ, Levitan RD, Enns MW, Morehouse R, Lam RW (2005) Therapeutic mechanism in seasonal affective disorder: do fluoxetine and light operate through advancing circadian phase? Chronobiol Int 22:937-943.

Nader N, Chrousos GP, Kino T (2010) Interactions of the circadian CLOCK system and the HPA axis. Trends Endocrinol Metab 21:277-286.

Natelson BH, Ottenweller JE, Cook JA, Pitman D, McCarty R, Tapp WN (1988) Effect of stressor intensity on habituation of the adrenocortical stress response. Physiol Behav 43:41-46.

Nebzydoski SJ, Pozzo S, Nemec L, Rankin MK, Gressley TF (2010) The effect of dexamethasone on clock gene mRNA levels in bovine neutrophils and lymphocytes. Vet Immunol Immunopathol 138:183-192.

Nievergelt CM, Kripke DF, Barrett TB, Burg E, Remick RA, Sadovnick AD, McElroy SL, Keck PE, Jr., Schork NJ, Kelsoe JR (2006) Suggestive evidence for association of the circadian genes PERIOD3 and ARNTL with bipolar disorder. Am J Med Genet B Neuropsychiatr Genet 141B:234-241.

Nygren LG, Olson L (1977) A new major projection from locus coeruleus: the main source of noradrenergic nerve terminals in the ventral and dorsal columns of the spinal cord. Brain Res 132:85-93.

Oliveira MJ, Monteiro MP, Ribeiro AM, Pignatelli D, Aguas AP (2009) Chronic exposure of rats to occupational textile noise causes cytological changes in adrenal cortex. Noise Health 11:118-123.

Oren I, Fleishman SJ, Kessel A, Ben-Tal N (2004) Free diffusion of steroid hormones across biomembranes: a simplex search with implicit solvent model calculations. Biophys J 87:768-779.

Oster H, Damerow S, Hut RA, Eichele G (2006a) Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes. J Biol Rhythms 21:350-361.

Oster H, Damerow S, Kiessling S, Jakubcakova V, Abraham D, Tian J, Hoffmann MW, Eichele G (2006b) The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. Cell Metab 4:163-173.

Partonen T, Treutlein J, Alpman A, Frank J, Johansson C, Depner M, Aron L, Rietschel M, Wellek S, Soronen P, Paunio T, Koch A, Chen P, Lathrop M, Adolfsson R, Persson ML, Kasper S, Schalling M, Peltonen L, Schumann G (2007) Three circadian clock genes Per2, Arntl, and Npas2 contribute to winter depression. Ann Med 39:229-238.

Patterson ZR, Khazall R, Mackay H, Anisman H, Abizaid A (2013) Central ghrelin signaling mediates the metabolic response of C57BL/6 male mice to chronic social defeat stress. Endocrinology 154:1080-1091.

Paxinos G, Franklin KBJ (1997) The mouse brain in stereotaxic coordinates. Academic Press, San Diego, CA.

Paxinos G, Watson C (1998) The brain in stereotaxic coordinates. Academic Press, San Diego, CA.

Peters S, Slattery DA, Uschold-Schmidt N, Reber SO, Neumann ID (2014) Dose-dependent effects of chronic central infusion of oxytocin on anxiety, oxytocin receptor binding and stress-related parameters in mice. Psychoneuroendocrinology 42:225-236.

Pezuk P, Mohawk JA, Wang LA, Menaker M (2012) Glucocorticoids as entraining signals for peripheral circadian oscillators. Endocrinology 153:4775-4783.

Picard D, Yamamoto KR (1987) Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. Embo J 6:3333-3340.

Pittendrigh CS (1993) Temporal organization: reflections of a Darwinian clock-watcher. Annu Rev Physiol 55:16-54.

Pizarro JM, Lumley LA, Medina W, Robison CL, Chang WE, Alagappan A, Bah MJ, Dawood MY, Shah JD, Mark B, Kendall N, Smith MA, Saviolakis GA, Meyerhoff JL (2004) Acute social defeat reduces neurotrophin expression in brain cortical and subcortical areas in mice. Brain Res 1025:10-20.

Pjrek E, Winkler D, Konstantinidis A, Willeit M, Praschak-Rieder N, Kasper S (2007) Agomelatine in the treatment of seasonal affective disorder. Psychopharmacology (Berl) 190:575-579.

Preitner N, Brown S, Ripperger J, Le-Minh N, Damiola F, Schibler U (2003) Orphan nuclear receptors, molecular clockwork, and the entrainment of peripheral oscillators. Novartis Found Symp 253:89-99; discussion 99-109.

Purba JS, Hoogendijk WJ, Hofman MA, Swaab DF (1996) Increased number of vasopressin- and oxytocin-expressing neurons in the paraventricular nucleus of the hypothalamus in depression. Arch Gen Psychiatry 53:137-143.

Raadsheer FC, Hoogendijk WJ, Stam FC, Tilders FJ, Swaab DF (1994) Increased numbers of corticotropin-releasing hormone expressing neurons in the hypothalamic paraventricular nucleus of depressed patients. Neuroendocrinology 60:436-444.

Raadsheer FC, van Heerikhuize JJ, Lucassen PJ, Hoogendijk WJ, Tilders FJ, Swaab DF (1995) Corticotropin-releasing hormone mRNA levels in the paraventricular nucleus of patients with Alzheimer's disease and depression. Am J Psychiatry 152:1372-1376.

Raison CL, Miller AH (2003) When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. Am J Psychiatry 160:1554-1565.

Ramirez-Zacarias JL, Castro-Munozledo F, Kuri-Harcuch W (1992) Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. Histochemistry 97:493-497.

Razzoli M, Carboni L, Andreoli M, Ballottari A, Arban R (2011) Different susceptibility to social defeat stress of BalbC and C57BL6/J mice. Behav Brain Res 216:100-108.

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.

Reber SO, Obermeier F, Straub RH, Falk W, Neumann ID (2006) Chronic intermittent psychosocial stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration. Endocrinology 147:4968-4976.

Reber SO, Birkeneder L, Veenema AH, Obermeier F, Falk W, Straub RH, Neumann ID (2007) Adrenal insufficiency and colonic inflammation after a novel chronic psycho-social stress paradigm in mice: implications and mechanisms. Endocrinology 148:670-682.

Reghunandanan V, Reghunandanan R (2006) Neurotransmitters of the suprachiasmatic nuclei. J Circadian Rhythms 4:2.

Reppert SM, Weaver DR (2002) Coordination of circadian timing in mammals. Nature 418:935-941.

Reul JM, de Kloet ER (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117:2505-2511.

Reul JM, Gesing A, Droste S, Stec IS, Weber A, Bachmann C, Bilang-Bleuel A, Holsboer F, Linthorst AC (2000) The brain mineralocorticoid receptor: greedy for ligand, mysterious in function. Eur J Pharmacol 405:235-249.

Reyes BA, Valentino RJ, Xu G, Van Bockstaele EJ (2005) Hypothalamic projections to locus coeruleus neurons in rat brain. Eur J Neurosci 22:93-106.

Robison B, Harbour, V.L., Amir, S. (2005) Repeated immobilization stress disrupts circadian rhythms of PER2 expression in the limbic forebrain. Neuroscience Meeting Planner, Society for Neuroscience Annual Meeting, Washington, DC Program No. 60.7.

Roenneberg T, Merrow M (2003) The network of time: understanding the molecular circadian system. Curr Biol 13:R198-207.

Rosol TJ, Yarrington JT, Latendresse J, Capen CC (2001) Adrenal gland: structure, function, and mechanisms of toxicity. Toxicol Pathol 29:41-48.

Ruby CL, Prosser RA, DePaul MA, Roberts RJ, Glass JD (2009) Acute ethanol impairs photic and nonphotic circadian phase resetting in the Syrian hamster. Am J Physiol Regul Integr Comp Physiol 296:R411-418.

Ruby NF, Burns DE, Heller HC (1999) Circadian rhythms in the suprachiasmatic nucleus are temperature-compensated and phase-shifted by heat pulses in vitro. J Neurosci 19:8630-8636.

Rupprecht R, Papadopoulos V, Rammes G, Baghai TC, Fan J, Akula N, Groyer G, Adams D, Schumacher M (2010) Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. Nat Rev Drug Discov 9:971-988.

Rybkin, II, Zhou Y, Volaufova J, Smagin GN, Ryan DH, Harris RB (1997) Effect of restraint stress on food intake and body weight is determined by time of day. Am J Physiol 273:R1612-1622.

Rygula R, Abumaria N, Flugge G, Hiemke C, Fuchs E, Ruther E, Havemann-Reinecke U (2006) Citalopram counteracts depressive-like symptoms evoked by chronic social stress in rats. Behav Pharmacol 17:19-29.

Sage D, Maurel D, Bosler O (2001) Involvement of the suprachiasmatic nucleus in diurnal ACTH and corticosterone responsiveness to stress. Am J Physiol Endocrinol Metab 280:E260-269.

Sage D, Ganem J, Guillaumond F, Laforge-Anglade G, Francois-Bellan AM, Bosler O, Becquet D (2004) Influence of the corticosterone rhythm on photic entrainment of locomotor activity in rats. J Biol Rhythms 19:144-156.

Sanchez MM, Aguado F, Sanchez-Toscano F, Saphier D (1998) Neuroendocrine and immunocytochemical demonstrations of decreased hypothalamo-pituitary-adrenal axis responsiveness to restraint stress after long-term social isolation. Endocrinology 139:579-587.

Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21:55-89.

Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, FitzGerald GA, Kay SA, Hogenesch JB (2004) A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron 43:527-537.

Sawchenko PE, Swanson LW, Vale WW (1984) Co-expression of corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat. Proc Natl Acad Sci U S A 81:1883-1887.

Sawchenko PE, Arias CA, Mortrud MT (1993) Local tetrodotoxin blocks chronic stress effects on corticotropin-releasing factor and vasopressin messenger ribonucleic acids in hypophysiotropic neurons. J Neuroendocrinol 5:341-348.

Schernhammer ES, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Colditz GA (2001) Rotating night shifts and risk of breast cancer in women participating in the nurses' health study. J Natl Cancer Inst 93:1563-1568.

Schibler U (2007) The daily timing of gene expression and physiology in mammals. Dialogues Clin Neurosci 9:257-272.

Schibler U, Sassone-Corsi P (2002) A web of circadian pacemakers. Cell 111:919-922.

Schwartz MW, Porte D, Jr. (2005) Diabetes, obesity, and the brain. Science 307:375-379.

Scott AJ, Monk TH, Brink LL (1997) Shiftwork as a Risk Factor for Depression: A Pilot Study. Int J Occup Environ Health 3:S2-S9.

Scott LV, Dinan TG (1998) Vasopressin and the regulation of hypothalamic-pituitary-adrenal axis function: implications for the pathophysiology of depression. Life Sci 62:1985-1998.

Scribner KA, Akana SF, Walker CD, Dallman MF (1993) Streptozotocin-diabetic rats exhibit facilitated adrenocorticotropin responses to acute stress, but normal sensitivity to feedback by corticosteroids. Endocrinology 133:2667-2674.

Selye H (1936) A syndrome produced by diverse nocuous agents. J Neuropsychiatry Clin Neurosci 10:230-231.

Selye H (1950) Stress and the general adaptation syndrome. Br Med J 1:1383-1392.

Sgoifo A, Meerlo P (2002) Animal models of social stress: implications for the study of stress related pathologies in humans. Stress 5:1-2.

Sgoifo A, Pozzato C, Meerlo P, Costoli T, Manghi M, Stilli D, Olivetti G, Musso E (2002) Intermittent exposure to social defeat and open-field test in rats: acute and long-term effects on ECG, body temperature and physical activity. Stress 5:23-35.

Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GT, Hastings MH, Reppert SM (2000) Interacting molecular loops in the mammalian circadian clock. Science 288:1013-1019.

Sherman TG, McKelvy JF, Watson SJ (1986) Vasopressin mRNA regulation in individual hypothalamic nuclei: a northern and in situ hybridization analysis. J Neurosci 6:1685-1694.

Silver R, LeSauter J, Tresco PA, Lehman MN (1996) A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. Nature 382:810-813.

Singewald GM, Nguyen NK, Neumann ID, Singewald N, Reber SO (2009) Effect of chronic psychosocial stress-induced by subordinate colony (CSC) housing on brain neuronal activity patterns in mice. Stress 12:58-69.

Slattery DA, Uschold N, Magoni M, Bar J, Popoli M, Neumann ID, Reber SO (2011) Behavioural consequences of two chronic psychosocial stress paradigms: anxiety without depression. Psychoneuroendocrinology 37:702-714.

Smale L, Lee T, Nunez AA (2003) Mammalian diurnality: some facts and gaps. J Biol Rhythms 18:356-366.

So AY, 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 106:17582-17587.

Solberg LC, Horton TH, Turek FW (1999) Circadian rhythms and depression: effects of exercise in an animal model. Am J Physiol 276:R152-161.

Son GH, Chung S, Kim K (2011) The adrenal peripheral clock: glucocorticoid and the circadian timing system. Front Neuroendocrinol 32:451-465.

Son GH, Chung S, Choe HK, Kim HD, Baik SM, Lee H, Lee HW, Choi S, Sun W, Kim H, Cho S, Lee KH, Kim K (2008) Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. Proc Natl Acad Sci U S A 105:20970-20975.

Sorrells SF, Caso JR, Munhoz CD, Sapolsky RM (2009) The stressed CNS: when glucocorticoids aggravate inflammation. Neuron 64:33-39.

Souetre E, Salvati E, Belugou JL, Pringuey D, Candito M, Krebs B, Ardisson JL, Darcourt G (1989) Circadian rhythms in depression and recovery: evidence for blunted amplitude as the main chronobiological abnormality. Psychiatry Res 28:263-278.

Srinivasan V, Zakaria R, Othman Z, Lauterbach EC, Acuna-Castroviejo D (2012) Agomelatine in depressive disorders: its novel mechanisms of action. J Neuropsychiatry Clin Neurosci 24:290-308.

Stephan FK, Zucker I (1972) Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. Proc Natl Acad Sci U S A 69:1583-1586.

Sterlemann V, Ganea K, Liebl C, Harbich D, Alam S, Holsboer F, Muller MB, Schmidt MV (2008) Long-term behavioral and neuroendocrine alterations following chronic social stress in mice: implications for stress-related disorders. Horm Behav 53:386-394.

Stratmann M, Schibler U (2006) Properties, entrainment, and physiological functions of mammalian peripheral oscillators. J Biol Rhythms 21:494-506.

Strecker GJ, Wuarin JP, Dudek FE (1997) GABAA-mediated local synaptic pathways connect neurons in the rat suprachiasmatic nucleus. J Neurophysiol 78:2217-2220.

Swaab DF, Fliers E, Partiman TS (1985) The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia. Brain Res 342:37-44.

Takahashi JS, Hong HK, Ko CH, McDearmon EL (2008) The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nat Rev Genet 9:764-775.

Takahashi K, Yamada T, Tsukita S, Kaneko K, Shirai Y, Munakata Y, Ishigaki Y, Imai J, Uno K, Hasegawa Y, Sawada S, Oka Y, Katagiri H (2013) Chronic mild stress alters circadian expressions of molecular clock genes in the liver. Am J Physiol Endocrinol Metab 304:E301-309.

Takumi T, Taguchi K, Miyake S, Sakakida Y, Takashima N, Matsubara C, Maebayashi Y, Okumura K, Takekida S, Yamamoto S, Yagita K, Yan L, Young MW, Okamura H (1998) A light-independent oscillatory gene mPer3 in mouse SCN and OVLT. Embo J 17:4753-4759.

Tamashiro KL, Nguyen MM, Fujikawa T, Xu T, Yun Ma L, Woods SC, Sakai RR (2004) Metabolic and endocrine consequences of social stress in a visible burrow system. Physiol Behav 80:683-693.

Tamashiro KL, Hegeman MA, Nguyen MM, Melhorn SJ, Ma LY, Woods SC, Sakai RR (2007) Dynamic body weight and body composition changes in response to subordination stress. Physiol Behav 91:440-448.

Tonsfeldt KJ, Chappell PE (2011) Clocks on top: the role of the circadian clock in the hypothalamic and pituitary regulation of endocrine physiology. Mol Cell Endocrinol 349:3-12.

Tornatzky W, Miczek KA (1993) Long-term impairment of autonomic circadian rhythms after brief intermittent social stress. Physiol Behav 53:983-993.

Torra IP, Tsibulsky V, Delaunay F, Saladin R, Laudet V, Fruchart JC, Kosykh V, Staels B (2000) Circadian and glucocorticoid regulation of Rev-erbalpha expression in liver. Endocrinology 141:3799-3806.

Torrellas A, Guaza C, Borrell J, Borrell S (1981) Adrenal hormones and brain catecholamines responses to morning and afternoon immobilization stress in rats. Physiol Behav 26:129-133.

Torres-Farfan C, Mendez N, Abarzua-Catalan L, Vilches N, Valenzuela GJ, Seron-Ferre M (2011) A circadian clock entrained by melatonin is ticking in the rat fetal adrenal. Endocrinology 152:1891-1900.

Torres-Farfan C, Rocco V, Monso C, Valenzuela FJ, Campino C, Germain A, Torrealba F, Valenzuela GJ, Seron-Ferre M (2006) Maternal melatonin effects on clock gene expression in a nonhuman primate fetus. Endocrinology 147:4618-4626.

Tschop M, Smiley DL, Heiman ML (2000) Ghrelin induces adiposity in rodents. Nature 407:908-913.

Tsigos C, Chrousos GP (2002) Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53:865-871.

Turnbull AV, Rivier C (1997) Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides. Proc Soc Exp Biol Med 215:1-10.

Turnbull AV, Rivier CL (1999) Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. Physiol Rev 79:1-71.

Uhl GR, Reppert SM (1986) Suprachiasmatic nucleus vasopressin messenger RNA: circadian variation in normal and Brattleboro rats. Science 232:390-393.

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

Ulrich-Lai YM, Arnhold MM, Engeland WC (2006a) 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:R1128-1135.

Ulrich-Lai YM, Figueiredo HF, Ostrander MM, Choi DC, Engeland WC, Herman JP (2006b) Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. Am J Physiol Endocrinol Metab 291:E965-973.

Uschold-Schmidt N, Nyuyki KD, Füchsl AM, Neumann ID, Reber SO (2012) Chronic psychosocial stress results in sensitization of the HPA axis to acute heterotypic stressors despite a reduction of adrenal in vitro ACTH responsiveness. Psychoneuroendocrinology 37:1676-1687.

Ushijima K, Morikawa T, To H, Higuchi S, Ohdo S (2006) Chronobiological disturbances with hyperthermia and hypercortisolism induced by chronic mild stress in rats. Behav Brain Res 173:326-330.

Valentino RJ, Van Bockstaele E (2008) Convergent regulation of locus coeruleus activity as an adaptive response to stress. Eur J Pharmacol 583:194-203.

van der Lely AJ, Tschop M, Heiman ML, Ghigo E (2004) Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev 25:426-457.

van der Pompe G, Antoni MH, Heijnen CJ (1996) Elevated basal cortisol levels and attenuated ACTH and cortisol responses to a behavioral challenge in women with metastatic breast cancer. Psychoneuroendocrinology 21:361-374.

Venihaki M, Majzoub JA (1999) Animal models of CRH deficiency. Front Neuroendocrinol 20:122-145.

Walker JJ, Terry JR, Lightman SL (2012) Origin of ultradian pulsatility in the hypothalamic-pituitary-adrenal axis. Proc Biol Sci 277:1627-1633.

Watanabe S, Ono K (1986) An experimental analysis of "empathic" response: Effects of pain reactions of pigeon upon other pigeon's operant behavior. Behav Processes 13:269-277.

Watts AG, Tanimura S, Sanchez-Watts G (2004) Corticotropin-releasing hormone and arginine vasopressin gene transcription in the hypothalamic paraventricular nucleus of unstressed rats: daily rhythms and their interactions with corticosterone. Endocrinology 145:529-540.

Weiser MJ, Osterlund C, Spencer RL (2011) Inhibitory effects of corticosterone in the hypothalamic paraventricular nucleus (PVN) on stress-induced adrenocorticotrophic hormone secretion and gene expression in the PVN and anterior pituitary. J Neuroendocrinol 23:1231-1240.

Welsh DK, Takahashi JS, Kay SA (2010) Suprachiasmatic nucleus: cell autonomy and network properties. Annu Rev Physiol 72:551-577.

Westlund KN, Bowker RM, Ziegler MG, Coulter JD (1983) Noradrenergic projections to the spinal cord of the rat. Brain Res 263:15-31.

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 139:443-450.

Windle RJ, Wood SA, Kershaw YM, Lightman SL, Ingram CD, Harbuz MS (2001) Increased corticosterone pulse frequency during adjuvant-induced arthritis and its relationship to alterations in stress responsiveness. J Neuroendocrinol 13:905-911.

Winkler D, Pjrek E, Praschak-Rieder N, Willeit M, Pezawas L, Konstantinidis A, Stastny J, Kasper S (2005) Actigraphy in patients with seasonal affective disorder and healthy control subjects treated with light therapy. Biol Psychiatry 58:331-336.

Wittmann M, Dinich J, Merrow M, Roenneberg T (2006) Social jetlag: misalignment of biological and social time. Chronobiol Int 23:497-509.

Woods SC, Seeley RJ, Porte D, Jr., Schwartz MW (1998) Signals that regulate food intake and energy homeostasis. Science 280:1378-1383.

Wotjak CT, Kubota M, Liebsch G, Montkowski A, Holsboer F, Neumann I, Landgraf R (1996) Release of vasopressin within the rat paraventricular nucleus in response to emotional stress: a novel mechanism of regulating adrenocorticotropic hormone secretion? J Neurosci 16:7725-7732.

Wotjak CT, Ludwig M, Ebner K, Russell JA, Singewald N, Landgraf R, Engelmann M (2002) Vasopressin from hypothalamic magnocellular neurons has opposite actions at the adenohypophysis and in the supraoptic nucleus on ACTH secretion. Eur J Neurosci 16:477-485.

Wust S, Federenko IS, van Rossum EF, Koper JW, Hellhammer DH (2005) Habituation of cortisol responses to repeated psychosocial stress-further characterization and impact of genetic factors. Psychoneuroendocrinology 30:199-211.

Xing Y, Parker CR, Edwards M, Rainey WE (2010) ACTH is a potent regulator of gene expression in human adrenal cells. J Mol Endocrinol 45:59-68.

Yamamoto T, Nakahata Y, Soma H, Akashi M, Mamine T, Takumi T (2004) Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. BMC Mol Biol 5:18.

Yamamoto T, Nakahata Y, Tanaka M, Yoshida M, Soma H, Shinohara K, Yasuda A, Mamine T, Takumi T (2005) Acute physical stress elevates mouse period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. J Biol Chem 280:42036-42043.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, Tei H (2000) Resetting central and peripheral circadian oscillators in transgenic rats. Science 288:682-685.

Yan L, Silver R (2002) Differential induction and localization of mPer1 and mPer2 during advancing and delaying phase shifts. Eur J Neurosci 16:1531-1540.

Yi CX, van der Vliet J, Dai J, Yin G, Ru L, Buijs RM (2006) Ventromedial arcuate nucleus communicates peripheral metabolic information to the suprachiasmatic nucleus. Endocrinology 147:283-294.

Yoder JM, Brandeland M, Engeland WC (2014) Phase-dependent resetting of the adrenal clock by ACTH in vitro. Am J Physiol Regul Integr Comp Physiol 306:R387-393.

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A 101:5339-5346.

Yu H, Wang DD, Wang Y, Liu T, Lee FS, Chen ZY (2012) Variant brain-derived neurotrophic factor Val66Met polymorphism alters vulnerability to stress and response to antidepressants. J Neurosci 32:4092-4101.

Zelena D, Haller J, Halasz J, Makara GB (1999) Social stress of variable intensity: physiological and behavioral consequences. Brain Res Bull 48:297-302.

Zhang J, Ge R, Matte-Martone C, Goodwin J, Shlomchik WD, Mamula MJ, Kooshkabadi A, Hardy MP, Geller D (2009) Characterization of a novel gain of function glucocorticoid receptor knock-in mouse. J Biol Chem 284:6249-6259.

Zhou JN, Riemersma RF, Unmehopa UA, Hoogendijk WJ, van Heerikhuize JJ, Hofman MA, Swaab DF (2001) Alterations in arginine vasopressin neurons in the suprachiasmatic nucleus in depression. Arch Gen Psychiatry 58:655-662.

Zimmermann E, Critchlow V (1967) Effects of diurnal variation in plasma corticosterone levels on adrenocortical response to stress. Proc Soc Exp Biol Med 125:658-663.

Zingg HH, Lefebvre D, Almazan G (1986) Regulation of vasopressin gene expression in rat hypothalamic neurons. Response to osmotic stimulation. J Biol Chem 261:12956-12959.

FULL TEXT PAPER 1

Time matters: pathological effects of repeated psychosocial stress during the active, but not inactive, phase of male mice

Manuela S Bartlang, Inga D Neumann[1], David A Slattery[1], Nicole Uschold-Schmidt[1], Dominik Kraus[1], Charlotte Helfrich-Förster and **Stefan O Reber[1]**

Department of Neurobiology and Genetics, University of Würzburg, 97074 Würzburg, Germany
[1]Department of Behavioural and Molecular Neurobiology, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany
(Correspondence should be addressed to S O Reber; Email: stefan.reber@biologie.uni-regensburg.de)

Abstract

Recent findings in rats indicated that the physiological consequences of repeated restraint stress are dependent on the time of day of stressor exposure. To investigate whether this is also true for clinically more relevant psychosocial stressors and whether repeated stressor exposure during the light phase or dark phase is more detrimental for an organism, we exposed male C57BL/6 mice to social defeat (SD) across 19 days either in the light phase between Zeitgeber time (ZT)1 and ZT3 (SDL mice) or in the dark phase between ZT13 and ZT15 (SDD mice). While SDL mice showed a prolonged increase in adrenal weight and an attenuated adrenal responsiveness to ACTH *in vitro* after stressor termination, SDD mice showed reduced dark phase home-cage activity on observation days 7, 14, and 20, flattening of the diurnal corticosterone rhythm, lack of social preference, and higher *in vitro* IFNγ secretion from mesenteric lymph node cells on day 20/21. Furthermore, the colitis-aggravating effect of SD was more pronounced in SDD than SDL mice following dextran sulfate sodium treatment. In conclusion, the present findings demonstrate that repeated SD effects on behavior, physiology, and immunology strongly depend on the time of day of stressor exposure. Whereas physiological parameters were more affected by SD during the light/inactive phase of mice, behavioral and immunological parameters were more affected by SD during the dark phase. Our results imply that repeated daily SD exposure has a more negative outcome when applied during the dark/active phase. By contrast, the minor physiological changes seen in SDL mice might represent beneficial adaptations preventing the formation of those maladaptive consequences.
Journal of Endocrinology (2012) **215,** 425–437

Introduction

Chronic stress in humans is known to be a risk factor for many affective disorders including anxiety and depression (for reviews, see Herbert (1997), and Shalev (2009)) as well as somatic disorders such as inflammatory bowel disease (Duffy *et al.* (1991) and Levenstein *et al.* (2000), for review, see Reber (2011)). Moreover, several rodent studies have shown that chronic or repeated stressor exposure induces anxiety- (Reber *et al.* 2007, Slattery *et al.* 2011) and depressive-like behavior (Berton *et al.* 2006). However, the mechanisms behind stress-related disorders or maladaptations are still far from being understood, due in part to a lack of adequate and translationally relevant stress paradigms (for review, see Reber (2011)). Chronic psychosocial stress paradigms represent the most promising approach in animal research to unravel the molecular basis for stress-promoted pathologies as they more accurately reflect the human situation (for review, see Zohar & Westenberg (2000)). In support, and unlike physical stressors, the hypothalamo-pituitary–adrenal (HPA) axis responses to repeated psychosocial stressors, such as intermittent social defeat (SD) in rats, do not habituate (Tornatzky & Miczek 1993). Intermittent SD refers to repeated exposure to a larger, dominant conspecific, which according to traditional definitions would seem to represent a repeated homotypic stressor. However, it is believed that repeated SD does not result in adaptation as it rather represents a heterotypic stressor for the defeated animal due to the behavioral differences displayed by the varying dominant conspecifics it is exposed to (Sgoifo *et al.* 2002).

Although the monoaminergic theories of depression have dominated scientific thinking over the past decades (for review, see Hirschfeld (2000)), newer theories have become more popular to explain the accumulating basic and clinical evidence (for review, see Slattery *et al.* (2004) and Katz *et al.* (2010)). One such theory states that a disturbed internal clock is causally involved in the development of depression (Winkler *et al.* (2005), for review, see Racagni *et al.* (2007)), which is supported by altered plasma cortisol rhythms (Deuschle *et al.* 1997) and sleep patterns observed in

 © 2012 Society for Endocrinology *Printed in Great Britain*
DOI: 10.1530/JOE-12-0267
Online version via http://www.endocrinology-journals.org

most depressed patients and the recent licensing of agomelatine, which re-synchronizes disrupted circadian rhythms (Yous *et al.* (1992), for review, see de Bodinat *et al.* (2010)). In line with this, it was recently shown in rodents that chronic unpredictable stress affects suprachiasmatic nucleus (SCN) functionality, the central pacemaker of the circadian system in mammals, by dampening the amplitude of circadian PERIOD2 (PER2) protein expression, one of the main clock proteins involved in internal rhythm generation (Jiang *et al.* 2011). In addition, various biological diurnal rhythms generated and fine-tuned by the SCN such as locomotor activity (Gorka *et al.* 1996), body temperature (Ushijima *et al.* 2006), heart rate (Grippo *et al.* 2003), sleep (Moreau *et al.* 1995), and plasma glucocorticoid (GC) levels (Ottenweller *et al.* 1994) are also affected by repeated exposure to non-psychosocial stressors.

Similar to GC levels, activity of the gut system is regulated in a circadian fashion and chronodisruption within the gastrointestinal tract was shown to result in several gastrointestinal diseases such as irritable bowel syndrome (for review, see Konturek *et al.* (2011)).

As peripheral organs express GC receptors, peripheral circadian rhythms like gastrointestinal oscillations can be shifted under stressful conditions (Balsalobre *et al.* 2000). Moreover, persistent stimulation of the HPA axis, e.g. by chronic stress is considered to be a crucial contributory factor to the incidence of spontaneous or aggravation of chemically induced colonic inflammation characterized by elevated interferon (IFN)γ levels, reduced colon length, and an increased histological damage score (Reber *et al.* (2006), for review, see Reber (2011)).

With respect to the latter parameter, HPA axis activity and, thus, GC levels peak at the start of the activity phase, i.e. in the early morning in humans and early evening in most rodent species. Given that HPA axis activity/rhythmicity is controlled by SCN efferents to the paraventricular nucleus of the hypothalamus (for review, see Nader *et al.* (2010)), the key activator of the HPA axis during stress, it is likely that stressor exposures at distinct times of the light–dark cycle may generate differential acute stress responses. This, in turn, would lead to differential consequences of chronic/intermittent stressor exposure, in terms of both outcome and severity, for an individual. In support, the few studies that have examined this hypothesis have shown that restraint stress has different effects on body weight development and adrenal gland weight, depending on the time of day of stressor exposure (Rybkin *et al.* (1997) and Perez-Cruz *et al.* (2009), for review, see Koolhaas *et al.* (2011)).

However, it is currently not known whether and to what extent a clinically more relevant chronic/intermittent psychosocial stressor affects biological rhythmicity and whether this is dependent on the time of day of stressor exposure. Therefore, the main aim of this study was to investigate whether selected behavioral, physiological, and immunological consequences of repeated SD exposure (2 h/day; 19 days) are dependent on the time of day of stressor exposure. For this purpose, male mice were exposed to SD either during the light (SDL) or dark (SDD) phase, and home-cage activity, social preference, anxiety-related behavior, basal plasma ACTH, and corticosterone (CORT) concentrations, as well as adrenal *in vitro* responsiveness to ACTH, IFNγ secretion from mesenteric lymph node cells, and the severity of dextran sulfate sodium (DSS)-induced colitis were assessed at different times of the day.

Materials and Methods

Animals

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19–22 g (experimental mice) or 30–35 g (used as residents during the stress procedure) were individually housed in standard polycarbonate mouse cages (16×22×14 cm; experimental mice) or polycarbonate observation cages (38×22×35 cm; residents) for at least 1 week before the start of the experiment. All mice were kept under standard laboratory conditions (22±2 °C; 60±5% humidity) and had free access to tap water and standard mouse diet. They were exposed to a 12 h light:12 h darkness cycle (light phase: 200±50 lux) with lights-on at 0700 h. The time of lights-on is defined as Zeitgeber time (ZT)0 and lights-off as ZT12. All experimental protocols were approved by the Committee on Animal Health and Care of the local government and performed according to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their sufferings.

Experimental procedures

After delivery, mice were weighed and randomly assigned to a single-housed control (SHC), a SDL, and a SDD group (Fig. 1). SHC mice remained undisturbed except for weighing on days 0 and 20 (experiment 1; Fig. 1A) and a weekly change of bedding (all experiments). Based on previous studies, SHC mice were considered to be the most appropriate controls in this paradigm (Singewald *et al.* 2009). SDL and SDD mice were exposed daily for 2 h to SD over 19 days (except for days 7 and 14; experiment 1, 2, and 4) or over 4 days (experiment 3), either at the beginning of the light phase (ZT1–ZT3, SDL mice) or at the beginning of the dark phase (ZT13–ZT15, SDD mice) (see Fig. 1). The experimental design of the SDL and SDD procedure (for detailed description, see below) was identical for all experiments. The majority of parameters, except behavior (social preference/avoidance test, SPAT; elevated plus-maze, EPM), were assessed in both the light and dark phase in order to determine whether repeated stressor exposure resulted in phase-dependent or -independent alterations.

Experiment 1 In order to assess whether submission latencies, the acute behavioral stress response (active behavior

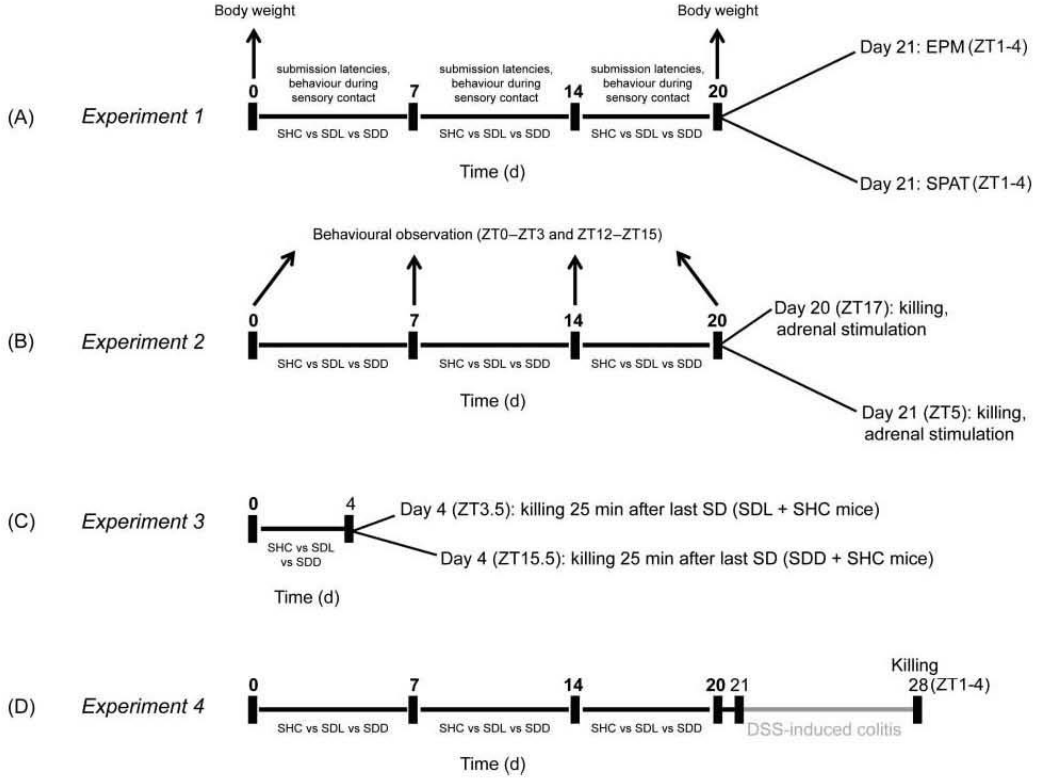


Figure 1 Schematic illustration of the experimental procedures. Description of the procedures performed in experiments 1-4 including their timeline. SHC, single-housed control; SDL, social defeat light; SDD, social defeat dark.

and time spent in contact zone during sensory contact), and social preference and/or anxiety are dependent on the time of day of stressor exposure, separate cohorts of animals were used for assessment of these parameters (Fig. 1A). In detail, submission latencies before and after 2 h of sensory contact and active behavior shown during sensory contact were assessed for SDL ($n=14$) and SDD ($n=15$) mice. Furthermore, SHC ($n=14$), SDL ($n=14$), and SDD ($n=14$) mice were weighed before the start (day 0) and following termination of the SDL/SDD paradigm (day 20) to investigate whether the time of day of stressor exposure differentially affects body weight. Subsequently, respective mice were either exposed to the SPAT (SHC: $n=9$; SDL: $n=10$; SDD: $n=9$) or the EPM (SHC: $n=8$; SDL: $n=9$; SDD: $n=9$) at the beginning of the light phase (ZT1–ZT4) on day 21. We chose the light phase for these tests because preliminary data (confirmed by the results from experiment 2) showed that locomotion during the light phase was not different in SDL and SDD mice (data not shown), in contrast to locomotion during the dark phase (see results from experiment 1B).

Experiment 2 To assess whether the time of day of stressor exposure has an effect on active home-cage behavior, climbing, locomotion, rearing, eating, drinking, grooming, and digging were scored in another set of SHC ($n=24$), SDL ($n=25$), and SDD ($n=23$) mice on days 0, 7, 14, and 20 in the light phase from ZT0 to ZT3 and in the dark phase from ZT12 to ZT15 (Fig. 1B). In order to investigate whether the time of day of stressor exposure influences morning and/or evening basal plasma CORT and ACTH levels, adrenal gland weight, adrenal *in vitro* responsiveness to ACTH, and anti-CD3-stimulated *in vitro* IFNγ secretion from mesenteric lymph node cells, SHC, SDL, and SDD mice used for behavioral observations were subsequently killed by decapitation under brief inhalation anesthesia either in the dark phase (day 20, ZT17; $n=7$–8 per group) or in the light phase (day 21, ZT5; $n=6$–7 per group), organs were removed and trunk blood was collected.

Experiment 3 Based on our findings from experiment 2, we assessed in a next step whether four daily sessions of repeated SD during either light (SDL) or dark (SDD) phase were sufficient to result in HPA axis habituation (Fig. 1C).

Therefore, separate groups of SHC ($n=16$), SDL ($n=8$), and SDD ($n=8$) mice were killed 25 min after the last defeat (SDL and respective SHC mice in the light phase at ~ZT3·5; SDD and respective SHC mice in the dark phase at ~ZT15·5) on day 4 of SD exposure. Trunk blood was collected for quantification of plasma ACTH levels.

Experiment 4 To assess whether the time of day of stressor exposure has an impact on the severity of a subsequent pharmacologically-induced colitis, SHC ($n=9$), SDL ($n=10$), and SDD ($n=9$) mice were treated with 1% DSS in their drinking water for 8 days (Reber *et al.* 2008), starting on day 21 (day 21–28) (Fig. 1D). After killing on day 28, colon length was measured, the histological damage was scored, and IFNγ secretion from mesenteric lymph node cells was determined.

SDL and SDD stress paradigms

During each SD session, the experimental mouse was taken from its home-cage and placed into a male resident's home-cage. After the first attack by the resident that resulted in submissive behavior from the intruder, both mice were separated from each other by a perforated partition wall. This protected the intruder from physical injuries by the resident, but allowed visual, olfactory, and auditory contact as described previously (Reber *et al.* 2006). The submission latency of every intruder was assessed in order to make sure that the level of aggression received during SD is comparable for SDL and SDD mice. During the subsequent 2 h of sensory contact, food pellets, but not tap water, were available for all experimental mice including SHC mice. Furthermore, the time spent in the 3·5 cm broad contact zone (zone adjacent to the partition wall) and the active behavior (climbing, locomotion, rearing, eating, grooming, and digging) was assessed for the intruder every 3 min (note: values represent the sum of all active behaviors per intruder mouse). These 3-min scores were then summed up for 30-min periods per experimental mouse in each 2 h SD session, averaged per treatment group, and expressed in % of total counts possible per 30 min interval, finally resulting in four 30-min values per treatment group (0–30, 30–60, 60–90, and 90–120 min). Following removal of the partition wall, direct social interaction between the resident and the experimental mouse was allowed until the first attack by the resident that resulted in submissive behavior from the intruder. Again, submission latency of the intruder was scored before placing the experimental mouse back into its home-cage. Each experimental mouse was exposed to a different resident every day to avoid habituation. SD was omitted on days 7 and 14 in every experiment, and, during experiment 2, home-cage behavior of experimental mice was observed and scored at the beginning of the light phase (ZT0–ZT3) and dark phase (ZT12–ZT15) on days 0, 7, 14, and 20 respectively. The behavior was scored in 3-min time intervals, thereby distinguishing between locomotion, rearing, eating, drinking, grooming, digging, and climbing (active behavior) and inactive behavior. These 3-min scores were then summed up for the whole 180-min period per mouse and averaged per treatment group.

Social preference/avoidance test

SHC, SDL, and SDD mice were exposed to the SPAT on day 21 between ZT1 and ZT4 as described in detail before (Slattery *et al.* 2011). Briefly, the experimental mouse was placed into the SPAT box (length: 45 cm; width: 27 cm; height: 27 cm; light intensity: 10–40 lux) for 30 s to habituate to the unfamiliar environment before a small empty wire mesh cage (length: 10 cm; width: 6·5 cm; height: 5 cm; nonsocial trial) was introduced to the SPAT box for 150 s. This initial period gives an indication of general anxiety (novel object exposure). Afterward, this empty cage was exchanged for an identical cage containing an unfamiliar male mouse (social trial) for another 150 s. The total time spent in the 8 cm broad contact zone around the wire mesh cage was recorded using EthoVision XT (Version 5.0.216, Noldus Information Technology, Wageningen, The Netherlands) during both 150 s trials. The box and empty cage were cleaned thoroughly before each test.

Elevated plus-maze

SHC, SDL, and SDD mice were tested on the EPM on day 21 between ZT1 and ZT4 for 5 min as described previously (Reber *et al.* 2007). Briefly, the EPM consisted of two open (6×30 cm) and two closed (6×30×17 cm) arms radiating from a central platform (6×6 cm) to form a plus-shaped figure, which was elevated 60 cm above the floor. Each mouse was placed on the central platform facing a closed arm at the start of the trial and the maze was cleaned thoroughly before each test. The number of entries into the closed arms (as a measure of general activity) and the time spent on the open arms (as a measure of anxiety-related behavior) were recorded by means of a video/computer setup to allow calculation of the percentage of time spent on open arms of the maze.

Quantification of plasma CORT and ACTH by ELISA

SHC, SDL, and SDD mice of experiment 2 (day 20, ZT17, or day 21, ZT5) and experiment 3 (day 4, SDL and respective SHC mice in the light phase ~ZT3·5; SDD and respective SHC mice in the dark phase at ~ZT15·5) were killed rapidly by decapitation following brief inhalation anesthesia. Approximately 500 µl trunk blood was collected in EDTA-coated tubes on ice (Sarstedt, Nümbrecht, Germany), centrifuged at 4 °C (2000 ***g***, 10 min), and finally stored at −20 °C until assayed using a commercially available ELISA for CORT (analytical sensitivity <1·631 nmol/l, intra-assay and interassay coefficients of variation ≤6·35%, IBL International, Hamburg, Germany) and ACTH (analytical sensitivity 0·22 pg/ml, intra-assay and interassay coefficients of variation ≤7·1%, IBL International).

Adrenal gland weight

SHC, SDL, and SDD mice of experiment 2 were killed either on day 20 (ZT17) or on day 21 (ZT5). After removal, the adrenals were pruned from fat and weighed separately. Subsequently, adrenal weights were summed (sum of respective left and right adrenal weight) per mouse and expressed as absolute adrenal weight (mg).

ACTH stimulation of adrenal explants in vitro

Adrenal stimulation was performed as described previously (Uschold-Schmidt *et al.* 2012). Briefly, left and right adrenals were separately weighed and stored in ice-cold DMEM/F-12 (Life Technologies, Inc.) containing 0·1% BSA until all mice were killed and adrenals removed. Afterward, each left and right adrenal gland was cut into two halves each containing cortical and medullary tissues. The halves were then weighed and pre-incubated in 200 µl DMEM/F-12 for 4 h (37 °C, 95% O_2, 5% CO_2) before any further treatment. Culture medium was then replaced and each half of one adrenal was incubated with either medium containing 0·9% saline (basal) or medium containing 0·9% saline plus ACTH (100 nM) for 6 h at 37 °C (95% O_2 and 5% CO_2). Incubation of adrenals with either saline or ACTH took place on day 21 from ZT1 to ZT7 when mice were killed on day 20 (ZT17) and on day 21/22 from ZT13 to ZT19 when mice were killed on day 21 (ZT5). After incubation, supernatants were carefully removed and stored at −20 °C until analyzed using a commercially available ELISA for CORT (IBL International). Basal as well as ACTH-induced CORT concentrations (ng/ml) in the supernatants were summed up afterwards for left and right adrenal per mouse respectively, and expressed in relation to respective adrenal explants' weight (sum of respective left and right adrenal explants) (ng/ml per mg).

Secretion of IFNγ from anti-CD3-stimulated mesenteric lymph node cells

Isolation and anti-CD3 stimulation of mesenteric lymph node cells from SHC, SDL, and SDD mice (experiment 2, day 20/21; experiment 4, day 28) were performed as described in detail before (Reber *et al.* 2006, 2008). Briefly, mesenteric lymph nodes (pooled from each experimental group) were harvested under sterile conditions and collected on ice in cell culture medium (RPMI 1640 supplemented with 10% fetal bovine serum (PAA, Cölbe, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA), and 3×10^{-5} M 2-mercaptoethanol (Sigma)). Lymph nodes were mechanically disrupted and filtered through a cell strainer (70 µm Nylon, Falcon, Becton Dickinson, Heidelberg, Germany). Cells were washed twice in cell culture medium and adjusted to a concentration of 10^6 cells/ml. Then, 2×10^5 (200 µl) lymph node cells were transferred to wells of a 96-well plate; to stimulate the cells, the wells were pre-coated with 100 µl of 2·5 µg/ml anti-CD3 antibody. Eight wells were loaded with the respective number of cells for each experimental group. After incubation for 24 h (37 °C, 5% CO_2), concentrations of the cytokine IFNγ were measured in the supernatants by ELISA (Thermo Fisher Scientific, Rockford, IL, USA) according to the respective protocols, using four wells per experimental group.

Determination of the severity of DSS-induced colitis

Acute colitis was induced by administering 1% DSS (36–50 kDa; ICN, Eschwege, Germany) in the drinking water *ad libitum* for 8 days as described previously (Reber *et al.* 2006, 2008), starting on day 21.

On day 28, inflammatory shortening of colonic length and the histological damage score were used to quantify the severity of the DSS-induced colonic inflammation as described previously (Reber *et al.* 2006, 2008). Briefly, the colon was removed, mechanically cleaned, and measured to 0·1 cm precision. Afterward, 1 cm of the distal third of the colon was cut longitudinally, laid on filter paper, and fixed in 10% formalin overnight. The next day, the fixed tissue was embedded in paraffin and cut longitudinally. Three 3 µm hematoxylin–eosin-stained sections taken 100 µm apart were evaluated by histological scoring performed by an investigator blind to treatment. For statistics, each individual score represented the mean of the three sections. Histology was scored as follows after (Obermeier *et al.* 2003, Reber *et al.* 2007, 2008): epithelium (0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas) and infiltration (0, no infiltration; 1, infiltrate around crypt basis; 2, infiltrate reaching to lamina muscularis mucosae; 3, extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema; 4, infiltration of the lamina submucosa). The total histological score represents the sum of the epithelium and infiltration score and ranges from 0 to 8.

Statistical analysis

For statistical comparisons, the software package SPSS (version 19) was used. One-way ANOVA (factor=treatment (SHC vs SDL vs SDD)) followed by Bonferroni *post hoc* test, if appropriate, was used for analysis of changes in body weight gain across SD, in the time spent on open arms and entries into closed arms during EPM testing, and in delta body weight gain, colon length, histological damage score, and IFNγ secretion from mesenteric lymph node cells during DSS treatment. ANOVA for repeated measures (rm ANOVA) followed by Bonferroni *post hoc* test, if appropriate, was used for analysis of the time spent in the contact zone during the SPAT (factor 1=trial (empty cage vs mouse in cage) and factor 2=treatment (SHC vs SDL vs SDD)) and of the number of active behaviors during home-cage observations (factor 1=day (0, 7, 14, and 20) and factor 2=treatment (SHC vs SDL vs SDD)). All comparisons depending on two

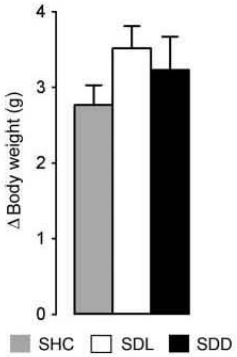


Figure 2 Effect of SD during light phase (SDL) and dark phase (SDD) on body weight. Body weight gain of SHC ($n=14$, grey bars), SDL ($n=14$, white bars), and SDD ($n=14$, black bars) mice across the stress procedure. Data represent mean weight gain (between day 0 and 20)+S.E.M.

factors (absolute adrenal weight, basal plasma ACTH, and CORT levels, IFNγ secretion from mesenteric lymph nodes (in mice not treated with DSS) (factor 1=treatment (SHC vs SDL vs SDD) and factor 2=time of day); *in vitro* adrenal CORT secretion (factor 1=treatment (SHC, SDL, and SDD) and factor 2=medium (ACTH vs saline)); submission latency (factor 1=treatment (SHC vs SDL vs SDD) and factor 2=interval (0–30 vs 30–60 vs 60–90 vs 90–120)) were performed using a two-way ANOVA followed by Bonferroni *post hoc* test if appropriate. Acute plasma ACTH responses to the fourth SD were analyzed separately for SDL and SDD mice (both vs respective SHC mice) by Mann–Whitney *U* test. Data are presented as mean+S.E.M. Significance was set at $P\leq 0{\cdot}05$.

Results

Neither SDL nor SDD affects body weight development (experiment 1)

The body weight gain between days 0 and 20 was not different between SHC, SDL, and SDD mice (Fig. 2).

Submission latencies and behavioral patterns shown during SDL and SDD are comparable (experiment 1)

SDL and SDD mice did not differ in the time spent active nor in the time spent in the contact zone during 2 h of sensory contact. Furthermore, the submission latency before and after sensory contact was comparable between SDL and SDD mice (Table 1).

SDD, but not SDL, decreases social preference (experiment 1)

The absolute time spent in the contact zone during the SPAT was dependent on the stimulus (empty cage or mouse; $F_{1,25}=15{\cdot}9$; $P\leq 0{\cdot}001$). *Post hoc* analysis showed that only SHC ($P\leq 0{\cdot}01$) and SDL ($P\leq 0{\cdot}01$), but not SDD, mice spent significantly more time in the social contact zone (mouse) compared with the nonsocial contact zone (empty cage) (Fig. 3A), thus showing social preference. The time spent exploring the empty cage was not different between the three groups.

Neither SDL nor SDD affects anxiety-like behavior (experiment 1)

In agreement with the SPAT results, neither the percentage of time spent in the open arms of the EPM, indicative of anxiety-like behavior, nor the number of closed arm entries, indicative of locomotor behavior, was statistically different between SHC, SDL, and SDD mice (Fig. 3B and C).

SDD, but not SDL, reduces dark phase activity on stress-free days 7, 14, and 20 (experiment 2)

Active behavior in the dark phase was found to be dependent on both the day of observation and the SD paradigm employed ($F_{2,69}=20{\cdot}9$; $P\leq 0{\cdot}001$). *Post hoc* analysis showed that SDD mice were significantly less active on days 7, 14, and 20 compared with SHC ($P\leq 0{\cdot}001$) and SDL ($P\leq 0{\cdot}001$) mice and compared with respective day 0 values ($P\leq 0{\cdot}001$; Fig. 4). By contrast, active behavior in the light phase was neither dependent on the day of observation nor on the SD paradigm employed (data not shown).

SDL and SDD increase adrenal gland weight (experiment 2)

Absolute adrenal gland weight was dependent on the SD paradigm employed ($F_{2,38}=19{\cdot}4$; $P\leq 0{\cdot}001$). *Post hoc* analysis revealed that, in comparison with SHC mice, SDL ($P\leq 0{\cdot}01$)

Table 1 Submission latency before and after sensory contact and analysis of behavior shown during sensory contact (experiment 1). Submission latencies and the acute behavioral stress response (active behavior and time spent in contact zone) of SDL ($n=14$) and SDD ($n=15$) mice. Data represent mean±S.E.M.

	SDL ($n=14$)	**SDD** ($n=15$)
Time spent in contact zone during sensory contact (%)		
0–30 min	17±0·013	20±0·016
30–60 min	18±0·017	22±0·021
60–90 min	20±0·018	20±0·018
90–120 min	19±0·021	23±0·016
Active behavior during sensory contact (%)		
0–30 min	82±0·030	84±0·023
30–60 min	81±0·029	80±0·027
60–90 min	79±0·033	79±0·025
90–120 min	76±0·037	79±0·023
Submission latency (s)		
Before SD	76·7±8·55	66·1±6·96
After SD	74·4±6·10	64·5±6·02

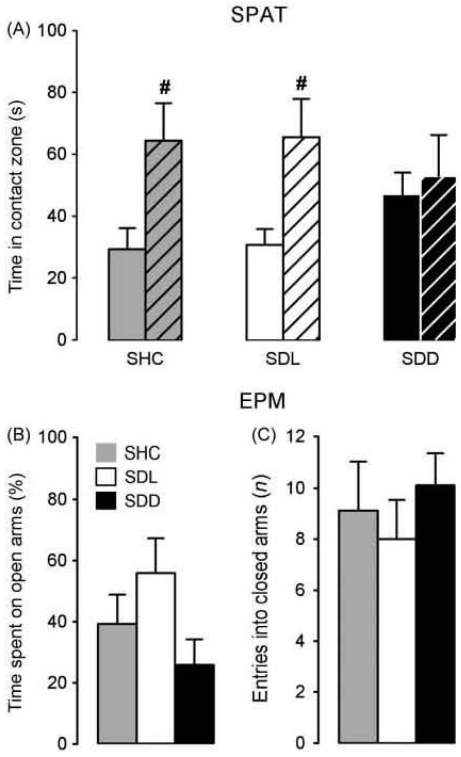


Figure 3 Effects of SD during the light phase (SDL) and dark phase (SDD) on social and general anxiety. To assess chronic psychosocial stress effects on social preference, SHC ($n=9$), SDL ($n=10$), and SDD ($n=9$) mice were exposed to the social preference/avoidance test (SPAT) on day 21 between ZT1 and ZT4 (A). Data show the absolute time (s) spent in the 8 cm broad contact zone around the wire mesh cage without (solid bars) and with (striped bars) a conspecific mouse present; with both trials lasting 150 s each. Data represent mean+S.E.M.; $^{\#}P\leq0{\cdot}05$ vs respective group in the non-social trial. To assess anxiety-like behavior, SHC ($n=8$, grey bars), SDL ($n=9$, white bars), and SDD ($n=9$, black bars) mice were exposed to the elevated plus-maze (EPM) on day 21 (ZT1–ZT4; B and C). Data show the % time on open arms (B) and the number of entries into closed arms (C). Data represent mean+S.E.M.

and SDD ($P\leq0{\cdot}01$) mice adrenal gland weight was significantly increased when measured in the mid-dark phase. However, when measured in the mid-light phase, adrenal gland weight was significantly increased in SDL, but not SDD, mice ($P\leq0{\cdot}001$) in comparison with respective SHC mice (Fig. 5A).

SDD and SDL result in a loss of plasma ACTH rhythmicity (experiment 2)

Plasma ACTH was found to be affected by both the time of day of trunk blood collection and the SD paradigm employed ($F_{2,39}=3{\cdot}79$; $P\leq0{\cdot}05$). *Post hoc* tests showed that SHC ($P\leq0{\cdot}001$), but not SDL and SDD, mice had significantly higher ACTH levels during the dark phase than the light phase.

Furthermore, separate statistical analysis (one-way ANOVA followed by Bonferroni *post hoc* analysis) of mice killed in the light phase showed that, in comparison with SHC mice, both SDL and SDD mice had significantly higher plasma ACTH levels (both $P\leq0{\cdot}05$ vs SHC; $F_{2,18}=6{\cdot}82$; $P\leq0{\cdot}01$). However, plasma ACTH levels of mice killed during the dark phase did not differ between the three groups (Fig. 5B).

SDD, but not SDL, results in a loss of plasma CORT rhythmicity (experiment 2)

Two-way ANOVA did not reveal any significant dependence of plasma CORT levels on the time of day or the SD paradigm employed. However, separate statistical analysis employing a Mann–Whitney *U* comparison showed that plasma CORT of SHC and SDL mice was significantly lower during the light phase than the dark phase ($P\leq0{\cdot}05$). This light/dark difference was absent in SDD mice showing high CORT levels during both the light and dark phases (Fig. 5C).

Four days of SDL, but not of SDD, results in adaptation of the acute SD-induced plasma ACTH response (experiment 3)

Statistical analysis of plasma ACTH levels determined 25 min after the last defeat on day 4 indicated that SDD mice had

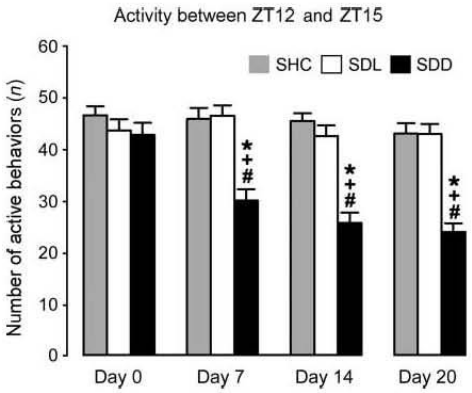


Figure 4 Effects of SD during light phase (SDL) and dark phase (SDD) on active behavior. To assess active home-cage behavior during the first 3 h following lights-off (ZT12–ZT15) on days 0, 7, 14, and 20, climbing, locomotion, rearing, eating, drinking, grooming, and digging behaviors were scored in the home-cage of SHC ($n=24$, grey bars), SDL ($n=25$, white bars), and SDD ($n=23$, black bars) mice. Data represent mean+S.E.M.; $^{*}P\leq0{\cdot}05$ vs respective SHC mice; $^{+}P\leq0{\cdot}05$ vs respective SDL mice; $^{\#}P\leq0{\cdot}05$ vs respective group on day 0.

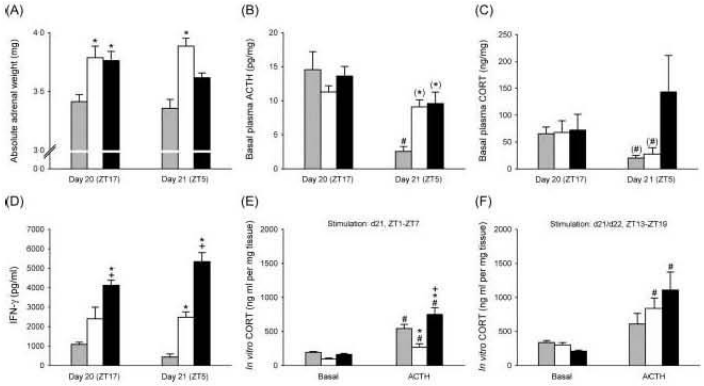


Figure 5 Effects of SD during the light phase (SDL) and dark phase (SDD) on adrenal weight, basal plasma ACTH and CORT, IFNγ, and adrenal *in vitro* ACTH responsiveness. Separate cohorts of SHC (grey bars), SDL (white bars), and SDD mice (black bars) were killed on day 20 (ZT17; 38 h/26 h after the last SD for SDL/SDD mice; $n=7–8$ per group) or on day 21 (ZT5; 50 h/38 h after the last SD for SDL/SDD mice; $n=6–7$ per group). Trunk blood was collected for quantification of basal plasma ACTH (B) and CORT (C). In each set the adrenal glands were removed, pruned from fat and weighed (A). Subsequently, adrenal explants were incubated with either medium containing saline (basal) or medium containing ACTH (100 nM) for 6 h (day 21, ZT1–ZT7; day 21/day 22, ZT13–ZT19). Afterwards, CORT concentrations in the supernatants (ng/ml) were quantified and expressed per mg of adrenal tissue (ng/ml per mg) (E and F). In addition, *in vitro* anti-CD3-stimulated IFNγ secretion from mesenteric lymph node cells was assessed (D). Data represent mean+S.E.M.; $^{*}P\leq0{\cdot}05$ vs respective SHC mice; $^{+}P\leq0{\cdot}05$ vs respective SDL mice; $^{\#}P\leq0{\cdot}05$ vs respective group on day 20, ZT17 (B and C) or vs respective basal (E and F); $^{(*)}P\leq0{\cdot}05$ (one-way ANOVA) vs respective SHC mice; $^{(\#)}P\leq0{\cdot}05$ (Mann–Whitney *U* test) vs respective group on day 20 (ZT17).

significantly higher plasma ACTH levels than respective SHC mice ($P\leq0{\cdot}01$; Fig. 6B). This effect was absent in SDL mice (Fig. 6A).

SDL, but not SDD, results in a reduction of adrenal in vitro *ACTH responsiveness (experiment 2)*

When adrenal explants were stimulated during the light phase, CORT secretion was found to be dependent on both the SD paradigm employed and the medium used (ACTH or saline) ($F_{2,40}=7{\cdot}19$; $P\leq0{\cdot}01$). *Post hoc* analyses revealed a significant increase in CORT secretion after *in vitro* ACTH stimulation in SHC ($P\leq0{\cdot}001$), SDL ($P\leq0{\cdot}05$), and SDD ($P\leq0{\cdot}001$) mice (Fig. 5E). However, this was significantly less pronounced in SDL compared with both SHC ($P\leq0{\cdot}01$) and SDD ($P\leq0{\cdot}001$) mice. By contrast, SDD mice showed a more pronounced ACTH-induced CORT secretion compared with SHC mice ($P\leq0{\cdot}05$).

When adrenal explants were stimulated during the dark phase, CORT secretion was dependent only on the medium used (ACTH or saline) ($F_{1,34}=26{\cdot}2$; $P\leq0{\cdot}001$). *Post hoc* analysis showed that ACTH-treated adrenal explants from SDL ($P\leq0{\cdot}01$) and SDD ($P\leq0{\cdot}001$) mice secreted significantly more CORT than respective saline-treated ones (Fig. 5F).

SDD results in a more pronounced elevation of IFNγ secretion from mesenteric lymph node cells than SDL (experiment 2)

IFNγ secretion from mesenteric lymph node cells was significantly dependent on the SD paradigm employed ($F_{2,16}=52{\cdot}1$; $P\leq0{\cdot}001$). When mice were killed during the dark phase, SDD mice showed a significantly higher IFNγ secretion compared with both SHC ($P\leq0{\cdot}001$) and SDL ($P\leq0{\cdot}05$) mice. When mice were killed during the light phase, both SDL ($P\leq0{\cdot}01$) and SDD ($P\leq0{\cdot}001$) mice showed a significantly higher IFNγ secretion than SHC mice. Again, this was more pronounced in SDD mice ($P\leq0{\cdot}001$ vs SDL) (Fig. 5D).

SDD causes a more severe aggravation of DSS colitis than SDL (experiment 4)

Body weight The body weight development between days 1 (i.e. day 21) and 8 (i.e. day 28) of DSS treatment was found to be dependent on the SD paradigm employed ($F_{2,25}=3{\cdot}97$; $P\leq0{\cdot}05$). *Post hoc* tests revealed that SDD mice lost significantly more body weight than SHC mice ($P\leq0{\cdot}05$; Fig. 7A).

Colon length The colon length following DSS treatment was dependent on the SD paradigm employed ($F_{2,25}=4{\cdot}31$;

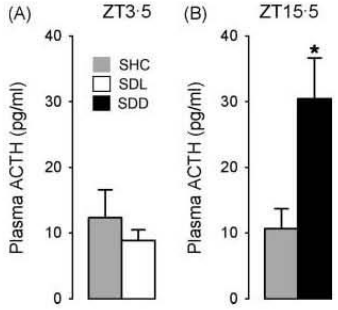


Figure 6 Effects of acute SD during the light phase (SDL) and dark phase (SDD) on plasma ACTH. To assess the effect of four SD sessions on HPA axis activity, SHC, SDL, and SDD mice were killed 25 min after the last defeat on day 4 (A: SDL and SHC mice in the light phase at ~ZT3·5, $n=8$ per group; B: SDD and SHC mice in the dark phase at ~ZT15·5; $n=8$ per group) and plasma ACTH was measured. Data represent mean+S.E.M.; $^{*}P\leq0{\cdot}05$ vs respective SHC mice.

$P\leq0{\cdot}05$). *Post hoc* analysis showed that SDD mice had a significantly shorter colon than SHC mice ($P\leq0{\cdot}05$; Fig. 7B).

Histological damage score The histological damage score of the colon was dependent on the SD paradigm employed ($F_{2,25}=5{\cdot}96$; $P\leq0{\cdot}01$). *Post hoc* analysis revealed that both SDL and SDD mice showed a significantly increased histological damage score compared with SHC mice ($P\leq0{\cdot}05$; Fig. 7C).

IFNγ secretion from mesenteric lymph node cells IFNγ secretion was dependent on the SD paradigm employed ($F_{2,9}=403$; $P\leq0{\cdot}001$). *Post hoc* analysis showed that IFNγ secretion from mesenteric lymph node cells was significantly higher in SDD mice compared with both SHC ($P\leq0{\cdot}001$) and SDL ($P\leq0{\cdot}001$) mice (Fig. 7D).

Discussion

In this study, we demonstrate that the effects of chronic/intermittent psychosocial stress on behavior, physiology, and immunology strongly depend on the time of day of stressor exposure, thus highlighting the importance of the circadian clock in this context. In more detail, while physiological parameters, such as absolute adrenal gland weight and adrenal ACTH responsiveness, were more affected by SD during the light phase, behavioral and immunological parameters like active home-cage behavior, social preference, IFNγ secretion from mesenteric lymph node cells, and severity of DSS colitis were more affected by dark phase SD. Thus, our findings clearly show that the same stressor has a more pronounced negative outcome when applied during the active phase of an organism and provide the first evidence, suggesting that the physiological changes that occur in response to intermittent stressor exposure can be beneficial (SDL) or maladaptive (SDD).

Though there have been studies showing decreased body weight gain following repeated/chronic stressor exposure (Sachser & Lick 1989, Berton *et al.* 1998), body weight in this study was not affected by SD, regardless of the time of day of stressor exposure. However, recent studies (Razzoli *et al.* 2011, Savignac *et al.* 2011, Slattery *et al.* 2011) have suggested that this parameter is not a reliable indicator of chronic stressor exposure.

In addition to body weight, assessment of adrenal gland weight and alterations in HPA axis activity are often used as hallmark signs of chronic stress. In line with the literature (Zelena *et al.* 2003, Reber *et al.* 2007), adrenal gland weight was increased in SDL and SDD mice in the mid-dark phase 1 day after stressor termination. However, this effect was only transient in SDD mice, as it disappeared 12 h later at a time after stressor termination where they were still enlarged in SDL mice. Support for a more pronounced effect of

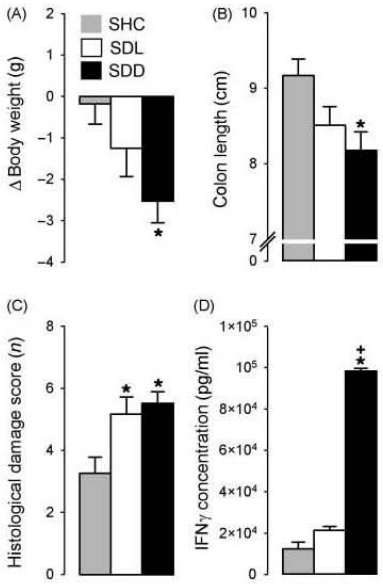


Figure 7 Effects of SD during the light phase (SDL) and dark phase (SDD) on the severity of DSS-induced colitis. To assess the consequences of SD on a chemically-induced colitis, SHC (grey bars, $n=9$), SDL (white bars, $n=10$), and SDD mice (black bars, $n=9$) were treated with 1% DSS in the drinking water from day 21 to day 28 in order to induce acute colitis. Body weight gain (A), colonic length (B), histological damage score of the colon (C) and IFNγ secretion from anti-CD3-stimulated mesenteric lymph node cells (D, pooled per group and measured in quadruplets) were quantified to assess colitis severity. Data represent mean+S.E.M.; $^{*}P\leq0{\cdot}05$ vs respective SHC mice; $^{+}P<0{\cdot}05$ vs respective SDL mice.

light phase compared with dark phase stressor exposure on the HPA axis also comes from a previous study showing a higher acute HPA axis response, indicated by higher plasma ACTH and CORT levels, to restraint stress in the former phase (Kant *et al.* 1986).

Given these discrepancies in adrenal weight, we next assessed the influence of SDL and SDD on plasma CORT and ACTH. Interestingly, basal plasma CORT concentrations were not affected in either phase by repeated SD, regardless of the time of day of repeated stressor exposure. This finding is contradictory to studies reporting either hypercorticism (for review, see Bartolomucci *et al.* (2005), Ushijima *et al.* (2006)) or hypocorticism (Sanchez *et al.* 1998, Reber *et al.* 2006, 2007) as a consequence of chronic/intermittent psychosocial stressor exposure. However, basal plasma CORT levels in the dark phase were significantly higher than during the light phase in SHC and SDL, but not SDD, mice indicating a disturbed diurnal plasma CORT rhythm following dark phase stressor exposure. This lack, or shift, of CORT rhythmicity exclusively in SDD mice is of special relevance, given that both SDL and SDD mice showed disturbed diurnal plasma ACTH patterns (elevated basal light phase levels), when compared with SHC mice. Together, these findings strongly suggest that adrenals of SDL, but not those of SDD, mice develop an adaptive reduction in at least light phase ACTH responsiveness in order to maintain low basal light phase plasma GC levels and, thus, a vital diurnal CORT rhythm. In contrast to the transient increase in SDD mice, the more persistent increase in adrenal mass seen in SDL mice might be crucial for maintaining this vital CORT rhythm by exhibiting a reduced ACTH sensitivity during the light phase.

As other factors can influence CORT release *in vivo*, we next assessed the effects of ACTH on CORT secretion of adrenal explants *in vitro*. Our *in vivo* hypotheses are supported by our *in vitro* data assessing adrenal gland ACTH responsivity. While adrenal explants of all three treatment groups (SHC, SDL, and SDD) responded to ACTH with a significant increase in CORT secretion, this effect was blunted in SDL when adrenals were stimulated during the light phase on day 21. Intriguingly, the response of the SDD was even greater than that of the SHC mice at this timepoint, which supports our claim that these mice do not generate an adaptive ACTH insensitivity. Importantly, peripheral organs such as the adrenal glands show their intrinsic rhythm of clock gene expression (likely a measure of the rhythmicity of the tissue) for at least a few days after they had been removed from an animal, indicating that this process is not centrally regulated by the SCN (Yamazaki *et al.* 2000, 2009). Thus, the CORT secretion of adrenal explants during *in vitro* ACTH stimulation does not reflect the potential of the adrenal to secret CORT at the moment of killing, but at the actual stimulation period.

In addition to maintaining low light phase plasma CORT levels following stressor termination, the reduction in light phase adrenal ACTH sensitivity allows SDL mice to adapt their HPA axis responses to the temporally predictable repeated SD sessions taking place from ZT1 to ZT3 over 19 consecutive days (except observation days). Besides the decrease in light phase adrenal ACTH sensitivity, HPA axis adaptations in SDL mice are further seen at the level of the pituitary gland on day 4 of SDL. In detail, plasma ACTH was not affected in SDL, but increased in SDD mice, 25 min after termination of the SD session on day 4 of repeated stressor exposure, further indicating that the process of adaptation in SDL mice develops reasonably early in the context of repeated stressor exposure. Herefore, the decrease in adrenal ACTH sensitivity in SDL mice during the light phase seems more likely to represent an adaptational process to this predictable SD stressor than a maladaptive consequence of chronic/repeated SD.

In addition to the physiological and neuroendocrine changes that were found to strongly depend on the time of day of the stressor exposure, home-cage behavioral analyses revealed profound differences in the dark, but not light, phase activity profiles of the different treatment groups. SDD, but not SDL, mice showed a significant decrease in dark phase activity on days 7, 14, and 20 when compared with SHC mice. At least for the SDD group, our data are consistent with a previous study, reporting a decrease in activity during the active, but not inactive, phase after chronic mild stress in rats (Gorka *et al.* 1996). Given that SDD mice were predictably exposed to SD for 19 consecutive days (except observation days) at the beginning of the dark phase, a reduction in dark phase activity is likely to indicate that SDD mice are expecting the next SD. As a consequence, they show less motivation and drive during that phase, which are approved hallmarks of affective disorders such as depression and anxiety (Willner 1990, Liebsch *et al.* 1998). The finding that these behavioral alterations were lacking in SDL mice gives further support for our hypothesis that these mice actively develop coping mechanisms, which enable them to adapt better to the situation than SDD mice.

Further support for this is shown by the fact that SDL, but not SDD, mice display normal social preference toward an unfamiliar male conspecific. Social avoidance has also been described following 10 days of repeated SD with 24-h sensory contact (Berton *et al.* 2006, Krishnan *et al.* 2007) and was associated with depressive-like and anxiety-related behavioral abnormalities and, thus, can be interpreted as a maladaptive behavioral consequence (Lagace *et al.* 2010, Slattery *et al.* 2011).

Importantly, and in agreement with the EPM results of this study, the time spent in the nonsocial contact zone was not affected by SDL or SDD, suggesting that irrespective of the time of exposure, anxiety-like behavior is not influenced by repeated SD. In support, lack of behavioral signs of anxiety has also been found following 19 days of repeated SD and/or overcrowding (Slattery *et al.* 2011), which is in contrast with elevated levels of anxiety as a consequence of chronic psychosocial stress paradigms such as chronic subordinate colony housing (Reber *et al.* 2007, Slattery *et al.* 2011).

Thus, these different social stress models have disparate behavioral and physiological effects akin to those seen following SDL and SDD respectively. Despite discrepancies in the above mentioned parameters, SD and/or overcrowding as well as chronic subordinate colony housing lead to the same immunological effects and aggravate DSS-induced colitis (Reber *et al.* 2006, 2008). Therefore, as the physiological and behavioral results of this study do not allow for a clear conclusion on whether they are adaptive or maladaptive, we investigated the immunological response to DSS-induced colitis, a model that is used to determine the maladaptive consequence of chronic psychosocial stress (Reber *et al.* (2006), for review, see Reber (2011)). Thus, SHC, SDL, and SDD mice were treated with DSS in their drinking water for 8 days subsequent to 19 days of SD. As expected, the severity of DSS-induced colitis was increased in SDL and SDD mice compared with SHC mice. In agreement with our and other previous data (Reber *et al.* (2007), for review, see Reber (2011), Savignac *et al.* (2011)), repeated/chronic psychosocial stress, induced by SD either during light or dark phase in this study, resulted in development of a mild spontaneous colonic inflammation, indicated by an increased histological damage score in both SDL and SDD mice. However, the inflammatory response was significantly more pronounced in SDD mice, indicated by a greater body weight loss, inflammatory reduction of colon length, and IFNγ secretion from anti CD3-stimulated mesenteric lymph node cells in SDD compared with SDL mice. This strongly suggests that SD during the dark phase has a stronger pro-inflammatory potential compared with SD during the light phase. Thus, these findings further suggest both maladaptive affective and somatic consequences of repeated SD during the dark (active) phase, in contrast to SDL, which results in beneficial adaptations preventing these maladaptive effects.

Importantly, SDL and SDD mice did not differ in submission latencies toward their respective residents, as well as in time spent in the contact zone and time spent active during the 2 h of sensory contact. Therefore, it is very unlikely that any of the above described behavioral, physiological, and immunological differences between SDL and SDD mice are simply due to different levels of aggression received during the light phase (inactive period) and dark phase (active period) SD.

General conclusion

Overall, our study is the first to demonstrate that chronic/intermittent psychosocial stress effects on behavior, physiology, and immunology strongly depend on the time of day of stressor exposure. While physiological parameters such as adrenal gland weight and adrenal ACTH responsiveness were more affected by SD during the light phase, behavioral and immunological parameters like active home-cage behavior during the dark phase, social preference, IFNγ secretion from mesenteric lymph node cells, and severity of a DSS-induced colitis were more affected by SD during the dark phase. In contrast to the physiological changes seen in SDL mice that are likely to represent beneficial adaptations, decreased interest in an unfamiliar conspecific, loss of general activity, a flattened diurnal CORT rhythm, and shift toward a more pro-inflammatory body milieu, occurring particularly in SDD mice, represent well-accepted hallmarks of affective disorders such as depression, and, thus, are considered to be maladaptive. Taken together, though further work is required to provide additional insight, our data suggest that equally severe stressors have a more pronounced negative outcome when applied during the active/dark phase. Moreover, they also suggest that the physiological changes seen in mice stressed during the rest period (SDL mice) might represent beneficial adaptations rather than maladaptive consequences.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the Deutsche Forschungsgemeinschaft (DFG FO-207/13-1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contribution statement

M S B, I D N, D A S, C H-F, and S O R involved in the conception and design of the experiments; M S B, N U-S, D K, and S O R involved in the collection, analysis, and interpretation of data; M S B, I D N, D A S, C H-F, and S O R involved in drafting the article or revising it critically for important intellectual content.

Acknowledgements

The authors are grateful to N Grunwald, A Havasi, S Peters, A Füchsl, and K Altmann for their excellent technical help and to Dr I Karatsoreos for his helpful comments regarding the manuscript.

References

Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schutz G & Schibler U 2000 Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science* **289** 2344–2347. (doi:10.1126/science.289.5488.2344)

Bartolomucci A, Palanza P, Sacerdote P, Panerai AE, Sgoifo A, Dantzer R & Parmigiani S 2005 Social factors and individual vulnerability to chronic stress exposure. *Neuroscience & Biobehavioral Reviews* **29** 67–81. (doi:10.1016/j.neubiorev.2004.06.009)

Berton O, Aguerre S, Sarrieau A, Mormede P & Chaouloff F 1998 Differential effects of social stress on central serotonergic activity and emotional reactivity in Lewis and spontaneously hypertensive rats. *Neuroscience* **82** 147–159. (doi:10.1016/S0306-4522(97)00282-0)

Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M *et al.* 2006 Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science* **311** 864–868. (doi:10.1126/science.1120972)

de Bodinat C, Guardiola-Lemaitre B, Mocaer E, Renard P, Munoz C & Millan MJ 2010 Agomelatine, the first melatonergic antidepressant: discovery, characterization and development. *Nature Reviews. Drug Discovery* **9** 628–642. (doi:10.1038/nrd3274)

Deuschle M, Schweiger U, Weber B, Gotthardt U, Korner A, Schmider J, Standhardt H, Lammers CH & Heuser I 1997 Diurnal activity and pulsatility of the hypothalamus–pituitary–adrenal system in male depressed patients and healthy controls. *Journal of Clinical Endocrinology and Metabolism* **82** 234–238. (doi:10.1210/jc.82.1.234)

Duffy LC, Zielezny MA, Marshall JR, Byers TE, Weiser MM, Phillips JF, Calkins BM, Ogra PL & Graham S 1991 Relevance of major stress events as an indicator of disease activity prevalence in inflammatory bowel disease. *Behavioral Medicine* **17** 101–110. (doi:10.1080/08964289.1991.9937553)

Gorka Z, Moryl E & Papp M 1996 Effect of chronic mild stress on circadian rhythms in the locomotor activity in rats. *Pharmacology, Biochemistry and Behavior* **54** 229–234. (doi:10.1016/0091-3057(95)02173-6)

Grippo AJ, Beltz TG & Johnson AK 2003 Behavioral and cardiovascular changes in the chronic mild stress model of depression. *Physiology & Behavior* **78** 703–710. (doi:10.1016/S0031-9384(03)00050-7)

Herbert J 1997 Fortnighly review, Stress, the brain, and mental illness. *BMJ* **315** 530–535. (doi:10.1136/bmj.315.7107.530)

Hirschfeld RM 2000 History and evolution of the monoamine hypothesis of depression. *Journal of Clinical Psychiatry* **61** (Suppl 6) 4–6.

Jiang WG, Li SX, Zhou SJ, Sun Y, Shi J & Lu L 2011 Chronic unpredictable stress induces a reversible change of PER2 rhythm in the suprachiasmatic nucleus. *Brain Research* **1399** 25–32. (doi:10.1016/j.brainres.2011.05.001)

Kant GJ, Mougey EH & Meyerhoff JL 1986 Diurnal variation in neuroendocrine response to stress in rats: plasma ACTH, β-endorphin, β-LPH, corticosterone, prolactin and pituitary cyclic AMP responses. *Neuroendocrinology* **43** 383–390. (doi:10.1159/000124553)

Katz MM, Bowden CL & Frazer A 2010 Rethinking depression and the actions of antidepressants: uncovering the links between the neural and behavioral elements. *Journal of Affective Disorders* **120** 16–23. (doi:10.1016/j.jad.2009.08.011)

Konturek PC, Brzozowski T & Konturek SJ 2011 Gut clock: implication of circadian rhythms in the gastrointestinal tract. *Journal of Physiology and Pharmacology* **62** 139–150.

Koolhaas JM, Bartolomucci A, Buwalda B, de Boer SF, Flugge G, Korte SM, Meerlo P, Murison R., Olivier B, Palanza P *et al.* 2011 Stress revisited: a critical evaluation of the stress concept. *Neuroscience & Biobehavioral Reviews* **35** 1291–1301. (doi:10.1016/j.neubiorev.2011.02.003)

Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ, Laplant Q, Graham A, Lutter M, Lagace DC *et al.* 2007 Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* **131** 391–404. (doi:10.1016/j.cell.2007.09.018)

Lagace DC, Donovan MH, DeCarolis NA, Farnbauch LA, Malhotra S, Berton O, Nestler EJ, Krishnan V & Eisch AJ 2010 Adult hippocampal neurogenesis is functionally important for stress-induced social avoidance. *PNAS* **107** 4436–4441. (doi:10.1073/pnas.0910072107)

Levenstein S, Prantera C, Varvo V, Scribano ML, Andreoli A, Luzi C, Arca M, Berto E, Milite G & Marcheggiano A 2000 Stress and exacerbation in ulcerative colitis: a prospective study of patients enrolled in remission. *American Journal of Gastroenterology* **95** 1213–1220. (doi:10.1111/j.1572-0241.2000.02012.x)

Liebsch G, Montkowski A, Holsboer F & Landgraf R 1998 Behavioural profiles of two Wistar rat lines selectively bred for high or low anxiety-related behaviour. *Behavioural Brain Research* **94** 301–310. (doi:10.1016/S0166-4328(97)00198-8)

Moreau JL, Scherschlicht R, Jenck F & Martin JR 1995 Chronic mild stress-induced anhedonia model of depression; sleep abnormalities and curative effects of electroshock treatment. *Behavioural Pharmacology* **6** 682–687. (doi:10.1097/00008877-199511000-00003)

Nader N, Chrousos GP & Kino T 2010 Interactions of the circadian CLOCK system and the HPA axis. *Trends in Endocrinology and Metabolism* **21** 277–286. (doi:10.1016/j.tem.2009.12.011)

Obermeier F, Dunger N, Strauch UG, Grunwald N, Herfarth H, Scholmerich J & Falk W 2003 Contrasting activity of cytosin–guanosin dinucleotide oligonucleotides in mice with experimental colitis. *Clinical and Experimental Immunology* **134** 217–224. (doi:10.1046/j.1365-2249.2003.02288.x)

Ottenweller JE, Servatius RJ & Natelson BH 1994 Repeated stress persistently elevates morning, but not evening, plasma corticosterone levels in male rats. *Physiology & Behavior* **55** 337–340. (doi:10.1016/0031-9384(94)90143-0)

Perez-Cruz C, Simon M, Flugge G, Fuchs E & Czeh B 2009 Diurnal rhythm and stress regulate dendritic architecture and spine density of pyramidal neurons in the rat infralimbic cortex. *Behavioural Brain Research* **205** 406–413. (doi:10.1016/j.bbr.2009.07.021)

Racagni G, Riva MA & Popoli M 2007 The interaction between the internal clock and antidepressant efficacy. *International Clinical Psychopharmacology* **22** (Suppl 2) S9–S14. (doi:10.1097/01.yic.0000277957.75852.c7)

Razzoli M, Carboni L, Andreoli M, Ballottari A & Arban R 2011 Different susceptibility to social defeat stress of BalbC and C57BL6/J mice. *Behavioural Brain Research* **216** 100–108. (doi:10.1016/j.bbr.2010.07.014)

Reber SO 2011 Stress and animal models of inflammatory bowel disease – an update on the role of the hypothalamo-pituitary–adrenal axis. *Psychoneuroendocrinology* **37** 1–19. (doi:10.1016/j.psyneuen.2011.05.014)

Reber SO, Obermeier F, Straub RH, Falk W & Neumann ID 2006 Chronic intermittent psychosocial stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration. *Endocrinology* **147** 4968–4976. (doi:10.1210/en.2006-0347)

Reber SO, Birkeneder L, Veenema AH, Obermeier F, Falk W, Straub RH & Neumann ID 2007 Adrenal insufficiency and colonic inflammation after a novel chronic psycho-social stress paradigm in mice: implications and mechanisms. *Endocrinology* **148** 670–682. (doi:10.1210/en.2006-0983)

Reber SO, Obermeier F, Straub RH, Veenema AH & Neumann ID 2008 Aggravation of DSS-induced colitis after chronic subordinate colony (CSC) housing is partially mediated by adrenal mechanisms. *Stress* **11** 225–234. (doi:10.1080/10253890701733351)

Rybkin II, Zhou Y, Volaufova J, Smagin GN, Ryan DH & Harris RB 1997 Effect of restraint stress on food intake and body weight is determined by time of day. *American Journal of Physiology* **273** R1612–R1622.

Sachser N & Lick C 1989 Social stress in guinea pigs. *Physiology & Behavior* **46** 137–144. (doi:10.1016/0031-9384(89)90246-1)

Sanchez MM, Aguado F, Sanchez-Toscano F & Saphier D 1998 Neuroendocrine and immunocytochemical demonstrations of decreased hypothalamo-pituitary–adrenal axis responsiveness to restraint stress after long-term social isolation. *Endocrinology* **139** 579–587. (doi:10.1210/en.139.2.579)

Savignac HM, Hyland NP, Dinan TG & Cryan JF 2011 The effects of repeated social interaction stress on behavioural and physiological parameters in a stress-sensitive mouse strain. *Behavioural Brain Research* **216** 576–584. (doi:10.1016/j.bbr.2010.08.049)

Sgoifo A, Pozzato C, Meerlo P, Costoli T, Manghi M, Stilli D, Olivetti G & Musso E 2002 Intermittent exposure to social defeat and open-field test in rats: acute and long-term effects on ECG, body temperature and physical activity. *Stress* **5** 23–35. (doi:10.1080/102538902900012387)

Shalev AY 2009 Posttraumatic stress disorder and stress-related disorders. *Psychiatric Clinics of North America* **32** 687–704. (doi:10.1016/j.psc.2009.06.001)

Singewald GM, Nguyen NK, Neumann ID, Singewald N & Reber SO 2009 Effect of chronic psychosocial stress-induced by subordinate colony (CSC) housing on brain neuronal activity patterns in mice. *Stress* **12** 58–69. (doi:10.1080/10253890802042082)

Slattery DA, Hudson AL & Nutt DJ 2004 Invited review: the evolution of antidepressant mechanisms. *Fundamental & Clinical Pharmacology* **18** 1–21. (doi:10.1111/j.1472-8206.2004.00195.x)

Slattery DA, Uschold N, Magoni M, Bar J, Popoli M, Neumann ID & Reber SO 2011 Behavioural consequences of two chronic psychosocial stress paradigms: anxiety without depression. *Psychoneuroendocrinology* **37** 702–714. (doi:10.1016/j.psyneuen.2011.09.002)

Tornatzky W & Miczek KA 1993 Long-term impairment of autonomic circadian rhythms after brief intermittent social stress. *Physiology & Behavior* **53** 983–993. (doi:10.1016/0031-9384(93)90278-N)

Uschold-Schmidt N, Nyuyki KD, Fuchsl AM, Neumann ID & Reber SO 2012 Chronic psychosocial stress results in sensitization of the HPA axis to acute heterotypic stressors despite a reduction of adrenal *in vitro* ACTH responsiveness. *Psychoneuroendocrinology* **37** 1676–1687. (doi:10.1016/j.psyneuen.2012.02.015)

Ushijima K, Morikawa T, To H, Higuchi S & Ohdo S 2006 Chronobiological disturbances with hyperthermia and hypercortisolism induced by chronic mild stress in rats. *Behavioural Brain Research* **173** 326–330. (doi:10.1016/j.bbr.2006.06.038)

Willner P 1990 Animal models of depression: an overview. *Pharmacology & Therapeutics* **45** 425–455. (doi:10.1016/0163-7258(90)90076-E)

Winkler D, Pjrek E, Praschak-Rieder N, Willeit M, Pezawas L, Konstantinidis A, Stastny J & Kasper S 2005 Actigraphy in patients with seasonal affective disorder and healthy control subjects treated with light therapy. *Biological Psychiatry* **58** 331–336. (doi:10.1016/j.biopsych.2005.01.031)

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M & Tei H 2000 Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **288** 682–685. (doi:10.1126/science.288.5466.682)

Yamazaki S, Yoshikawa T, Biscoe EW, Numano R, Gallaspy LM, Soulsby S, Papadimas E, Pezuk P, Doyle SE, Tei H *et al.* 2009 Ontogeny of circadian organization in the rat. *Journal of Biological Rhythms* **24** 55–63. (doi:10.1177/0748730408328438)

Yous S, Andrieux J, Howell HE, Morgan PJ, Renard P, Pfeiffer B, Lesieur D & Guardiola-Lemaitre B 1992 Novel naphthalenic ligands with high affinity for the melatonin receptor. *Journal of Medicinal Chemistry* **35** 1484–1486. (doi:10.1021/jm00086a018)

Zelena D, Mergl Z, Foldes A, Kovacs KJ, Toth Z & Makara GB 2003 Role of hypothalamic inputs in maintaining pituitary–adrenal responsiveness in repeated restraint. *American Journal of Physiology. Endocrinology and Metabolism* **285** E1110–E1117.

Zohar J & Westenberg HG 2000 Anxiety disorders: a review of tricyclic antidepressants and selective serotonin reuptake inhibitors. *Acta Psychiatrica Scandinavica* **403** 39–49. (doi:10.1111/j.1600-0447.2000.tb10947.x)

Received in final form 20 August 2012
Accepted 20 September 2012
Made available online as an Accepted Preprint
20 September 2012

FULL TEXT PAPER 2

Chronobiology International, 2014; 31(9): 996–1007
© Informa Healthcare USA, Inc.
ISSN: 0742-0528 print / 1525-6073 online
DOI: 10.3109/07420528.2014.940085

informa
healthcare

ORIGINAL ARTICLE

Repeated psychosocial stress at night, but not day, affects the central molecular clock

Manuela S. Bartlang[1]*, Sergey A. Savelyev[2]*, Anne-Sofie Johansson[2], Stefan O. Reber[3], Charlotte Helfrich-Förster[1]#, and Gabriella B. S. Lundkvist[2,4]#

[1]*Department of Neurobiology and Genetics, Biocenter, University of Würzburg, Würzburg, Germany,* [2]*Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden,* [3]*Laboratory for Molecular Psychosomatics, Clinic for Psychosomatic Medicine and Psychotherapy, University of Ulm, Ulm, Germany, and* [4]*Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden*

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

We have recently demonstrated that the outcome of repeated social defeat (SD) on behavior, physiology and immunology is more negative when applied during the dark/active phase as compared with the light/inactive phase of male C57BL/6 mice. Here, we investigated the effects of the same stress paradigm, which combines a psychosocial and novelty stressor, on the circadian clock in transgenic PERIOD2::LUCIFERASE (PER2::LUC) and wildtype (WT) mice by subjecting them to repeated SD, either in the early light phase (social defeat light = SDL) or in the early dark phase (social defeat dark = SDD) across 19 days. The PER2::LUC rhythms and clock gene mRNA expression were analyzed in the suprachiasmatic nucleus (SCN) and the adrenal gland, and PER2 protein expression in the SCN was assessed. SDD mice showed increased PER2::LUC rhythm amplitude in the SCN, reduced *Per2* and *Cryptochrome1* mRNA expression in the adrenal gland, and increased PER2 protein expression in the posterior part of the SCN compared with single-housed control (SHC) and SDL mice. In contrast, PER2::LUC rhythms in the SCN of SDL mice were not affected. However, SDL mice exhibited a 2-hour phase advance of the PER2::LUC rhythm in the adrenal gland compared to SHC mice. Furthermore, plasma levels of brain-derived neurotrophic factor (BDNF) and BDNF mRNA in the SCN were elevated in SDL mice. Taken together, these results show that the SCN molecular rhythmicity is affected by repeated SDD, but not SDL, while the adrenal peripheral clock is influenced mainly by SDL. The observed increase in BDNF in the SDL group may act to protect against the negative consequences of repeated psychosocial stress.

Keywords: Adrenal gland, circadian clock, clock gene, Period2, social defeat, suprachiasmatic nucleus

INTRODUCTION

It is well established that the hypothalamic–pituitary–adrenal (HPA) axis is under strong circadian influence driven by the master circadian pacemaker: the suprachiasmatic nucleus (SCN) (Moore & Eichler, 1972; Yang et al., 2009). Under homeostatic conditions, the SCN causes a circadian release pattern of glucocorticoids (GC; cortisol in humans, corticosterone in most rodent species) with the highest levels associated with waking up, to prepare the organism for the onset of activity (Kalsbeek et al., 2012). This is achieved by an alternating activity of stimulatory and inhibitory SCN inputs to the paraventricular nucleus (PVN), resulting in a rhythmic release of corticotrophin-releasing hormone that evokes a circadian release of adrenocorticotropic hormone (ACTH) from the anterior pituitary (Carnes et al., 1988, 1989). ACTH, in turn, activates the synthesis and secretion of GC (Kalsbeek et al., 2012). Besides this humoral pathway, a neuronal SCN – adrenal gland connection via the autonomic nervous system modulates GC release from the adrenal glands by modifying the sensitivity of the adrenal cortex to ACTH in a diurnal manner (Barclay et al., 2012). In addition to the central activation of the HPA axis, the molecular clock in the adrenal gland itself contributes to the circadian GC release through rhythmic expression of steroidogenic enzymes such as Steroidogenic Acute Regulatory Protein (StAR) (Oster et al., 2006; Son et al., 2008).

Acute stressful situations transiently disrupt the daily circadian rhythm of GC and ACTH by an immediate increase of the circulating levels of these stress

Submitted May 13, 2014, Returned for revision June 11, 2014, Accepted June 26, 2014

*Equal contribution first author.
#Equal contribution last author.
Correspondence: Gabriella B. S. Lundkvist, Department of Neuroscience, Karolinska Institutet, Retzius väg 8, 17177 Stockholm, Sweden. Tel: +46 8 524 878 79. E-mail: gabriella.schmitz.lundkvist@ki.se

RIGHTSLINK

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

hormones in order to adapt to the stressful situation. Since peripheral organs express GC receptors (GR) that stimulate the transcription of the canonical clock genes *Period* (*Per*)*1* and *Per2* after binding of GC, acute stress may shift the peripheral clocks so that these can respond quickly and properly to the stressful situation (Balsalobre et al., 2000, So et al., 2009, Yamamoto et al., 2005). In contrast, the SCN belongs to those rare areas that do not express GR; implying that the master clock maintains its intrinsic rhythm properties independent of GC signaling (Balsalobre et al., 2000). Therefore, short-term stress is most likely not capable of perturbing the master clock and, thus, the SCN can reset the peripheral clocks to their original phase after termination of acute stressor exposure (Balsalobre et al., 2000). Although no GR have been shown in the SCN, an indirect GC feedback from the periphery via different GR-containing brain areas such as the PVN, the dorsomedial nucleus of the hypothalamus or the raphe nuclei is likely (Kiessling et al., 2010; Malek et al., 2007). Such indirect feedback may be responsible for mediating effects of chronic or repeated stressor exposure on the SCN. Accordingly, chronic restraint stress in mice as well as chronic unpredictable stress in rats reduces PER2 protein expression in the SCN (Jiang et al., 2011; Kinoshita et al., 2012), thus indicating an impact of chronic stressor exposure on SCN function. Besides GC, pro-inflammatory cytokines and trophic factors such as brain-derived neurotrophic factor (BDNF) likely provide feedback from the periphery to the central circadian pacemaker, since the interferon (IFN)-γ receptor (Cermakian et al., 2013; Lundkvist et al., 1998) and the BDNF receptor TrkB (Liang et al., 1998) were detected in the SCN. Numerous studies have established a link between chronic/repeated stressor exposure and brain BDNF levels (Smith et al., 1995b; Tsankova et al., 2006).

Recently, we have shown that repeated social defeat (SD), a stress paradigm combining a psychosocial and novelty stressor, has differential effects on behavioral, physiological and immunological parameters depending on the time of day of stressor exposure (Bartlang et al., 2012). SD during the early dark phase (SDD) resulted in a blunted activity rhythm, flattened GC rhythm and a lack of social preference towards an unfamiliar male mouse, whereas SD during the early light phase (SDL) did not. Furthermore, SDD resulted in a higher anti-CD3-stimulated *in vitro* IFN-γ secretion from mesenteric lymph node cells, and an aggravated colitis following dextran sulfate sodium treatment compared with SDL mice. These findings suggest that repeated SD exposure has more severe consequences when performed in the early dark/active phase, as compared to the early light/inactive phase. However, it has never been assessed whether SD during early day or early night differentially affects the rhythmicity in the central and peripheral molecular clocks. Thus, in the present study, we elucidated whether repeated SD at day versus night affects the SCN and the adrenal gland differentially. Furthermore, we examined plasma BDNF and BDNF mRNA levels in the SCN following SDL and SDD.

MATERIALS AND METHODS

Animals

Male transgenic knock-in PER2::LUC (Yoo et al., 2004) and C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 21–28 g (experimental intruder mice, age 3–4 months) or male CD1 mice weighing 34–39 g (used as dominant residents during the stress procedure, age 6–7 months, Charles River, Sulzfeld, Germany) were individually housed in standard polycarbonate mouse cages ($17.5 \times 35.5 \times 13$ cm, experimental mice or $41 \times 15 \times 25$ cm, residents) for 1 week before the start of the experiment. Transgenic PER2::LUC mice contain a PER2::LUCIFERASE fusion protein enabling continuous recording of the PER2 protein rhythm by measuring luciferase-derived bioluminescence (Yoo et al., 2004). The PER2::LUC mice were bred from in-house colonies maintained at the Department of Neuroscience, Karolinska Institutet. All mice were kept under standard laboratory conditions ($23 \pm 1\,^{\circ}$C; $55 \pm 5\%$ humidity) and had free access to tap water and standard mouse diet. They were exposed to a 12:12-h light/dark cycle (light phase: 250 ± 50 lux) with lights-on at 06:00. The time of lights-on is defined as Zeitgeber Time (ZT) 0 and lights-off as ZT 12. All experimental protocols were approved by the review board "Norra Djurförsöksetiska Nämnden", Stockholm, and performed according to international guidelines on the ethical use of animals (Portaluppi et al., 2010).

SDL and SDD paradigms

The stress paradigm is visualized in Figure 1 and was performed as described before (Bartlang et al., 2012) by exposing a subordinate male mouse (intruder) to a dominant older male mouse (resident) for 2 h, either at ZT 1–3 (SDL mice) or ZT 13–15 (SDD mice), across 19 days. The details of these procedures are outlined in the Supplemental Materials and Methods.

Experimental procedures

Experiment 1 (Figure 1): In order to assess whether amplitude, phase and period of the PER2::LUC rhythm in the SCN and adrenal gland are dependent on the time of day of stressor exposure, SHC ($n = 6$), SDL ($n = 6$) and SDD ($n = 6$) mice were sacrificed subsequent to the SD paradigm on day 21 and day 22 between ZT 7 and ZT 11. The animals were collected equally across the experimental groups (SHC, SDL, SDD) to avoid masking due to long sampling time span. The tissues were dissected, cultured and placed under photomultiplier tube assemblies. Bioluminescence, corresponding to the amount of PER2 protein, was continuously recorded during 5–10 days.

© Informa Healthcare USA, Inc.

RIGHTSLINK

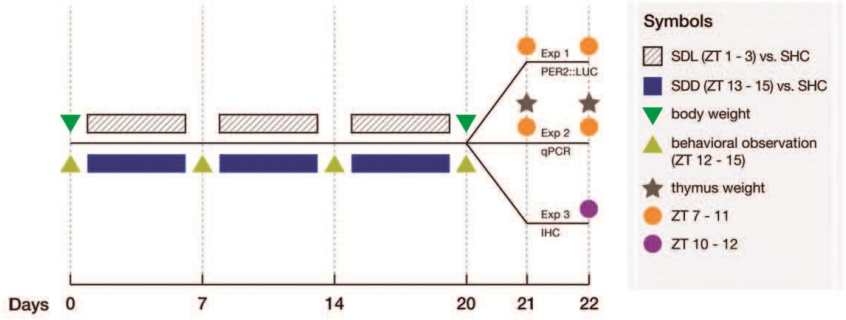


FIGURE 1. Schematic illustration of the stress paradigm. Behavioral observations as well as body and thymus weight assessment were performed in Experiment 2. SHC, single-housed control; SDL, social defeat light; SDD, social defeat dark.

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

Experiment 2 (Figure 1): To assess whether the time of day of stressor exposure has an impact on central and peripheral mRNA clock gene expression, SHC ($n=10$), SDL ($n=9$) and SDD ($n=9$) mice were anesthetized with isofluorane and decapitated on day 21 and 22 between ZT 7 and ZT 11 and trunk blood was collected for quantification of plasma BDNF levels. The animals were collected equally across the experimental groups (SHC, SDL, SDD) to avoid masking due to long sampling time span. The SCN and the right adrenal gland were removed from each mouse (the right adrenal gland is slightly smaller and, thus, more appropriate for organotypic tissue culture (Droste et al., 2003)). RNA was extracted and mRNA levels of *Per2* (SCN, adrenal gland), *Cryptochrome (Cry) 1* (SCN, adrenal gland), *Brain and muscle Arnt-like protein-1 (Bmal 1)* (SCN, adrenal gland) and *Bdnf* (SCN) were quantified by means of qPCR. In addition, the thymus was dissected and weighed. The thymus was chosen for analysis based on previous studies that report a reduced thymus weight following psychosocial stress (Engler & Stefanski, 2003; Reber et al., 2007). The body weight of these mice was assessed before SD started (day 0) and following termination of the SD paradigm (day 20). On days without stressor exposure (days 0, 7, 14 and 20), active home-cage behavior of SHC, SDL, and SDD mice was observed in the dark phase between ZT 12 and ZT 15.

Experiment 3 (Figure 1): To assess whether the time of day of stressor exposure affects PER2-positive neurons in the SCN, SHC ($n=4$), SDL ($n=4$) and SDD ($n=6$) mice were anesthetized with carbon dioxide on day 22 between ZT10 and ZT12 when PER2 is maximally expressed (Field et al., 2000). Subsequently, mice were perfused with paraformaldehyde (PFA) (4% in PBS; Merck KGaA, Darmstadt, Germany). An injection needle connected to a pumping system was inserted into the left ventricle of the heart. The right atrium was cut open before an isotonic saline solution (0.9% NaCl, Braun Melsungen AG, Melsungen, Germany) followed by PFA solution was pumped through the body. The brains were removed and further processed for the quantification of the mPER2 protein expression by immunohistochemistry (IHC).

Organotypic tissue culturing and bioluminescence recording

SHC, SDL and SDD mice were anesthetized with isofluorane followed by decapitation either on day 21 or on day 22 in the afternoon between ZT 7 and ZT 11. The right adrenal gland (the right adrenal gland is slightly smaller and, thus, more appropriate for organotypic tissue culture (Droste et al., 2003)) was collected and the surrounding fat was removed. The bilateral SCN was dissected from 250 µm coronal vibratome-cut slices. The tissues were cultured organotypically on a membrane (Millipore, Billerica, MA) in culture medium (DMEM 2902, Sigma-Aldrich, St. Louis, MO). The procedure of slice preparation and organotypic tissue culturing was previously described (Savelyev et al., 2011; Yamazaki et al., 2000). Bioluminescence corresponding to the amount of PER2 protein was recorded with photon multiplier tube assemblies during 5–10 days. For a detailed description of the procedure, see Supplemental Materials and Methods.

Period

The period of one complete cycle was defined as the time between two consecutive peaks (i.e. the maximum of photon counts within one cycle). Five consecutive peaks were used for analyzing the average period.

Phase

Phase was determined as the time when the second peak in tissue culture occurred.

Amplitude

The amplitude was assessed by subtracting the lowest (trough) photon count value from the highest (peak) photon count value within one cycle. The subtraction was performed from trough-to-peak and from peak-to-trough, thus giving two values (half-cycles) within

RIGHTSLINK

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

one cycle. For amplitude analysis of the SCN and adrenal gland day 1–7 and day 1–5 were used, respectively, due to the different duration in sustained oscillation in these tissues.

Clock gene assessment by qPCR

After dissection tissues were immediately placed in RNAlater RNA Stabilization Reagent (Qiagen, GmbH, Hilden, Germany), incubated over night at 4 °C and stored at −80 °C until assayed using the real-time qPCR technique. RNA was extracted and reversely transcribed following preparation of cDNA (for details, see Supplemental Materials and Methods). For PCR, cDNA templates were added in triplicates to reaction mixtures using Platinum SYBR Green qPCR Supermix UDG (Invitrogen, Life Technologies, Carlsbad, CA). An ABI Prism 7000 real-time thermocycler was used (Applied Biosystems, Palo Alto, CA). Threshold cycle (Ct) values from the exponential phase of the PCR amplification plot for each target transcript (*Per2*, *Cry1*, *Bmal1* and *Bdnf*) were normalized to encoding glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), after confirming that *Gapdh* expression did not show circadian variation. For primer sequences, see Table S2. Abundance was quantified according to the formula $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). Data are presented as relative quantification (RQ).

Analysis of mPER2 immunostaining by immunohistochemistry

After perfusion, brains were removed, fixed, embedded in ovalbumine gelatin and 200 µM coronal sections were prepared (see Supplemental Materials and Methods). The sections were then mounted and scanned into 2.3 µm thick optical cross-sections with a confocal microscope (Zeiss, LSM 510) with a resolution of 1024 × 1024 pixel (12 bit) and analyzed using Image J Version 1.42 (Wayne Rasband, National Institutes of Health, Bethesda, MD). For quantification, the 12 bit confocal pictures were converted into 8 bit (256 pixel) pictures. The left and right SCN were analyzed separately by calculating the sum of the stained area of the SCN for every optical slice for every animal. The resulting values were summed up for both hemispheres, multiplied by the size of one pixel (0.8789 μm^2), averaged over each treatment group, and expressed as mean sum of stained area for the entire SCN [μm^2]. For quantification of the mPER2-positive neurons in the rostral – caudal distribution, the number of scans was summed up for both hemispheres for each animal. The resulting values were averaged over each treatment group, and expressed as mean number of scans for the entire SCN [*n*].

For depicting the mean staining intensity/zone for the entire SCN, the left and right SCN were analyzed separately by calculating the mean staining intensity for each of the three zones (anterior, medial, posterior) of the SCN and subtracting the mean staining intensity of the background (area of 100 pixel × 40 pixel) from these values. Negative values were evaluated as 0 (i.e. no staining). The resulting values were averaged over each hemisphere and each treatment group, respectively, and expressed as mean staining intensity/zone for the entire SCN [pixel].

Quantification of plasma BDNF by ELISA

SHC, SDL and SDD mice were anesthetized with isofluorane followed by decapitation on day 21 and 22 between ZT 7 and ZT 11. Approximately 200 µl trunk blood was collected in heparin-coated Vacutainer tubes (Becton Dickinson and Company, Franklin Lakes, NJ) on ice, centrifuged at 4 °C (5000 rpm, 10 min) and finally stored at −20 °C until assayed using a commercially available ELISA for BDNF (analytical sensitivity <2 pg/ml, Biorbyt Ltd., Cambridge, UK).

Statistics

For statistical comparisons, the software package SPSS (IBM, Armonk, NY, version 19) was used. An one-way Analysis of Variance [ANOVA; factor = treatment (SHC versus SDL versus SDD)] followed by Fisher's least significant difference (LSD) *post-hoc* test was used for analysis of PER2::LUC rhythms (phase, period), clock gene and BDNF mRNA, immunohistochemical PER2 staining, plasma BDNF, delta body weight and thymus weight. The phase of PER2::LUC rhythms in the right adrenal gland was analyzed separately for SHC versus SDL mice by applying Student's *t*-test. ANOVA for repeated measures (rm ANOVA) followed by LSD *post-hoc* test was used for analysis of the amplitude of PER2::LUC rhythms and the active behavior during home-cage observation [PER2::LUC rhythms: factor 1 = day 1–7 (SCN), 1–5 (adrenal), observation: factor 1 = day 0, 7, 14, 20 and factor 2 = treatment (SHC versus SDL versus SDD)]. Data are presented as means + /± SEM. Significance was set at $p \leq 0.05$.

RESULTS

Home-cage activity

Home-cage activity was found to be dependent on both the SD paradigm employed and the day of observation ($F_{6,75} = 18.45$; $p \leq 0.001$). *Post hoc* analysis showed that SDD mice exhibited a reduced activity in the early dark phase (ZT12–15) on stress-free days 7, 14 and 20 compared with SHC ($p \leq 0.001$) and SDL ($p \leq 0.05$) animals (Supplemental Figure 1A). SDL mice showed reduced activity on day 7 in comparison with SHC mice ($p \leq 0.001$). Both SDL and SDD mice showed decreased home-cage activity on days 7, 14 and 20 ($p \leq 0.01$ and $p \leq 0.001$, respectively) when compared with day 0 (basal levels) values.

Body weight and thymus weight

Analysis of delta body weight (d1–d20) showed no statistical differences between SHC, SDL and SDD

© Informa Healthcare USA, Inc.

RIGHTSLINK

mice (Supplemental Figure 1B). The thymus weight on day 21 and day 22 was found to be dependent on the SD paradigm employed ($F_{2,25}=3.52$; $p\leq0.05$). *Post hoc* analysis revealed that SDD, but not SDL, mice had a significantly decreased thymus weight ($p\leq0.05$) compared with SHC mice (Supplemental Figure 1C).

SDD increases the PER2::LUC rhythm amplitude in the SCN

Figure 2(A) illustrates averaged PER2::LUC protein rhythms in the SCN from SHC, SDL and SDD mice. The amplitude of PER2::LUC protein expression in the SCN was found to be dependent on the SD paradigm employed and the time in culture ($F_{12,78}=4.69$; $p\leq0.001$). *Post hoc* analysis showed that SDD mice exhibited higher PER2::LUC amplitude as compared with SHC and SDL mice ($p\leq0.05$) (Figure 2B). All three treatment groups showed a reduction of PER2::LUC amplitude over time ($p\leq0.05$) as previously reported (Yoo et al., 2004). PER2::LUC was maximally expressed between ZT 12 and ZT 13. Neither the phase (Figure 2C, Table S1) nor the period (Figure 2D, Table S1) of PER2::LUC protein expression was affected by either SD paradigm.

SDL induces a phase advance of the PER2::LUC rhythm in the adrenal gland

Averaged traces of PER2::LUC protein expression in the right adrenal gland from SHC, SDL and SDD mice are presented in Figure 3(A). The amplitude of PER2::LUC protein expression was found to be dependent on the time in culture ($F_{4,56}=88.92$; $p\leq0.001$). *Post hoc* analysis revealed that all three treatment groups showed a reduction of PER2::LUC amplitude over time ($p\leq0.05$) (Figure 3B), which is in accordance with a previous study (Yoo et al., 2004). Molecular rhythms in peripheral tissues are often approximately 6 h phase delayed in comparison with the SCN (Yamamoto et al., 2004). In accordance, the adrenal PER2::LUC rhythm was

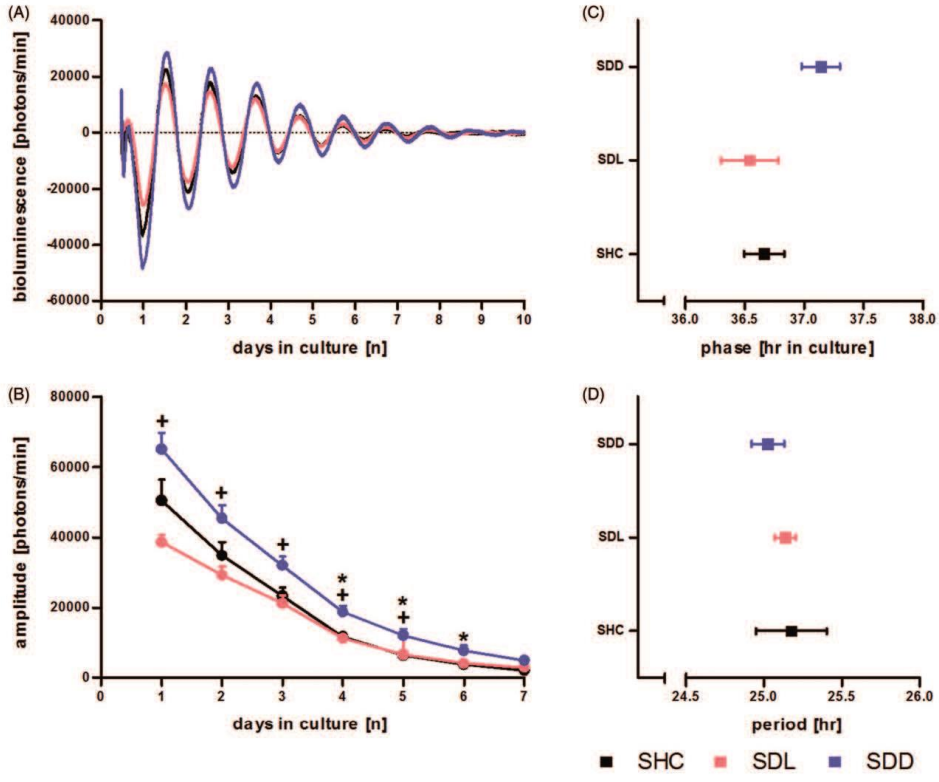


FIGURE 2. Effects of SD on PER2::LUC protein expression in the SCN. (A) Mean detrended traces of bioluminescence recordings of PERIOD2::LUCIFERASE (PER2::LUC) expression in SCN slices from SHC (black lines), SDL (red lines) and SDD (blue lines) mice, respectively. (B) Mean amplitude, (C) mean phase and (D) mean period of the PER2::LUC rhythm in the SCN of SHC ($n=6$; black lines/squares), SDL ($n=5$; red lines/squares) and SDD ($n=5$; blue lines/squares) mice. Data are presented as mean+/± SEM. *$p\leq0.05$ versus respective SHC mice; +$p\leq0.05$ versus respective SDL mice.

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

RIGHTSLINK

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

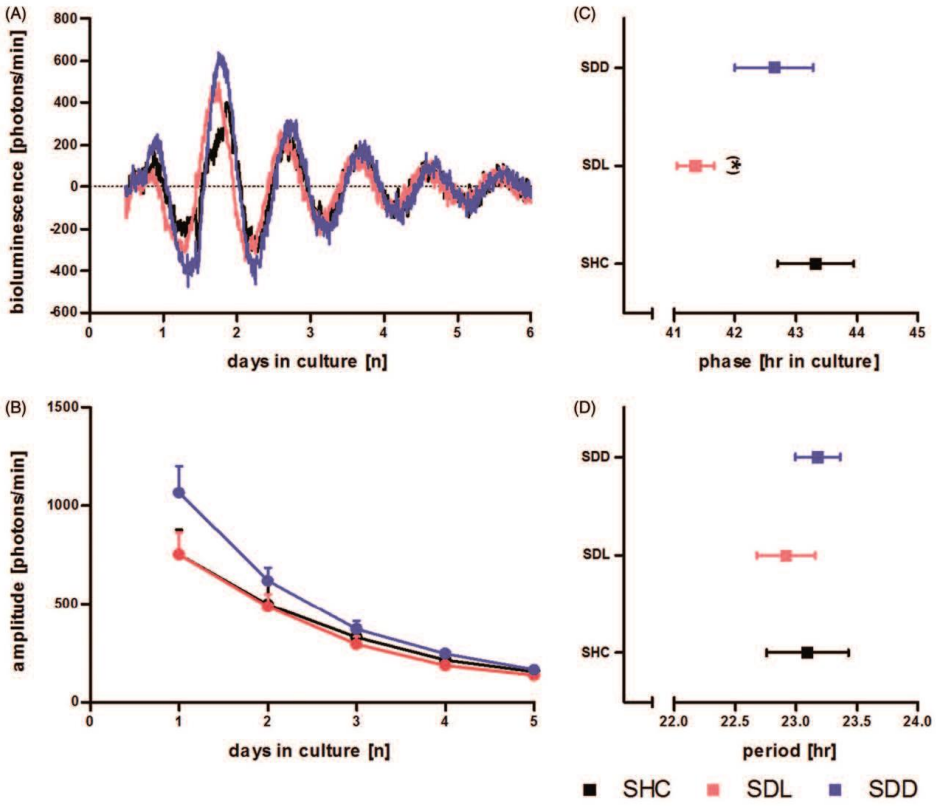


FIGURE 3. Effects of SD on PER2::LUC protein expression in the adrenal gland. (A) Mean detrended traces of bioluminescence recordings of PERIOD2::LUCIFERASE (PER2::LUC) expression in the right adrenal gland from SHC (black lines), SDL (red lines) and SDD (blue lines) mice, respectively. (B) Mean amplitude, (C) mean phase and (D) mean period of the PER2::LUC rhythm in the right adrenal gland of SHC ($n=5-6$; black lines/squares), SDL ($n=6$; red lines/squares) and SDD ($n=6$; blue lines/squares) mice. Data are presented as mean +/± SEM. (*)$p \leq 0.05$ (*t*-test) versus respective SHC mice.

maximally expressed at ZT 19, i.e. 6–7 h after the SCN peak. The phase of PER2::LUC protein expression showed a strong trend towards a significant dependence on the SD paradigm employed ($F_{2,15}=3.33$; $p=0.06$). Separate statistical analysis employing Student's *t*-test showed that SDL mice exhibited a significant phase advance of 2 h of PER2::LUC protein expression ($p \leq 0.01$) in comparison with SHC mice (Figure 3C, Table S1). In contrast, the period of PER2::LUC protein expression was not affected by the SD paradigm employed (Figure 3D, Table S1).

SDD reduces Per2 and Cry1 mRNA levels in the adrenal gland

SCN: *Per2*, *Cry1* and *Bmal1* mRNA levels were statistically not different between the three groups (Figure 4A–C).

Adrenal gland: *Per2* mRNA levels were found to be dependent on the SD paradigm employed ($F_{2,21}=3.67$; $p \leq 0.05$). *Post hoc* analysis confirmed that SDD mice showed lower *Per2* mRNA levels ($p \leq 0.05$) compared with SHC and SDL mice (Figure 4D). *Cry1* mRNA levels were dependent on the SD paradigm employed ($F_{2,19}=3.41$; $p \leq 0.05$). *Post hoc* analysis showed that *Cry1* mRNA levels were lower following SDD ($p \leq 0.05$) compared with SHC mice (Figure 4E). *Bmal1* mRNA levels were statistically not different between the three groups (Figure 4F).

SDD results in altered immunostaining of PER2 positive neurons in the SCN

The sum of stained area of the entire SCN was dependent on the SD paradigm employed ($F_{2,9}=4.89$; $p \leq 0.05$). *Post hoc* analysis revealed that SDD mice showed a reduced PER2-positive stained area in the entire SCN compared with SDL mice ($p \leq 0.05$) (Figure 5A). The mean number of scans in rostro-caudal direction of the entire SCN was dependent on the SD

© Informa Healthcare USA, Inc.

RIGHTSLINK

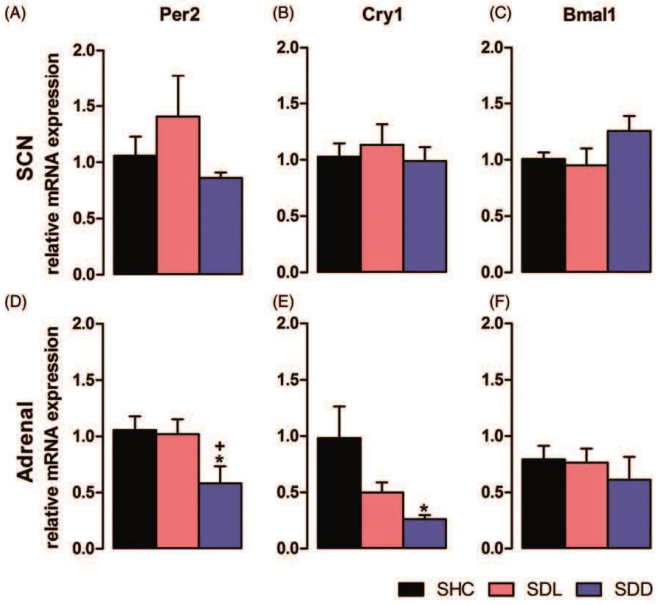


FIGURE 4. Effects of SD on relative expression of clock gene mRNA. Relative levels of clock gene transcripts *murine Period (mPer)2, murine Cryptochrome (mCry) 1* and *murine Brain and muscle Arnt-like protein-1 (mBmal1)* in the SCN (A–C) from SHC (black bars), SDL (red bars) and SDD (blue bars) mice ($n=5$/group) and in the right adrenal gland (D–F) from SHC ($n=9-10$; black bars), SDL ($n=6-7$; red bars) and SDD ($n=7$; blue bars) mice. Data are presented as mean + SEM. $^*p\leq0.05$ versus respective SHC mice; $+p\leq0.05$ versus respective SDL mice.

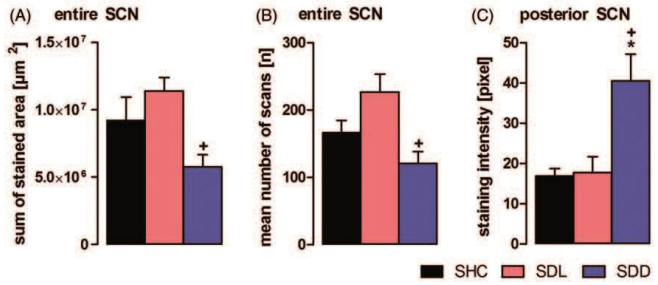


FIGURE 5. Effects of SD on PER2 positive neurons in the SCN. (A) Sum of stained area [μm^2] of the entire SCN of SHC ($n=4$; black bars), SDL ($n=4$; red bars) and SDD ($n=4$; blue bars) mice. (B) Mean number of scans [n] of the entire SCN of SHC ($n=4$; black bars), SDL ($n=4$; red bars) and SDD ($n=4$; blue bars) mice. (C) Mean staining intensity [pixel] in the posterior SCN of SHC ($n=3$; black bars), SDL ($n=3$; red bars) and SDD ($n=6$; blue bars) mice. Data are presented as mean + SEM. $^*p\leq0.05$ versus respective SHC mice; $+p\leq0.05$ versus respective SDL mice.

paradigm employed ($F_{2,9}=4.70$; $p\leq0.05$). *Post hoc* analysis showed that following SDD the number of scans was decreased compared with SDL mice ($p\leq0.05$) (Figure 5B). The staining intensity of the posterior SCN was found to be dependent on the SD paradigm employed ($F_{2,9}=5.15$; $p\leq0.05$). *Post hoc* analysis revealed that SDD mice showed an increased staining intensity in the posterior SCN compared with SHC and SDL mice ($p\leq0.05$) (Figure 5C).

Plasma BDNF and BDNF mRNA in the SCN are increased in SDL mice

Plasma BDNF was found to be dependent on the SD paradigm employed ($F_{2,22}=9.58$; $p\leq0.001$). *Post hoc* analysis showed that SDL mice displayed significantly increased BDNF levels in comparison to SHC ($p\leq0.01$) and SDD ($p\leq0.001$) mice (Figure 6A). BDNF mRNA in the SCN was also dependent on the SD paradigm employed ($F_{2,11}=4.47$; $p\leq0.05$). *Post hoc* analysis revealed that BDNF mRNA in the SCN was significantly increased in SDL compared with SHC and SDD mice ($p\leq0.05$) (Figure 6B).

DISCUSSION

In the present study, we demonstrate that the effects of chronic/intermittent psychosocial stress on the molecular circadian clocks strongly depend on the time of day of stressor exposure. In more detail, while repeated SD during the dark phase (SDD mice) affected the central circadian pacemaker, the same stressor applied during the light phase (SDL mice) had an impact on the peripheral adrenal clock and BDNF levels without altering the SCN. Thus, our findings provide the first

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

RIGHTSLINK

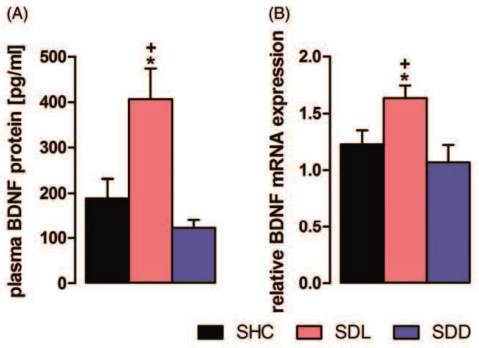


FIGURE 6. Effects of SD on BDNF levels. (A) Plasma BDNF of SHC ($n=10$; black bars), SDL ($n=7$; red bars) and SDD ($n=8$; blue bars) mice. (B) BDNF mRNA in the SCN of SHC ($n=5$; black bars), SDL ($n=4$; red bars) and SDD ($n=5$; blue bars) mice. Data are presented as mean + SEM. $^*p \leq 0.05$ versus respective SHC mice; $+p \leq 0.05$ versus respective SDL mice.

evidence that repeated stress applied at different times of the light–dark cycle differentially affects the central and peripheral circadian clocks.

Validation of the stress model in PER2::LUC mice

Home-cage activity in the dark phase on days 7, 14 and 20 was reduced in the SDD group compared to SHC and SDL mice, which is in line with our own previous study (Bartlang et al., 2012). In contrast to the previous study in which SDL mice showed no behavioral alterations at any days observed, SDL mice in the current study showed reduced dark phase activity on day 7. Also, SHC mice showed a tendency towards increased activity on day 7. However, since the decreased home-cage activity in the SDL group was restored to control levels on days 14 and 20, and, given that no differences in body weight gain were found between the SDL and SHC groups, our results are overall in accordance with our previous study showing that repeated SDD (night stress) has more profound effects than repeated SDL (day stress). In addition, we observed a reduced weight of thymus from SDD, but not SDL, mice that is a reliable indicator of chronic stress (Nyuyki et al., 2012). Taken together, we consider the SD paradigm valid also in the PER2::LUC mice.

SDD, but not SDL, affects PER2 in the SCN

Repeated SDD resulted in significantly increased amplitude of the PER2::LUC rhythm in the SCN compared with SHC and SDL mice. An increased amplitude of circadian rhythmicity in diverse tissues such as the SCN can be induced by restricted feeding (Nelson & Halberg, 1986). Furthermore, drugs used for the treatment of bipolar disorder, such as valproic acid and lithium, have been shown to increase the amplitude of the PER2::LUC rhythm (Johansson et al., 2011; Li et al., 2012). Increasing the amplitude is considered to be beneficial as it may help to restore attenuated circadian rhythmicity and make the clock more robust, thus driving and synchronizing downstream clock functions with higher efficiency (Gudmundsson et al., 1999; Nelson & Halberg, 1986). However, increased circadian amplitudes may also be associated with negative consequences. It has been suggested that higher amplitudes in blood pressure rhythmicity enhances the risk for stroke and nephropathy (Otsuka et al., 1997) as well as the frequency and duration of obstructive apneas at night time (Stephenson, 2004). Thus, in view of our previous study showing negative alterations in behavioral and immunological parameters following repeated SDD, an increased PER2::LUC rhythm amplitude in the SCN of SDD mice could represent a direct result of stress-induced alterations in the brain. Another possibility is that the increased PER2::LUC amplitude compensates for attenuated peripheral output rhythms as previously reported in SDD mice (Bartlang et al., 2012), thus providing increased driving and synchronizing force to peripheral clock tissues to counteract these impaired output disturbances. We discuss this possibility further in the context of GC signaling below.

In accordance with the higher PER2::LUC rhythm amplitude, we also observed an increase in PER2 immunostaining in the posterior SCN from C57BL/6 SDD mice. Since no differences in staining intensity in the anterior and medial SCN (data not shown) could be observed, obviously no relocation of PER2 protein takes place. Interestingly, the sum of PER2 staining within the SCN and the number of PER2 positive scans in the rostro-caudal direction were decreased following repeated SDD. The observed increase in PER2 staining in the posterior SCN might counteract the reduced elongation of PER2 protein in the rostro-caudal direction, thus producing a stronger PER2::LUC signal with an increased amplitude. No significant stress effects on PER2 staining were observed in the SCN following SDL, further supporting the more severe effect of stressor exposure at early night compared to early day. We were surprised that no changes in overall mRNA levels of *Per2* could be detected in the SCN. However, since IHC demonstrated a local increase of PER2 protein expression in the posterior SCN, and not a general PER2 protein increase in the entire nucleus, changes in locally produced mRNA may be masked in whole SCN mRNA extracts. As PER2::LUC recording is an extremely sensitive recording assay counting single photons, the increased PER2 protein staining in the posterior SCN was most likely not masked in the PER2::LUC recordings.

We have previously shown that SDD leads to increased GC levels at ZT 5, thus resulting in a flattened or phase shifted diurnal variation, as well as dampened active behavior at the beginning of the dark phase (Bartlang et al., 2012); suggesting disturbances in circadian output. Although the SCN itself lacks GR

© Informa Healthcare USA, Inc.

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

RIGHTSLINK

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

(Balsalobre et al., 2000), indirect GC feedback from the periphery via different GR-containing brain areas has been proposed (Kiessling et al., 2010; Malek et al., 2007). This indirect feedback may also be transmitted to the SCN via serotonergic projections from the raphe nucleus via circadian transcription of tryptophan hydroxylase-2, an enzyme associated with serotonin synthesis that is dependent on GC (Malek et al., 2007). Such feedback may be responsible for conveying the effects of repeated/chronic stressor exposure on the SCN. Indeed, a different stress paradigm, chronic unpredictable stress, which represents a reliable model to induce depression-related behavior, resulted in reduced PER2 protein expression in the rat SCN (Jiang et al., 2011). In contrast, we found that SDD increased the amplitude of PER2::LUC rhythms and PER2 protein, although a reduction in social preference, seen in SDD mice, in other studies is interpreted as depressive-like behavior (Krishnan et al., 2007; Lagace et al., 2010). Differences between the two stress paradigms may explain these contrasting results. In our SD paradigm the stressor is repetitive but not continuous, since the mice were exposed to the stressor only at a certain time point each day. Therefore, we speculate that the stressor could hypothetically act as a "Zeitgeber" and the increased daytime GC levels following repeated SDD could indirectly feed back to the SCN via signals from GR-containing brain areas and affect its intrinsic rhythm. This timely regulated GC signal could function as a synchronizer and indirectly increase the coherence between individual SCN cells. This increased coherence, in turn, may compensate for the impaired behavioral and endocrine rhythms seen in SDD mice by enhancing the oscillatory drive in the master pacemaker.

SDD reduces clock gene mRNA in the adrenal gland

Amongst peripheral clocks, the adrenal gland has an unique function since the local oscillator in the adrenal cortex is implicated in synchronization processes of other subordinated oscillators, such as the liver and kidney (Balsalobre et al., 2000). The rhythmic expression of several clock genes, such as *Per2*, in the adrenal gland is tightly linked to steroidogenesis, and the clock-controlled protein expression of StAR represents one of the underlying mechanisms for rhythmic GC secretion (Oster et al., 2006; Son et al., 2008). GCs, in turn, stimulate the expression of several clock genes, including *Per2*, via the glucocorticoid-responsive element (GRE), in liver, heart and kidney (So et al., 2009; Yamamoto et al., 2005). Within this context, we were surprised to find a decreased *Per2* and *Cry1* mRNA expression in SDD mice. GR in the adrenal gland have been demonstrated (Ceccatelli et al., 1989; Loose et al., 1980); however, since the adrenal gland is the GC-producing organ, it is likely that it responds differently to GC than other peripheral tissues, which may explain the observed decrease in clock gene expression. In support, it has been proposed that the GR in the adrenal gland has a lower affinity to GC compared to the GR in other peripheral organs such as the kidney (Loose et al., 1980). Patterns of clock gene expression are tissue-specific (Ko & Takahashi, 2006), but within the adrenal gland the expression levels and phasing of the cycling pattern of clock genes differ between the adrenal medulla and the cortex (Dickmeis, 2009). While all clock genes, except for *Clock* and *Cry2*, exhibit a robust circadian rhythm in expression in the adrenal cortex (Oster et al., 2006), the medulla merely expresses *Per1*, *Per3*, *Cry2* and *Bmal1* rhythmically (Bittman et al., 2003; Torres-Farfan et al., 2006). Likewise, light-induced increases in Per1 and Per2 mRNA were found in all cortical layers of the adrenal gland but not within the adrenal medulla (Ishida et al., 2005). Thus, the GC producing outer layers of the cortex are probably the main sites of the intrinsic adrenal pacemaker, and the PER2::LUC rhythms that we record most likely originate from the adrenal cortex. The observed decrease in *Per2* mRNA may therefore reflect decreased, non-rhythmic *Per2* expression in the medulla, suggesting that the intrinsic adrenal pacemaker, located in the cortex, was not affected by SDD.

In contrast to clock genes in the adrenal gland, clock gene mRNA in the SCN was not affected by repeated SDD or SDL, which is in accordance with Takahashi et al., who reported that chronic mild stress in mice affects the circadian expression of molecular clock genes such as *Bmal1* and *Per1* in the liver but not in the SCN (Takahashi et al., 2013). However, although the recorded PER2::LUC rhythms did not indicate a phase shift in protein expression, we cannot exclude a phase shift of clock gene mRNA rhythms in SD mice, as we only measured mRNA at one time point. Further studies that include more time points and the assessment of protein levels will be essential to investigate this possibility.

SDL phase advances the adrenal gland

Repeated SDL resulted in a significant phase advance of approximately 2 h of the PER2::LUC rhythm in the right adrenal gland in comparison with SHC mice, thus indicating an altered entrainment of the adrenal clock. This finding is consistent with a recent study showing a significant phase advance of the adrenal PER2 rhythm while basal plasma GC was unchanged following 2 weeks of chronic subordination stress in mice (Razzoli et al., 2014). We previously reported that repeated SDL results in a prolonged increase in adrenal gland weight, an attenuated adrenal responsiveness to ACTH *in vitro*, and a lack of the diurnal variation in plasma ACTH after stressor termination. Despite the lacking ACTH rhythm, SDL mice maintained an adequate day/night rhythm in plasma GC (Bartlang et al., 2012). Since SDL mice show no signs of depressive-like behavior, the phase advance in the adrenal gland function might therefore represent beneficial adaptations to the predictable stressor (Bartlang et al., 2012), helping those animals to maintain a normal GC rhythm despite a disturbed ACTH rhythm.

RIGHTSLINK

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

SDL increases BDNF in the SCN and plasma

We found an increase of BDNF mRNA in the SCN of repeated SDL mice. Contrasting effects of stressor exposure on BDNF expression levels have been reported depending on the brain area investigated. While chronic immobilization stress in rats reduced BDNF protein levels in the hippocampus, an increase could be observed in the amygdala (Lakshminarasimhan & Chattarji, 2012). In addition to BDNF mRNA in the SCN, plasma levels of BDNF (measured at ZT 7–11) were elevated in SDL, but not SDD, mice. Importantly, it has been shown in several species that plasma BDNF concentrations reflect levels in the brain (Klein et al., 2011). BDNF can cross the blood–brain barrier, thus being transported from the brain to the blood and vice versa (Forsgren et al., 2011; Rasmussen et al., 2009). Circulating BDNF is known to derive from both central and peripheral sources, whereas the brain produces almost 75% (Rasmussen et al., 2009). Endocrine organs such as the pituitary gland (Smith et al., 1995a) may provide an additional source for circulating BDNF. Furthermore, BDNF protein expression has been detected in the human and rat adrenal cortex (Schober et al., 1999; Szekeres et al., 2010). Our findings suggest that BDNF may contribute to the diurnal differences in sensitivity to SD, and could protect against the negative consequences of repeated stressor exposure. In rats and mice depressive-like symptoms, induced by social defeat stress, can be prevented by activation of the BDNF receptor TrkB in the hippocampus (Duclot & Kabbaj, 2013), and, activation of TrkB by the TrkB agonist 7,8-dihydroxyflavone blocks the recall of fear in female mice after extinction training (Baker-Andresen et al., 2013). Moreover, antidepressant-like properties of centrally administered BDNF have been reported (Siuciak et al., 1997), and chronic antidepressant treatment increases BDNF expression in the hippocampus (Nibuya et al., 1995; Russo-Neustadt et al., 1999).

In the SCN, BDNF plays a crucial role in photic entrainment and is required for glutamatergic signals to reset the SCN clock (Kim et al., 2006; Michel et al., 2006), and TrkB-deficient mice exhibit decreased light-induced phase shifts (Allen & Earnest, 2005). BDNF further regulates astroglial plasticity in the SCN in a circadian fashion, which plays a crucial role in synchronization processes of circadian rhythms (Girardet et al., 2013). In a recent study, we found that BDNF via its receptor TrkB, protects mice against the negative consequences of an acute acoustic stressor, acoustic noise trauma, and that mice were significantly more susceptible to noise stress in the night as compared to day (Meltser et al., 2014). Thus, the increased BDNF mRNA levels in the SCN and increased BDNF in plasma may represent neural adaptations that prevent stress-induced alterations in the SCN and, in turn, facilitate changes in adrenal ACTH sensitivity via a phase advanced PER2::LUC rhythm.

© Informa Healthcare USA, Inc.

General conclusion

In conclusion, our data show that repeated psychosocial stressor exposure has different effects on the molecular rhythmicity when applied at day versus night, and is in accordance with our previous study showing that repeated SDD (night stress) has more profound consequences on behavioral and immunological parameters as compared with repeated SDL (day stress). SDD alters the molecular rhythm of the master SCN pacemaker, whereas SDL phase advances the adrenal clock and increases levels of BDNF. The increased BDNF levels following repeated SDL may protect against the negative consequences of the repeated stress. Although our findings contribute to the understanding of the impact of stress at different times during the day, our study is primarily descriptive and further investigation is needed to determine the mechanisms underlying these circadian differences in stress responses.

ACKNOWLEDGMENTS

The authors are grateful to Dr T. Yoshii and Dr C. Wülbeck for excellent technical help and to Dr D. Slattery for his helpful comments regarding the manuscript.

DECLARATION OF INTEREST

All authors declare that there are no conflicts of interest.

This study was funded by Söderström-Königska Foundation, NIH (IR21DC013172), Swedish Society of Medicine and Deutsche Forschungsgemeinschaft FO-207/13-1.

REFERENCES

Allen GC, Earnest DJ. (2005). Overlap in the distribution of TrkB immunoreactivity and retinohypothalamic tract innervation of the rat suprachiasmatic nucleus. Neurosci Lett. 376: 200–4.

Baker-Andresen D, Flavell CR, Li X, Bredy TW. (2013). Activation of BDNF signaling prevents the return of fear in female mice. Learn Mem. 20:237–40.

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

Barclay JL, Tsang AH, Oster H. (2012). Interaction of central and peripheral clocks in physiological regulation. Prog Brain Res. 199:163–81.

Bartlang MS, Neumann ID, Slattery DA, 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.

Bittman EL, Doherty L, Huang L, Paroskie A. (2003). Period gene expression in mouse endocrine tissues. Am J Physiol Reg Integ Compar Physiol. 285:R561–9.

Carnes M, Kalin NH, Lent SJ, et al. (1988). Pulsatile ACTH secretion: Variation with time of day and relationship to cortisol. Peptides. 9:325–31.

Carnes M, Lent S, Feyzi J, Hazel D. (1989). Plasma adrenocorticotropic hormone in the rat demonstrates three different rhythms within 24 h. Neuroendocrinology. 50:17–25.

RIGHTSLINK

Ceccatelli S, Dagerlind A, Schalling M, et al. (1989). The glucocorticoid receptor in the adrenal gland is localized in the cytoplasm of adrenaline cells. Acta Physiol Scand. 137:559–60.
Cermakian N, Lange T, Golombek D, et al. (2013). Crosstalk between the circadian clock circuitry and the immune system. Chronobiol Int. 30:870–88.
Dickmeis T. (2009). Glucocorticoids and the circadian clock. J Endocrinol. 200:3–22.
Droste SK, Gesing A, Ulbricht S, et al. (2003). Effects of long-term voluntary exercise on the mouse hypothalamic-pituitary-adrenocortical axis. Endocrinology. 144:3012–23.
Duclot F, Kabbaj M. (2013). Individual differences in novelty seeking predict subsequent vulnerability to social defeat through a differential epigenetic regulation of brain-derived neurotrophic factor expression. J Neurosci. 33:11048–60.
Engler H, Stefanski V. (2003). Social stress and T cell maturation in male rats: Transient and persistent alterations in thymic function. Psychoneuroendocrinology. 28:951–69.
Field MD, Maywood ES, O'Brien JA, et al. (2000). Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanisms. Neuron. 25:437–47.
Forsgren S, Grimsholm O, Dalen T, Rantapaa-Dahlqvist S. (2011). Measurements in the blood of BDNF for RA patients and in response to anti-TNF treatment help us to clarify the magnitude of centrally related pain and to explain the relief of this pain upon treatment. Int J Inflam. 2011:650685.
Girardet C, Lebrun B, Cabirol-Pol MJ, et al. (2013). Brain-derived neurotrophic factor/TrkB signaling regulates daily astroglial plasticity in the suprachiasmatic nucleus: Electron-microscopic evidence in mouse. Glia. 61:1172–7.
Gudmundsson A, Goodman B, Lent S, et al. (1999). Effects of estrogen replacement therapy on the circadian rhythms of serum cortisol and body temperature in postmenopausal women. Exp Gerontol. 34:809–18.
Ishida A, Mutoh T, Ueyama T, et al. (2005). Light activates the adrenal gland: Timing of gene expression and glucocorticoid release. Cell Metab. 2:297–307.
Jiang WG, Li SX, Zhou SJ, et al. (2011). Chronic unpredictable stress induces a reversible change of PER2 rhythm in the suprachiasmatic nucleus. Brain Res. 1399:25–32.
Johansson AS, Brask J, Owe-Larsson B, et al. (2011). Valproic acid phase shifts the rhythmic expression of Period2::Luciferase. J Biol Rhythms. 26:541–51.
Kalsbeek A, van der Spek R, Lei J, et al. (2012). Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. Mol Cell Endocrinol. 349:20–9.
Kiessling S, Eichele G, Oster H. (2010). Adrenal glucocorticoids have a key role in circadian resynchronization in a mouse model of jet lag. J Clin Invest. 120:2600–9.
Kim YI, Choi HJ, Colwell CS. (2006). Brain-derived neurotrophic factor regulation of N-methyl-D-aspartate receptor-mediated synaptic currents in suprachiasmatic nucleus neurons. J Neurosci Res. 84:1512–20.
Kinoshita C, Miyazaki K, Ishida N. (2012). Chronic stress affects PERIOD2 expression through glycogen synthase kinase-3beta phosphorylation in the central clock. Neuroreport. 23:98–102.
Klein AB, Williamson R, Santini MA, et al. (2011). Blood BDNF concentrations reflect brain-tissue BDNF levels across species. Int J Neuropsychopharmacol. 14:347–53.
Ko CH, Takahashi JS. (2006). Molecular components of the mammalian circadian clock. Hum Mol Genet. 15:R271–7.
Krishnan V, Han MH, Graham DL, et al. (2007). Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell. 131:391–404.
Lagace DC, Donovan MH, DeCarolis NA, et al. (2010). Adult hippocampal neurogenesis is functionally important for stress-induced social avoidance. Proc Natl Acad Sci USA. 107:4436–41.
Lakshminarasimhan H, Chattarji S. (2012). Stress leads to contrasting effects on the levels of brain derived neurotrophic factor in the hippocampus and amygdala. PLoS One. 7:e30481.
Li J, Lu WQ, Beesley S, et al. (2012). Lithium impacts on the amplitude and period of the molecular circadian clockwork. PLoS One. 7:e33292.
Liang FQ, Sohrabji F, Miranda R, et al. (1998). Expression of brain-derived neurotrophic factor and its cognate receptor, TrkB, in the rat suprachiasmatic nucleus. Exp Neurol. 151:184–93.
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.
Loose DS, Do YS, Chen TL, Feldman D. (1980). Demonstration of glucocorticoid receptors in the adrenal cortex: Evidence for a direct dexamethasone suppressive effect on the rat adrenal gland. Endocrinology. 107:137–46.
Lundkvist GB, Robertson B, Mhlanga JD, et al. (1998). Expression of an oscillating interferon-gamma receptor in the suprachiasmatic nuclei. Neuroreport. 9:1059–63.
Malek ZS, Sage D, Pevet P, Raison S. (2007). Daily rhythm of tryptophan hydroxylase-2 messenger ribonucleic acid within raphe neurons is induced by corticoid daily surge and modulated by enhanced locomotor activity. Endocrinology. 148:5165–72.
Meltser I, Cederroth CR, Basinou V, et al. (2014). TrkB-mediated protection against circadian sensitivity to noise trauma in the murine cochlea. Curr Biol. 24:658–63.
Michel S, Clark JP, Ding JM, Colwell CS. (2006). Brain-derived neurotrophic factor and neurotrophin receptors modulate glutamate-induced phase shifts of the suprachiasmatic nucleus. Eur J Neurosci. 24:1109–16.
Moore RY, Eichler VB. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res. 42:201–6.
Nelson W, Halberg F. (1986). Meal-timing, circadian rhythms and life span of mice. J Nutr. 116:2244–53.
Nibuya M, Morinobu S, Duman RS. (1995). Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. J Neurosci. 15:7539–47.
Nyuyki KD, Beiderbeck DI, Lukas M, et al. (2012). Chronic subordinate colony housing (CSC) as a model of chronic psychosocial stress in male rats. PLoS One. 7:e52371.
Oster H, Damerow S, Kiessling S, et al. (2006). The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. Cell Metab. 4:163–73.
Otsuka K, Cornelissen G, Halberg F, Oehlerts G. (1997). Excessive circadian amplitude of blood pressure increases risk of ischaemic stroke and nephropathy. J Med Eng Technol. 21:23–30.
Portaluppi F, Smolensky MH, Touitou Y. (2010). Ethics and methods for biological rhythm research on animals and human beings. Chronobiol Int. 27:1911–29.
Rasmussen P, Brassard P, Adser H, et al. (2009). Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. Exp Physiol. 94:1062–9.
Razzoli M, Karsten C, Yoder JM, et al. (2014). Chronic subordination stress phase advances adrenal and anterior pituitary clock gene rhythms. Am J Physiol Reg Integr Comp Physiol. 307:R198–205.
Reber SO, Birkeneder L, Veenema AH, et al. (2007). Adrenal insufficiency and colonic inflammation after a novel chronic psycho-social stress paradigm in mice: Implications and mechanisms. Endocrinology. 148:670–82.
Russo-Neustadt A, Beard RC, Cotman CW. (1999). Exercise, antidepressant medications, and enhanced brain derived neurotrophic factor expression. Neuropsychopharmacology. 21:679–82.
Savelyev SA, Larsson KC, Johansson AS, Lundkvist GB. (2011). Slice preparation, organotypic tissue culturing and luciferase

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

RIGHTSLINK

recording of clock gene activity in the suprachiasmatic nucleus. J Vis Exp. (48):2439.
Schober A, Wolf N, Kahane N, et al. (1999). Expression of neurotrophin receptors trkB and trkC and their ligands in rat adrenal gland and the intermediolateral column of the spinal cord. Cell Tissue Res. 296:271–9.
Siuciak JA, Lewis DR, Wiegand SJ, Lindsay RM. (1997). Antidepressant-like effect of brain-derived neurotrophic factor (BDNF). Pharmacol Biochem Behav. 56:131–7.
Smith MA, Makino S, Kim SY, Kvetnansky R. (1995a). Stress increases brain-derived neurotropic factor messenger ribonucleic acid in the hypothalamus and pituitary. Endocrinology. 136:3743–50.
Smith MA, Makino S, Kvetnansky R, Post RM. (1995b). Effects of stress on neurotrophic factor expression in the rat brain. Ann N Y Acad Sci. 771:234–9.
So AY, Bernal TU, Pillsbury ML, et al. (2009). Glucocorticoid regulation of the circadian clock modulates glucose homeostasis. Proc Natl Acad Sci USA. 106:17582–7.
Son GH, Chung S, Choe HK, et al. (2008). Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. Proc Natl Acad Sci USA. 105:20970–5.
Stephenson R. (2004). A theoretical study of the effect of circadian rhythms on sleep-induced periodic breathing and apnoea. Respir Physiol Neurobiol. 139:303–19.
Szekeres M, Nadasy GL, Turu G, et al. (2010). Angiotensin II-induced expression of brain-derived neurotrophic factor in human and rat adrenocortical cells. Endocrinology. 151: 1695–703.
Takahashi K, Yamada T, Tsukita S, et al. (2013). Chronic mild stress alters circadian expressions of molecular clock genes in the liver. Am J Physiol Endocrinol Metab. 304:E301–9.
Torres-Farfan C, Rocco V, Monso C, et al. (2006). Maternal melatonin effects on clock gene expression in a nonhuman primate fetus. Endocrinology. 147:4618–26.
Tsankova NM, Berton O, Renthal W, et al. (2006). Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat Neurosci. 9:519–25.
Yamamoto T, Nakahata Y, Soma H, et al. (2004). Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. BMC Mol Biol. 5:18.
Yamamoto T, Nakahata Y, Tanaka M, 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, et al. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. Science. 288: 682–5.
Yang S, Liu A, Weidenhammer A, et al. (2009). The role of mPer2 clock gene in glucocorticoid and feeding rhythms. Endocrinology. 150:2153–60.
Yoo SH, Yamazaki S, Lowrey PL, et al. (2004). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci USA. 101:5339–46.

Supplementary material available online

Supplemental Materials and Methods, Tables S1, S2, Figure 1(A–C).

Chronobiol Int Downloaded from informahealthcare.com by Universitaetsbibliothek Regensburg on 10/11/14
For personal use only.

© Informa Healthcare USA, Inc.

RIGHTSLINK

FULL TEXT PAPER 3 (MANUSCRIPT, SUBMITTED)

Chronobiology International

Preview

From: yvantouitou@upmc.fr

To: Manuela.Bartlang@klinik.uni-regensburg.de

CC:

Subject: Chronobiology International - Account Created in Manuscript Central

Body: 14-Oct-2014

Dear Miss Bartlang,

A manuscript titled Repeated psychosocial stress at night, but not day, delays the entrained activity rhythm of male mice (LCBI-2014-0243) has been submitted by Miss Manuela Bartlang to Chronobiology International.

Manuscripts I Have Co-Authored

Manuscript ID	Manuscript Title	Date Created	Date Submitted	Status
LCBI-2014-0243	Repeated psychosocial stress at night, but not day, delays the entrained activity rhythm of male mice [View Submission]	14-Oct-2014	14-Oct-2014	ADM: Portaluppi, Francesco • Under Review

Repeated psychosocial stress at night, but not day, delays the entrained activity rhythm of male mice

Manuela S Bartlang[1*], Henrik Oster[2], Charlotte Helfrich-Förster[1]

[1]University of Würzburg, Biocenter, Theodor-Boveri-Institute, Neurobiology and Genetics, 97074 Würzburg, Germany

[2] University of Lübeck, Chronophysiology Group, Medical Department 1, 23562 Lübeck, Germany

*present address: University of Regensburg, Faculty for Medicine, Franz-Josef-Strauß-Allee 11, 93053 Regensburg

Correspondence to: Charlotte Helfrich-Förster, Biocenter, Theodor-Boveri-Institute, Neurobiology and Genetics, Am Hubland, 97074 Würzburg, Germany. Tel: +49 931 31-88823. E-mail: charlotte.foerster@biozentrum.uni-wuerzburg.de

Running head: Stress and the circadian clock

Abstract

We have recently shown that molecular rhythms in the murine suprachiasmatic nucleus (SCN) are affected by repeated social defeat (SD) during the dark/active phase (social defeat dark = SDD), while repeated SD during the light/inactive phase (social defeat light = SDL) had no influence on the PERIOD2::LUCIFERASE rhythm in the SCN. Given that *in vivo* biotelemetry is the gold standard for measuring stress-responsive changes in behavioral and physiological responses, we applied this method for assessing the effects of the same stress paradigm on two output rhythms of the circadian clock, i.e. activity and core body temperature, in wildtype (WT) and clock-deficient *Period (Per)1/2* double-mutant mice during and following repeated SDL and SDD. In general, stress had more pronounced effects on activity compared to body temperature rhythms. Throughout the SD procedure, activity and body temperature were markedly increased during the 2 hr of stressor exposure at *Zeitgeber* time (ZT)1 – ZT3 (SDL mice) and ZT13 – ZT15 (SDD mice), which was compensated by decreased activity during the remaining dark phase (SDL and SDD mice) and light phase (SDL mice) in WT and *Per1/2* mutant mice. Considerable differences in the activity between SDL and SDD mice were seen in the entrained post-stress period. While the normal activity peak at ZT13 was absent in the majority of SDD mice in the entrained post-stress condition, some SDD mice exhibited a delayed activity peak compared to single-housed control (SHC) and SDL mice. This effect was absent in *Per1/2* mutant SDD mice, implementing that the delay in SDD WT mice results from an aberrant circadian clock. Taken together, the present findings demonstrate that SDD, but not SDL, has long lasting consequences for the functional output of the biological clock, that appear to depend on the circadian genes *Per1* and *Per2*.

Key words:

Activity rhythms, temperature rhythms, social defeat, *Per1/2* mutants

Introduction

Due to the earth's rotation around its own axis, virtually all inhabitants are exposed to periodic environmental changes. In order to provide temporal homeostasis with the environment, organisms evolved an internal time-keeping system, i.e. the circadian clock. In mammals, the clock system is composed of a central pacemaker residing in the suprachiasmatic nucleus (SCN) of the hypothalamus and multiple subsidiary clocks in peripheral tissues. Both the SCN and the peripheral organs possess an endogenous clock machinery, thus enabling the formation of self-sustained circadian rhythms in the absence of external cues. However, in order to stay tuned to geophysical time, the circadian system requires daily synchronization of the endogenous oscillation to the solar day. This is accomplished using environmental timing cues, whereof the 24-hr light/dark (LD) cycle is the most important one (Dibner et al., 2010, Kwon et al., 2011).

The generation of circadian rhythms at the molecular level depends upon the activity of so called clock genes, which form interlocking feedback loops of gene transcription and translation. The positive limb of the core feedback loop involves the proteins CLOCK and BMAL1, which heterodimerize and act as transcriptional activators of *Period* (*Per1-3)* and *Cryptochrome* (*Cry1*, *Cry2*) genes. Their respective protein products, PER and CRY, function as the negative limb by forming heterodimers that interfere with the CLOCK/BMAL1 complex to repress their own transcription. Additional feedback loops involving the nuclear receptor genes *RevErbα/β* and *Rorα* stabilize and regulate the resetting properties of the aforementioned core feedback loop (Reppert and Weaver, 2002). Of note, negative elements of the core clock machinery can partly compensate each other. Although resulting in aberrant circadian periodicity, clock oscillation sustained when a single gene within the *Per* or *Cry* family was mutated (Bae et al., 2001, van der Horst et al., 1999, Zheng et al., 2001). In contrast, *Cry1/Cry2* double-mutant mice (van der Horst et al., 1999) and *Per1/2* double-mutants (Zheng et al., 2001) loose rhythmicity immediately after release into constant darkness (DD), suggesting a complete functional disruption of the clock.

It has been shown that glucocorticoids (GCs) – steroid hormones secreted into the blood stream upon stressful stimuli and in a circadian fashion under basal conditions – can reset the rhythmic

phase of peripheral clocks by a rapid induction of *Per1* and *Per2* through the GC response element (GRE) in their promoter region without affecting the central clock in the SCN (Balsalobre et al., 1998). In consistence, various early studies indicated that stress does not appear to have a robust effect on the central circadian pacemaker as assessed by free-running activity rhythms (Meerlo et al., 1997, Moberg and Clark, 1976, Van Reeth et al., 1991). However, stress can have some masking effects on circadian parameters. Decreased amplitudes of body temperature rhythms following social stress (Meerlo et al., 1997) or foot shocks (Kant et al., 1991) have been reported in rats. Furthermore, surgery stress was found to increase the circadian amplitude of heart rate and temperature rhythms in rats (Harper et al., 1996). Intriguingly, recent studies reveal dampened PER2, CLOCK, and BMAL1 protein expression in the SCN upon chronic unpredictable stress (Jiang et al., 2011) and decreased PER2 protein expression following repeated restraint stress (Kinoshita et al., 2012), indicating that certain stressors are capable of perturbing clock core function in the SCN.

In contrast to light, which induces *Per* gene expression only at early or late night (Albrecht et al., 1997, Shearman et al., 1997), GC-dependent phase resetting processes can be induced at any time of the LD cycle (Balsalobre et al., 2000). Yet, exposure of rats to novel environment or restraint stress, as psychological means to raise GC levels, resulted in elevated hypothalamic-pituitary-adrenal (HPA) axis responses at early day compared to early night. Vice versa, physical stressors, such as hypoglycemic shock, elicit the largest response at the onset of activity, demonstrating a stressor-specific circadian gating of the stress system (Dickmeis, 2009, Jacobson, 2005). In line, we have previously shown that repeated social defeat (SD) has different effects on the central clock in the SCN and peripheral output rhythms, depending on the time of day of stressor exposure. SD during the early dark phase (*Zeitgeber* time (ZT)13 – ZT15; social defeat dark = SDD), but not during the early light phase (ZT1 – ZT3; social defeat light = SDL), resulted in a blunted activity rhythm and flattened or shifted GC rhythm (Bartlang et al., 2012) and increased PER2 protein expression and PER2 rhythm amplitude in the SCN (Bartlang et al., 2014).

Based on these findings, the present study sought to investigate the consequences of repeated SDL and SDD on two output rhythms of the clock, i.e. the activity and body temperature. Therefore, wildtype (WT) and *Per1/2* double-mutant mice were implanted subcutaneously with small transmitters and body temperature and activity were recorded under baseline, stress, and post-stress conditions.

Materials and Methods

Animals

Male C57BL/6N and C57BL/6J mice (Charles River, Sulzfeld, Germany) as well as male *Per1/2* double-mutant mice were used as experimental animals exposed to SD (see below). The C57BL/6N substrain was used in our previous study (Bartlang et al., 2012), whereas the C57BL/6J substrain served as adequate control group for the *Per1/2* double-mutants that carried this genetic background. *Per1/Per2* mutant mice were bred from in-house colonies maintained at the Central Animal Facility, University of Lübeck, Germany. All mice were kept under standard laboratory conditions (23 ± 1°C; 55 ± 5% humidity) and had free access to tap water and standard mouse diet. Unless otherwise noted, they were exposed to a 12:12-hr LD cycle (light phase: 250 ± 50 lux) with lights-on at 07:00. The time of lights-on is defined as ZT0 and lights-off as ZT12. All animals were individually housed in standard polycarbonate cages (17,5 x 35,5 x 13 cm, experimental mice or 41 x 15 x 25 cm, residents) for 1 week before the start of the experiment. Animals weighed 17 – 22 g when exposed as intruders to male dominant resident CD1 mice weighing 30 – 35 g (Charles River, Sulzfeld, Germany). All experimental protocols were approved by the Committee on Animal Health and Care of the local government and performed according to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their sufferings.

Experimental design

Intra-abdominal telemetry transmitters were surgically implanted into the peritoneal cavity of experimental mice. Based on a previous study, baseline body temperature and activity data collection started 9 days after implantation of the transmitters at ZT0 (Vlach et al., 2000). 21 days after implantation, mice were assigned to a single-housed control (SHC), a SDL, and a SDD group, according to basal activity. SHC mice remained undisturbed except for a weekly change of bedding at the beginning of the dark phase. Based on previous studies, SHC mice were considered to be the most appropriate controls in this paradigm (Singewald et al., 2009). SDL and SDD mice were exposed to

SD for 2 hr daily across 19 days (except for days 7 and 14), either at the beginning of the light phase (ZT1 – ZT3, SDL mice) or at the beginning of the dark phase (ZT13 – ZT15, SDD mice). Subsequent to the last SD on day 19, data collection was continued for 14 days in the post-stress period, either in LD or DD conditions.

SDL and SDD paradigms

The stress paradigm was performed as described before (Bartlang et al., 2012, Bartlang et al., 2014). Briefly, during each SD session, the experimental mouse was taken from its home cage and placed into a male resident's home cage. After the first attack by the resident that resulted in submissive behavior from the intruder, both mice were separated from each other by a perforated partition wall. This protected the intruder from physical injuries by the resident, but allowed visual, olfactory, and auditory contact as described previously (Reber et al., 2006). During the subsequent 2 hr of sensory contact, food pellets, but not tap water, were available for all experimental mice including SHC mice. Following removal of the partition wall, direct social interaction between the resident and the experimental mouse was allowed until the first attack by the resident that resulted in submissive behavior from the intruder. Each experimental mouse was exposed to a different resident every day to avoid habituation.

Surgical implantation of transmitters

A small G2-E-Mitter (16.5 mm x 6.5 mm; 1.1 g, med-NATIC GmbH, München, Germany), containing a microchip as well as an antenna enclosed in bioglass, was sterilized with 70 % ethanol and kept in saline until implantation under semi-sterile conditions. Before insertion, transmitters were programmed with the ID-number of the respective mouse to avoid interference from neighbouring mice during data collection. For surgery, experimental mice (intruder) were anesthetized with isofluorane (Baxter GmbH, Unterschleißheim, Germany). After removal of the fur at the back, each transmitter was implanted subcutaneously in the abdominal region via a 1.5 cm long skin incision at the back of the mouse. The skin of the back was sutured with 2 – 3 stitches and iodine was applied to

the wound. During surgery body temperature was maintained by a heating pad. To avoid post-surgical infections, mice received 0.1 ml antibiotics (SC, Baytril®2.5 % Bayer Vital GmbH, Leverkusen, Germany).

Telemetry system and telemetric data collection

The telemetry monitoring system (Mini Mitter, Bend, USA, provided by med-NATIC GmbH, München, Germany) is composed of a passive (i.e. battery-free) telemetry transmitter (G2-E-Mitter), a receiver (model ER-4000, placed underneath the cage) and a data acquisition system with accompanying software (Vital View, Minimitter, 2007, Bend, Oregon, USA), which converts the transmitter signals directly into core body temperature and activity. The transmitters were calibrated by the producer in the range from 33 to 41°C and had an accuracy of 0.1 °C. The data acquisition system was programmed to sample body temperature and activity at 1-min time intervals around the clock. Data collection began 9 days after surgery at ZT0 and continued for eight weeks. Based on the fact that routine control procedures, such as placement into a clean cage, can affect behavior and induce an activation of the stress system (Balcombe et al., 2004, Duke et al., 2001), days on which cages were changed (day 1, 8, and 15) were excluded from the analysis (see Fig. 1).

Data analysis and statistics

Analysis of activity and body temperature was performed using Microsoft Excel. Normalized average activity profiles and the phases of the activity peaks were calculated as described in (Schlichting et al., 2014). Free-running period lengths in DD were determined using χ^2-periodogram analysis and actograms were depicted using the ImageJ plugin ActogramJ (Schmid et al., 2011). Average activity levels were calculated from mean activity counts of single mice during daytime (ZT0 to ZT6 (1st half) and ZT6 to ZT12 (2nd half)) and nighttime (ZT12 to ZT18 (1st half) and ZT18 to ZT0 (2nd half)) for basal, stress, day 20 and post-stress (LD and DD) periods, respectively. Average body temperature levels for the different periods (basal, stress, day 20 and post-stress (LD and DD) were calculated as described for activity above.

182 For statistical comparisons, the software package SPSS (IBM, Armonk, NY, version 19) was used.
183 Data were tested for normal distribution applying a one-sample Kolmogorov–Smirnov test. To test for
184 significant differences in normally distributed datasets, one-way ANOVA or ANOVA for repeated
185 measures (rm ANOVA) was applied, followed by a *post hoc* pairwise comparison with LSD
186 correction. Data are presented as means + / ± SEM. Significance was set at $p \leq 0.05$.
187

Results

Figure 1 shows typical activity (A, B) and body temperature recordings (C, D) of WT (C57BL/6J) and *Per1/Per2* mutant mice that were subjected to SD in the morning (SDL) or evening (SDD), respectively, under LD conditions. The periods of stressor exposure can be easily recognized by the elevated activity and body temperature levels during the acute stress episodes and the reduced levels during the remaining night. One half of the animals were kept in LD after the 19 days of stressor exposure (A, C), while the other half was transferred into DD (B, D) to assess the properties of the free-running circadian clock. For analyzing the effects of repeated stressor exposure on activity and body temperature, we divided the recording period in the following sections: basal, stress, immediately after the stress (day 20), post-stress in LD and post-stress in DD (Fig. 1).

Basal activity and body temperature

Activity of both WT strains showed three activity bouts, a sharp one after lights-off, a broader one in the first half of the night, and one of intermediate broadness at the end of the night (Fig. 1A, 2A, 4A). This trimodal pattern was principally reflected in the temperature profile, although it was less clear (Fig. 1C, 3A, 5A). In contrast, *Per1/Per2* mutant mice only showed one activity bout that coincided with the first sharp activity bout of WT mice, but it was much broader (Fig. 1A, 6A). The same was evident for the body temperature profile (Fig. 1C, 7A).

Activity and body temperature during the stress episodes

During SD exposure the trimodal activity pattern of WT mice was profoundly disrupted (Fig. 1A, 2B, 4B). Furthermore, as already observable in the actograms (Fig. 1A), SD exposure affected the activity levels of WT and *Per1/Per2* mutant mice in a similar way. Activity levels in the first half of the light phase (ZT0 to ZT6) on days of SD exposure were dependent on the employed SD paradigm (SHC, SDL or SDD) for C57BL/6N ($F_{2, 51} = 191.04$; $p \leq 0.001$), C57BL/6J ($F_{2, 51} = 175.67$; $p \leq 0.001$), and *Per1/Per2* mutant ($F_{2, 39} = 3.30$; $p \leq 0.05$) mice. *Post hoc* analysis revealed increased activity of WT SDL mice between ZT0 – ZT6 ($p \leq 0.001$) compared to both SHC and SDD mice (Fig. 2B+C, 4B+C),

while *Per1/2* double-mutant SDL mice showed increased activity ($p \leq 0.05$) compared to SHC mice only (Fig. 6B+C). The activity level in the second half of the light phase (ZT6 to ZT12) on days of SD exposure was again dependent on the SD paradigm for C57BL/6N ($F_{2, 51} = 8.71$; $p \leq 0.001$), C57BL/6J ($F_{2, 51} = 8.19$; $p \leq 0.001$), and *Per1/Per2* mutant ($F_{2, 39} = 6.48$; $p \leq 0.01$) mice. *Post hoc* tests showed that SDL mice exhibited decreased activity compared with SHC ($p \leq 0.01$) and SDD mice ($p \leq 0.05$) (Fig. 2B+C, 4B+C, 6B+C). Activity in the first half of the dark phase (ZT12 to ZT18) was similarly dependent on the SD paradigm for C57BL/6N ($F_{2, 51} = 58.42$; $p \leq 0.001$), C57BL/6J ($F_{2, 51} = 52.84$; $p \leq 0.001$) and *Per1/Per2* mutant ($F_{2, 39} = 25.56$; $p \leq 0.001$) mice. *Post hoc* analysis revealed reduced active behavior of SDL mice compared with SHC ($p \leq 0.01$) and SDD ($p \leq 0.001$) mice and increased activity of SDD mice compared with SHC animals ($p \leq 0.01$) (Fig. 2B+C, 4B+C, 6B+C).

Finally, activity in the second half of the dark phase (ZT18 to ZT0) was dependent on the SD paradigm for C57BL/6N ($F_{2, 51} = 20.09$; $p \leq 0.001$), C57BL/6J ($F_{2, 51} = 13.96$; $p \leq 0.001$), and *Per1/Per2* mutant ($F_{2, 39} = 9.85$; $p \leq 0.001$) mice. *Post hoc* tests revealed that both SDL ($p \leq 0.05$) and SDD ($p \leq 0.001$) mice showed less active behavior when compared with SHC mice (Fig. 2B+C, 4B+C, 6B+C).

Body temperature was affected by SD in a similar manner as activity, although the effects were less pronounced and not always significant, especially not in the mutants. Body temperature of C57BL/6N mice in the first half of the light phase (ZT0 to ZT6) was dependent on the SD paradigm ($F_{2, 51} = 6.85$; $p \leq 0.01$). *Post hoc* analysis revealed increased body temperature of SDL mice in comparison to SHC animals ($p \leq 0.001$) (Fig. 3B+C). Body temperature of C57BL/6J mice was dependent on the SD paradigm in the first half of the light phase (ZT0 to ZT6) ($F_{2, 51} = 11.48$; $p \leq 0.001$), in the first half of the dark phase (ZT12 to ZT18) ($F_{2, 51} = 5.86$; $p \leq 0.01$), and in the second half of the dark phase (ZT18 to ZT0) ($F_{2, 51} = 5.83$; $p \leq 0.01$). *Post hoc* tests revealed increased body temperature of SDL mice between ZT0 – ZT6 compared with SHC ($p \leq 0.001$) and SDD ($p \leq 0.01$) mice, while body temperature of SDL mice was decreased between ZT12 to ZT18 compared with SHC ($p \leq 0.01$) and SDD ($p \leq 0.01$) mice. Body temperature between ZT18 – ZT0 was decreased in SDL ($p \leq 0.01$) and SDD ($p \leq 0.01$) mice compared to SHC mice, as revealed by *post hoc* analysis (Fig. 5B+C). Body

temperature of *Per1/2* double-mutant mice was not dependent on the SD paradigm, regardless of the time of assessment (first half of the light phase, second half of the light phase, first half of the dark phase, second half of the dark phase; Fig. 7B+C).

Activity and body temperature immediately after SD stress (day 20)

Already on the first day following SD exposure the animals returned to more pre-stress-like activity patterns. The above-described differences in activity levels between SHC, SDL, and SDD mice disappeared in the two WT strains and the *Per1/2* double-mutants (Fig. 2D, 4D, 6D). Activity peaks in the mutants after lights-off were neither dependent on the SD paradigm nor on the time of assessment (basal period, day 20 and post-stress; Fig. 8). In contrast, the activity pattern of the two WT strains on day 20 was still different from that during the basal period (Fig. 2D, 4D). Most WT SDL and SDD mice lacked the first activity bout, and if it was present, it occurred significantly later as before the 19 days of stressor exposure (Fig. 8). Employment of rmANOVA showed that the first activity peak was dependent on both the SD paradigm and the time of assessment ($F_{4, 28} = 12.55$; $p \leq 0.001$). *Post hoc* analysis revealed that both SDL and SDD C57BL/6N mice had a significantly delayed first activity peak on day 20 compared to SHC mice ($p \leq 0.01$) and in comparison to the basal period ($p \leq 0.001$). The same was evident for C57BL/6J mice ($F_{4, 26} = 6.74$; $p \leq 0.001$). In addition, the second activity peak was affected by the SD paradigm and the time of assessment in C57BL/6N mice ($F_{4, 48} = 5.30$; $p \leq 0.001$) and by the time of assessment in C57BL/6J mice ($F_{4, 48} = 5.30$; $p \leq 0.001$). *Post hoc* analysis showed that C57BL/6N SDD mice exhibited a delayed second activity peak on day 20 compared to SHC ($p \leq 0.01$) and SDL ($p \leq 0.001$) mice and compared to the basal period ($p \leq 0.001$), while C57BL/6J SDD mice showed a delayed second activity peak on day 20 compared to the basal period ($p \leq 0.01$).

The same tendency appeared to be present in body temperature, but most patterns were too irregular to allow clear determination of temperature peak phases.

Post-stress activity and body temperature in LD

The above-described differences in the presence and timing of the first activity peak persisted during the entire post-stress period in SDD, but not in SDL WT mice. Evaluation of actograms from SDD animals yielded a complete loss of the first activity bout in 62.5 % (C57BL/6N) and 75 % (C57BL/6J) of the animals, resulting in a bimodal nocturnal activity pattern. Furthermore, ANOVA with *post hoc* analysis revealed that the remaining 25 – 37.5 % of C57BL/6J ($F_{4, 26} = 6.74$; $p \leq 0.001$) and C57BL/6N ($F_{4, 28} = 12.55$; $p \leq 0.001$) SDD mice showed a delayed first activity peak compared to both SHC ($p \leq 0.001$) and SDL ($p \leq 0.01$) mice as well as compared to basal levels ($p \leq 0.01$) (Fig. 8). Concerning the second activity peak at around ZT17, a significant dependence on the SD paradigm and the time of assessment was observed for C57BL/6N mice ($F_{4, 48} = 5.30$; $p \leq 0.001$). *Post hoc* analysis revealed that SDD C57BL/6N mice showed a delayed activity peak compared to SDL mice ($p \leq 0.05$) and basal levels ($p \leq 0.01$).

In contrast, SDL mice did not differ from SHC mice with respect to the nocturnal trimodal activity pattern and the phase of the activity peaks. Once more, the same tendency appeared to be present in body temperature, but it was more difficult to assess.

Post-stress activity and body temperature in DD

Directly upon transfer into DD conditions, *Per1/2* double-mutants lost their circadian rhythmicity, as expected, whereas the two WT strains free-ran with a period slightly shorter than 24 hr (Fig. 1B, D). During free-run, the trimodal activity pattern of the WT mice was retained in C57BL/6N mice (Fig. 2F), but became less obvious in C57BL/6J mice (Fig. 4F). Most importantly, the first activity bout remained absent in SDD mice (Fig. 2F), indicating that repeated SDD had long-term effects on the circadian clock of male WT mice.

Slight period differences were found in stressed C57BL/6N mice as compared to unstressed SHC mice: The period was dependent on the SD paradigm ($F_{2, 24} = 14.67$; $p \leq 0.001$) and *post hoc* analysis revealed that SDL ($p \leq 0.001$) (23.71 ± 0.03 hr) and SDD ($p \leq 0.01$) (23.76 ± 0.03 hr) mice exhibited a significant shorter period in comparison to SHC (23.90 ± 0.02 hr) mice. This effect was absent in C57BL/6J mice.

297 **Discussion**

298 To date, only a few rodent studies have addressed long-lasting effects of repeated/chronic stress on
299 circadian functionality. Twenty to thirty percent of rats repeatedly subjected to different stressors
300 revealed circadian changes, manifested in altered entrainment, advanced phase or changes in the
301 period length (Barrington et al., 1993). Moreover, rats exposed to a chronic mild stress procedure for
302 four weeks showed distinct and prolonged disturbances of the diurnal and circadian activity rhythms
303 (Gorka et al., 1996). Further, a long-lasting decrease in the amplitude of circadian rhythms for heart
304 rate and core body temperature has been reported following repeated SD in rats (Tornatzky and
305 Miczek, 1993). Strikingly, although it is well known that stress effects are determined by the time of
306 day of assessment (Janssens et al., 1995, Verma et al., 2010), it remains largely unknown whether
307 stress-induced changes in circadian rhythms also depend on the time of day of stressor presentation.
308 Our data provide first evidence that the circadian system is influenced in the long term by repeated SD
309 in such a way that the time of day, at which the stressor is applied, determines stress effects on the
310 circadian clock.
311 Consistent with previous reports (Sgoifo et al., 2001, 2002), no habituation in stress-induced activity
312 and body temperature rises in response to the repeatedly applied psychosocial stressor took place in
313 the present study. Therefore, we could pool all days of SD exposure (except days 1, 8, and 15, where
314 cages were changed). While SDL mice showed increased activity between ZT0 – ZT6, due to the
315 stressor exposure, activity was significantly decreased in this group in the second half of the light
316 phase (ZT6 to ZT12) and during the whole dark phase. In contrast, increased active behavior of SDD
317 mice between ZT12 – ZT18, attributable to the stressor exposure at that time, was balanced by less
318 activity during the second half of the dark phase (ZT18 – ZT0). The considerable additional activity
319 decrease in SDL mice during the second half of the light phase (ZT6 to ZT12) is likely to compensate
320 the elevated activity in the first half of their normally inactive period. Given that SDL and SDD
321 *Per1/2* mutants exhibited identical changes with respect to activity compared to WT animals, the acute
322 alterations throughout the stress procedure are not a result of an altered internal clock, but rather a
323 direct response to the social stressor. Body temperature was generally less affected by the stress

procedure than activity. This was also due for the post-stress period and is probably caused by the necessity to keep homeothermy.

During the post-stress period, SDL and SDD mice quickly returned to their normal activity range and also the free-running period appeared only marginally affected by stressor exposure in C57BL/6N, but not C57BL/6J, mice, suggesting a slightly different stress responsiveness of different C57BL/6 substrains. Nevertheless, the activity pattern of SDD mice was considerably different in both substrains as compared to SDL and SHC mice: The majority of SDD animals showed a bimodal, instead of a trimodal, nocturnal activity pattern and this effect was even visible under DD conditions. For the remaining (still trimodal) WT SDD mice, the first activity peak after lights-off was significantly delayed compared to SHC and SDL animals in the entrained post-stress period, indicating a phase delay of the endogenous clock following repeated stressor exposure during the early dark (i.e. active) phase. C57BL/6N SDD mice further showed a delayed second activity peak. This effect was not visible in C57BL/6J animals, suggesting again that the C57BL/6N substrain is more susceptible to SD stress than the C57BL/6J. Importantly, in our previous study, though not significant, the phase of the PERIOD2::LUCIFERASE (PER2::LUC) rhythm in the SCN of SDD mice tended towards a phase delay (Bartlang et al., 2014). The lack of significance is probably due to the fact that PER2::LUC mice are bred on a C57BL/6J background, which seems to be less responsive to the repeated SD as compared to the C57BL/6N substrain.

Following our hypothesis of an altered internal clock in WT SDD mice, we predicted no differences between clock-deficient *Per1/2* double-mutant SHC and SDD mice. Indeed, *Per1/Per2* mutant SDD mice showed a normal activity rhythm in the post-stress period under LD conditions that was comparable to SHC and SDL mice. As is known from the literature (Zheng et al., 2001), *Per1/Per2* double-mutant mice completely lost their rhythm when exposed to the post-stress period under DD conditions in the present study. This effect was irrespective of previous SD exposure, demonstrating the superordinating character of the LD cycle on the predictable daily social stress input.

Based on the fact that rhythmic activity is a reliable indicator of circadian pacemaker functioning, changes in the circadian phase due to repeated SD can be inferred to reflect SCN activity. In support,

we have recently reported increased PER2::LUC rhythm amplitude and increased PER2 protein expression in the SCN of C57BL/6J and C57BL/6N mice, respectively (Bartlang et al., 2014). In consistence, a recent study revealed increased *Per1* and *Per2* expression in the SCN of rats with an extreme, posttraumatic stress disorder-like, behavioral response upon exposure to predator scent stress. These changes could be reversed by treatment with the antidepressant agomelatine (Koresh et al., 2012). On the contrary, suppressed PER2 levels in the SCN following chronic restraint stress could be normalized by the mood-stabilizer lithium (Kinoshita et al., 2012). Irrespective of whether the changes are upwards or downwards, these studies point towards a role of the circadian *Per2* gene/protein in mood-related disorders. Interestingly, well-accepted hallmarks of affective disorders such as decreased interest in an unfamiliar conspecific, loss of general activity, and flattened diurnal CORT rhythmicity have become evident following repeated SDD (Bartlang et al., 2012). In support, polymorphisms in the *Per2* gene and abnormal circadian rhythms have been associated with increased vulnerability to major depressive disorder (MDD) and seasonal affective disorder (Lavebratt et al., 2010, McClung, 2007, Partonen et al., 2007). Thus, it can be hypothesized that rhythm disturbances at least sensitize and individual to depression. Likewise, shift work, involving disruptions in circadian output rhythms, represents an acknowledged risk factor for a plethora of devastating somatic health problems such as breast and colorectal cancer (Schernhammer et al., 2003, 2006), gastric ulcers (Segawa et al., 1987), diabetes (Morikawa et al., 2005), stroke (Karlsson et al., 2005), and coronary heart disease (Tenkanen et al., 1998). Akin to SD in our study, shift working can be regarded as a psychosocial stressor in humans and might include circadian disruption and altered phase angles of entrainment (Castanon-Cervantes et al., 2010). One proposed mechanism underlying the pathologies associated with circadian desynchrony, due to frequent jet lag exposure or shift work, is regulation of inflammation by the immune system (Castanon-Cervantes et al., 2010). For instance, experimental chronic jet lag in rodents has been shown to lead to a dramatic increase in the progression of dextran sodium sulfate (DSS)-induced colitis (Preuss et al., 2008). Furthermore, an absent interferon-gamma rhythm has been demonstrated for *Per2* mutant mice (Arjona and Sarkar, 2006), and altered sleep-wake cycles in humans increase inflammatory cytokines such as interleukin-6 and tumor necrosis

factor-alpha (Mullington et al., 2009, Shearer et al., 2001). Intriguingly, interferon-gamma secretion from mesenteric lymph node cells and severity of a DSS-induced colitis were significantly more pronounced in SDD than SDL mice (Bartlang et al., 2012). Thus, it is tempting to speculate that SDD mice resemble shift-workers, which might help in identifying novel and reliable markers of the underlying pathology of shift work.

Possible mechanisms underlying the more negative outcome of SD exposure during the dark versus the light phase might include the increased sensitivity of the adrenal cortex to adrenocorticotropic hormone in the active compared to the inactive phase (Buijs et al., 1999, Kalsbeek et al., 2011). In addition, based on the phase response curve (Dibner et al., 2010), resetting processes of the biological clock are likely to happen during early night, at a time that interferes with the time of SDD exposure. Thus, a resetting of the circadian clock, required for the appropriate timing of physiological and behavioral processes, might not be possible in mice repeatedly exposed to the stressor at the beginning of the dark phase.

General conclusion

In summary, our results indicate that psychosocial stressor exposure during the dark (i.e. active) phase has long-lasting consequences for the functional output of the biological clock. It is reasonable to speculate that these rhythm disturbances are ultimately linked to the maladaptive consequences of SDD (Bartlang et al., 2012), resembling symptoms of shift-work disorder. Though, our data point towards an involvement of the circadian gene *Per2,* and possibly *Per1*, in the time-of-day-dependent response to stress, more detailed studies including the analysis of other clock genes mutants are required to further clarify this point.

Acknowledgements

The authors are grateful to Dr. S. Peters, L. Lucke, M. Stieglitz, L. Onderka, and to A. Kühn for excellent technical help.

Declaration of interest

All authors declare that there are no conflicts of interest. This study was supported by the Deutsche Forschungsgemeinschaft (DFG FO-207/13-1). HO is a Lichtenberg fellow of the Volkswagen Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

412 **References**

413

414 Albrecht U, Sun ZS, Eichele G, Lee CC. (1997). A differential response of two putative mammalian
415 circadian regulators, mper1 and mper2, to light. *Cell* 91:1055-1064.
416 Arjona A, Sarkar DK. (2006). The circadian gene mPer2 regulates the daily rhythm of IFN-gamma. *J*
417 *Interferon Cytokine Res* 26:645-649.
418 Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. (2001). Differential functions of
419 mPer1, mPer2, and mPer3 in the SCN circadian clock. *Neuron* 30:525-536.
420 Balcombe JP, Barnard ND, Sandusky C. (2004). Laboratory routines cause animal stress. *Contemp*
421 *Top Lab Anim Sci* 43:42-51.
422 Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schutz G, Schibler
423 U. (2000). Resetting of circadian time in peripheral tissues by glucocorticoid signaling.
424 *Science* 289:2344-2347.
425 Balsalobre A, Damiola F, Schibler U. (1998). A serum shock induces circadian gene expression in
426 mammalian tissue culture cells. *Cell* 93:929-937.
427 Barrington J, Jarvis H, Redman JR, Armstrong SM. (1993). Limited effect of three types of daily
428 stress on rat free-running locomotor rhythms. *Chronobiol Int* 10:410-419.
429 Bartlang MS, Neumann ID, Slattery DA, Uschold-Schmidt N, Kraus D, Helfrich-Förster C, Reber SO.
430 (2012). Time matters: pathological effects of repeated psychosocial stress during the active,
431 but not inactive, phase of male mice. *J Endocrinol* 215:425-437.
432 Bartlang MS, Savelyev SA, Johansson AS, Reber SO, Helfrich-Förster C, Lundkvist GB. (2014).
433 Repeated psychosocial stress at night, but not day, affects the central molecular clock.
434 *Chronobiol Int* 31:996-1007.
435 Buijs RM, Wortel J, Van Heerikhuize JJ, Feenstra MG, Ter Horst GJ, Romijn HJ, Kalsbeek A. (1999).
436 Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal
437 (cortex) pathway. *Eur J Neurosci* 11:1535-1544.
438 Castanon-Cervantes O, Wu M, Ehlen JC, Paul K, Gamble KL, Johnson RL, Besing RC, Menaker M,
439 Gewirtz AT, Davidson AJ. (2010). Dysregulation of inflammatory responses by chronic
440 circadian disruption. *J Immunol* 185:5796-5805.
441 Dibner C, Schibler U, Albrecht U. (2010). The mammalian circadian timing system: organization and
442 coordination of central and peripheral clocks. *Annu Rev Physiol* 72:517-549.
443 Dickmeis T. (2009). Glucocorticoids and the circadian clock. *J Endocrinol* 200:3-22.
444 Duke JL, Zammit TG, Lawson DM. (2001). The effects of routine cage-changing on cardiovascular
445 and behavioral parameters in male Sprague-Dawley rats. *Contemp Top Lab Anim Sci* 40:17-
446 20.
447 Gorka Z, Moryl E, Papp M. (1996). Effect of chronic mild stress on circadian rhythms in the
448 locomotor activity in rats. *Pharmacol Biochem Behav* 54:229-234.
449 Harper DG, Tornatzky W, Miczek KA. (1996). Stress induced disorganization of circadian and
450 ultradian rhythms: comparisons of effects of surgery and social stress. *Physiol Behav* 59:409-
451 419.
452 Jacobson L. (2005). Hypothalamic-pituitary-adrenocortical axis regulation. *Endocrinol Metab Clin*
453 *North Am* 34:271-292, vii.
454 Janssens CJ, Helmond FA, Wiegant VM. (1995). The effect of chronic stress on plasma cortisol
455 concentrations in cyclic female pigs depends on the time of day. *Domest Anim Endocrinol*
456 12:167-177.
457 Jiang WG, Li SX, Zhou SJ, Sun Y, Shi J, Lu L. (2011). Chronic unpredictable stress induces a
458 reversible change of PER2 rhythm in the suprachiasmatic nucleus. *Brain Res* 1399:25-32.
459 Kalsbeek A, van der Spek R, Lei J, Endert E, Buijs RM, Fliers E. (2011). Circadian rhythms in the
460 hypothalamo-pituitary-adrenal (HPA) axis. *Mol Cell Endocrinol* 349:20-29.
461 Kant GJ, Bauman RA, Pastel RH, Myatt CA, Closser-Gomez E, D'Angelo CP. (1991). Effects of
462 controllable vs. uncontrollable stress on circadian temperature rhythms. *Physiol Behav*
463 49:625-630.

464 Karlsson B, Alfredsson L, Knutsson A, Andersson E, Toren K. (2005). Total mortality and cause-
465 specific mortality of Swedish shift- and dayworkers in the pulp and paper industry in 1952-
466 2001. *Scand J Work Environ Health* 31:30-35.
467 Kinoshita C, Miyazaki K, Ishida N. (2012). Chronic stress affects PERIOD2 expression through
468 glycogen synthase kinase-3beta phosphorylation in the central clock. *Neuroreport* 23:98-102.
469 Koresh O, Kozlovsky N, Kaplan Z, Zohar J, Matar MA, Cohen H. (2012). The long-term
470 abnormalities in circadian expression of Period 1 and Period 2 genes in response to stress is
471 normalized by agomelatine administered immediately after exposure. *Eur*
472 *Neuropsychopharmacol* 22:205-221.
473 Kwon I, Choe HK, Son GH, Kim K. (2011). Mammalian molecular clocks. *Exp Neurobiol* 20:18-28.
474 Lavebratt C, Sjoholm LK, Partonen T, Schalling M, Forsell Y. (2010). PER2 variantion is associated
475 with depression vulnerability. *Am J Med Genet B Neuropsychiatr Genet* 153B:570-581.
476 McClung CA. (2007). Circadian genes, rhythms and the biology of mood disorders. *Pharmacol Ther*
477 114:222-232.
478 Meerlo P, van den Hoofdakker RH, Koolhaas JM, Daan S. (1997). Stress-induced changes in circadian
479 rhythms of body temperature and activity in rats are not caused by pacemaker changes. *J Biol*
480 *Rhythms* 12:80-92.
481 Moberg GP, Clark CR. (1976). Effect of adrenalectomy and dexamethasone treatment on circadian
482 running in the rat. *Pharmacol Biochem Behav* 4:617-619.
483 Morikawa Y, Nakagawa H, Miura K, Soyama Y, Ishizaki M, Kido T, Naruse Y, Suwazono Y,
484 Nogawa K. (2005). Shift work and the risk of diabetes mellitus among Japanese male factory
485 workers. *Scand J Work Environ Health* 31:179-183.
486 Mullington JM, Haack M, Toth M, Serrador JM, Meier-Ewert HK. (2009). Cardiovascular,
487 inflammatory, and metabolic consequences of sleep deprivation. *Prog Cardiovasc Dis* 51:294-
488 302.
489 Partonen T, Treutlein J, Alpman A, Frank J, Johansson C, Depner M, Aron L, Rietschel M, Wellek S,
490 Soronen P, Paunio T, Koch A, Chen P, Lathrop M, Adolfsson R, Persson ML, Kasper S,
491 Schalling M, Peltonen L, Schumann G. (2007). Three circadian clock genes Per2, Arntl, and
492 Npas2 contribute to winter depression. *Ann Med* 39:229-238.
493 Preuss F, Tang Y, Laposky AD, Arble D, Keshavarzian A, Turek FW. (2008). Adverse effects of
494 chronic circadian desynchronization in animals in a "challenging" environment. *Am J Physiol*
495 *Regul Integr Comp Physiol* 295:R2034-2040.
496 Reber SO, Obermeier F, Straub RH, Falk W, Neumann ID. (2006). Chronic intermittent psychosocial
497 stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced
498 colitis and impairs regeneration. *Endocrinology* 147:4968-4976.
499 Reppert SM, Weaver DR. (2002). Coordination of circadian timing in mammals. *Nature* 418:935-941.
500 Schernhammer ES, Kroenke CH, Laden F, Hankinson SE. (2006). Night work and risk of breast
501 cancer. *Epidemiology* 17:108-111.
502 Schernhammer ES, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Fuchs CS, Colditz GA.
503 (2003). Night-shift work and risk of colorectal cancer in the nurses' health study. *J Natl*
504 *Cancer Inst* 95:825-828.
505 Schlichting M, Grebler R, Peschel N, Yoshii T, Helfrich-Förster C. (2014). Moonlight detection by
506 Drosophila's endogenous clock depends on multiple photopigments in the compound eyes. *J*
507 *Biol Rhythms* 29:75-86.
508 Schmid B, Helfrich-Förster C, Yoshii T. (2011). A new ImageJ plug-in "ActogramJ" for
509 chronobiological analyses. *J Biol Rhythms* 26:464-467.
510 Segawa K, Nakazawa S, Tsukamoto Y, Kurita Y, Goto H, Fukui A, Takano K. (1987). Peptic ulcer is
511 prevalent among shift workers. *Dig Dis Sci* 32:449-453.
512 Sgoifo A, Pozzato C, Costoli T, Manghi M, Stilli D, Ferrari PF, Ceresini G, Musso E. (2001). Cardiac
513 autonomic responses to intermittent social conflict in rats. *Physiol Behav* 73:343-349.
514 Sgoifo A, Pozzato C, Meerlo P, Costoli T, Manghi M, Stilli D, Olivetti G, Musso E. (2002).
515 Intermittent exposure to social defeat and open-field test in rats: acute and long-term effects
516 on ECG, body temperature and physical activity. *Stress* 5:23-35.

517 Shearer WT, Reuben JM, Mullington JM, Price NJ, Lee BN, Smith EO, Szuba MP, Van Dongen HP,
518 Dinges DF. (2001). Soluble TNF-alpha receptor 1 and IL-6 plasma levels in humans subjected
519 to the sleep deprivation model of spaceflight. *J Allergy Clin Immunol* 107:165-170.
520 Shearman LP, Zylka MJ, Weaver DR, Kolakowski LF, Jr., Reppert SM. (1997). Two period
521 homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron*
522 19:1261-1269.
523 Singewald GM, Nguyen NK, Neumann ID, Singewald N, Reber SO. (2009). Effect of chronic
524 psychosocial stress-induced by subordinate colony (CSC) housing on brain neuronal activity
525 patterns in mice. *Stress* 12:58-69.
526 Tenkanen L, Sjoblom T, Harma M. (1998). Joint effect of shift work and adverse life-style factors on
527 the risk of coronary heart disease. *Scand J Work Environ Health* 24:351-357.
528 Tornatzky W, Miczek KA. (1993). Long-term impairment of autonomic circadian rhythms after brief
529 intermittent social stress. *Physiol Behav* 53:983-993.
530 van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A,
531 Eker AP, van Leenen D, Buijs R, Bootsma D, Hoeijmakers JH, Yasui A. (1999). Mammalian
532 Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* 398:627-630.
533 Van Reeth O, Hinch D, Tecco JM, Turek FW. (1991). The effects of short periods of immobilization
534 on the hamster circadian clock. *Brain Res* 545:208-214.
535 Verma P, Hellemans KG, Choi FY, Yu W, Weinberg J. (2010). Circadian phase and sex effects on
536 depressive/anxiety-like behaviors and HPA axis responses to acute stress. *Physiol Behav*
537 99:276-285.
538 Vlach KD, Boles JW, Stiles BG. (2000). Telemetric evaluation of body temperature and physical
539 activity as predictors of mortality in a murine model of staphylococcal enterotoxic shock.
540 *Comp Med* 50:160-166.
541 Zheng B, Albrecht U, Kaasik K, Sage M, Lu W, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, Lee
542 CC. (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian
543 clock. *Cell* 105:683-694.
544

545

Figure legends

Figure 1. Representative individual activity (A+B) and body temperature profiles (C+D) of C57BL/6J and *Per1/2* mutant SDL and SDD mice under LD (A+C) or DD (B+D) conditions. The white bar indicates the light phase (ZT0 to ZT12), and the dark bar indicates the dark phase (ZT12 to ZT0). For SDL and SDD mice, the different episodes (i.e. basal, stress, d20, post-stress under LD or DD) are labeled in the actograms/temperature profiles with the days of SD exposure marked in red (SDL) and blue (SDD), respectively.

Figure 2. Average activity profiles of C57BL/6N SHC, SDL, and SDD mice during the basal (A), stress (B), immediately after stress (D), post-stress in LD (E) and post-stress in DD (F) periods. **C**: Average activity profiles were calculated for each treatment group, i.e. SHC (A-D: n = 19; E: n = 10; F: n = 9), SDL (A-D: n = 18; E: n = 9; F: n = 9), SDD (A-D: n = 17; E: n = 8; F: n = 9). The black bar indicates the dark phase (ZT12 to ZT0), the white bars represent the light phase (ZT0 to ZT12). For days of SD exposure, activity levels for both halves of the light phase and dark phase were calculated as mean activity, respectively (C). Data are presented as mean +/± SEM. * $p \leq 0.05$ versus respective SHC mice; + $p \leq 0.05$ versus respective SDD mice.

Figure 3. Mean body temperature of C57BL/6N SHC, SDL, and SDD mice during the basal (A), stress (B), immediately after stress (D), post-stress in LD (E) and post-stress in DD (F) periods. **C**: Average body temperature profiles were calculated for each treatment group, i.e. SHC (A-D: n = 19; E: n = 10; F: n = 9), SDL (A-D: n = 18; E: n = 9; F: n = 9), SDD (A-D: n = 17; E: n = 8; F: n = 9). Arrows point to the three temperature peaks whenever clearly visible. The black bar indicates the dark phase (ZT12 to ZT0), the white bar represents the light phase (ZT0 to ZT12). Data are presented as mean +/± SEM. * $p \leq 0.05$ versus respective SHC mice.

Figure 4. Average activity profiles of C57BL/6J SHC, SDL, and SDD mice during the basal (A), stress (B), immediately after stress (D), post-stress in LD (E) and post-stress in DD (F) periods. Average activity profiles were calculated for each treatment group, i.e. SHC (A-D: n = 20; E: n = 10; F: n = 10), SDL (A-D: n = 17; E: n = 8; F: n = 9), SDD (A-D: n = 17; E: n = 8; F: n = 9). For days of SD exposure, activity levels for both halves of the light phase and dark phase were calculated as mean activity, respectively (C). Data are presented as mean +/± SEM. * $p \leq 0.05$ versus respective SHC mice; + $p \leq 0.05$ versus respective SDD mice. Labeling as in Fig. 2.

Figure 5. Mean body temperature of C57BL/6J SHC, SDL, and SDD mice during the basal (A), stress (B), immediately after stress (D), post-stress in LD (E) and post-stress in DD (F) periods. Average body temperature profiles were calculated for each treatment group, i.e. SHC (A-D: n = 20; E: n = 10; F: n = 10), SDL (A-D: n = 17; E: n = 8; F: n = 9), SDD (A-D: n = 17; E: n = 8; F: n = 9). For days of SD exposure, mean temperatures for both halves of the light phase and dark phase were calculated, respectively (C). Data are presented as mean +/± SEM. * $p \leq 0.05$ versus respective SHC mice; + $p \leq 0.05$ versus respective SDD mice. Labeling as in Fig. 3.

Figure 6. Average activity profiles of Per1/2 double-mutant SHC, SDL, and SDD mice during the basal (A), stress (B), immediately after stress (D), post-stress in LD (E) and post-stress in DD (F) periods. Average activity profiles were calculated for each treatment group, i.e. SHC (A-D: n = 15; E: n = 7; F: n = 8), SDL (A-D: n = 12; E: n = 4; F: n = 8), SDD (A-D: n = 15; E: n = 7; F: n = 8). For days of SD exposure, activity levels for both halves of the light phase and dark phase were calculated as mean activity, respectively (C). Data are presented as mean +/± SEM. * $p \leq 0.05$ versus respective SHC mice; + $p \leq 0.05$ versus respective SDD mice. Labeling as in Fig. 2.

Figure 7. Mean body temperature of Per1/2 double-mutant SHC, SDL, and SDD mice during the basal (A), stress (B), immediately after stress (D), post-stress in LD (E) and post-stress in DD (F) periods. Average body temperature profiles were calculated for each treatment group, i.e. SHC

599 (A-D: n = 15; E: n = 7; F: n = 8), SDL (A-D: n = 12; E: n = 4; F: n = 8), SDD (A-D: n = 15; E: n = 7;
600 F: n = 8). For days of SD exposure, mean temperatures for both halves of the light phase and dark
601 phase were calculated, respectively (C). Data are presented as mean +/± SEM. Labeling as in Fig. 3.
602
603 **Figure 8. Phase of activity peaks of C57BL/6N, C57BL/6J, and *Per1/2* double-mutant SHC**
604 **(white), SDL (grey), and SDD (black) mice.** The average phase of each activity peak during the dark
605 phase was calculated for each treatment group for basal (C57BL/6N: SHC: n = 10, SDL: n = 9, SDD:
606 n = 8; C57BL/6J: SHC: n = 10, SDL: n = 8, SDD: n = 8; *Per1/2* double-mutants: SHC: n = 7, SDL: n
607 = 4, SDD: n = 7), day 20 (C57BL/6N: SHC: n = 10, SDL: n = 4 (Peak 1) / 9 (Peak 2+3), SDD: n = 3
608 (Peak 1) / 8 (Peak 2+3); C57BL/6J: SHC: n = 10, SDL: n = 4 (Peak 1) / 8 (Peak 2+3), SDD: n = 3
609 (Peak 1) / 8 (Peak 2+3); *Per1/2* double-mutants: SHC: n = 7, SDL: n = 4, SDD: n = 7), and entrained
610 post-stress periods (C57BL/6N: SHC: n = 10, SDL: n = 9, SDD: n = 3 (Peak 1) / 8 (Peak 2+3);
611 C57BL/6J: SHC: n = 10, SDL: n = 7-8, SDD: n = 2 (Peak 1) / 8 (Peak 2+3); *Per1/2* double-mutants:
612 SHC: n = 7, SDL: n = 4, SDD: n = 7). Note: for statistical analysis of the three activity peaks
613 employing rmANOVA only WT SDL and SDD mice could be included where all three measures
614 (basal, day 20, post-stress) were available.
615 The black bar indicates the dark phase (ZT12 to ZT0), the white bars represent the light phase (ZT0 to
616 ZT12). Data are presented as mean ± SEM. * $p \leq 0.05$ versus respective SHC mice; + $p \leq 0.05$ versus
617 respective SDL mice; # $p \leq 0.05$ versus respective basal levels.

Figure 1

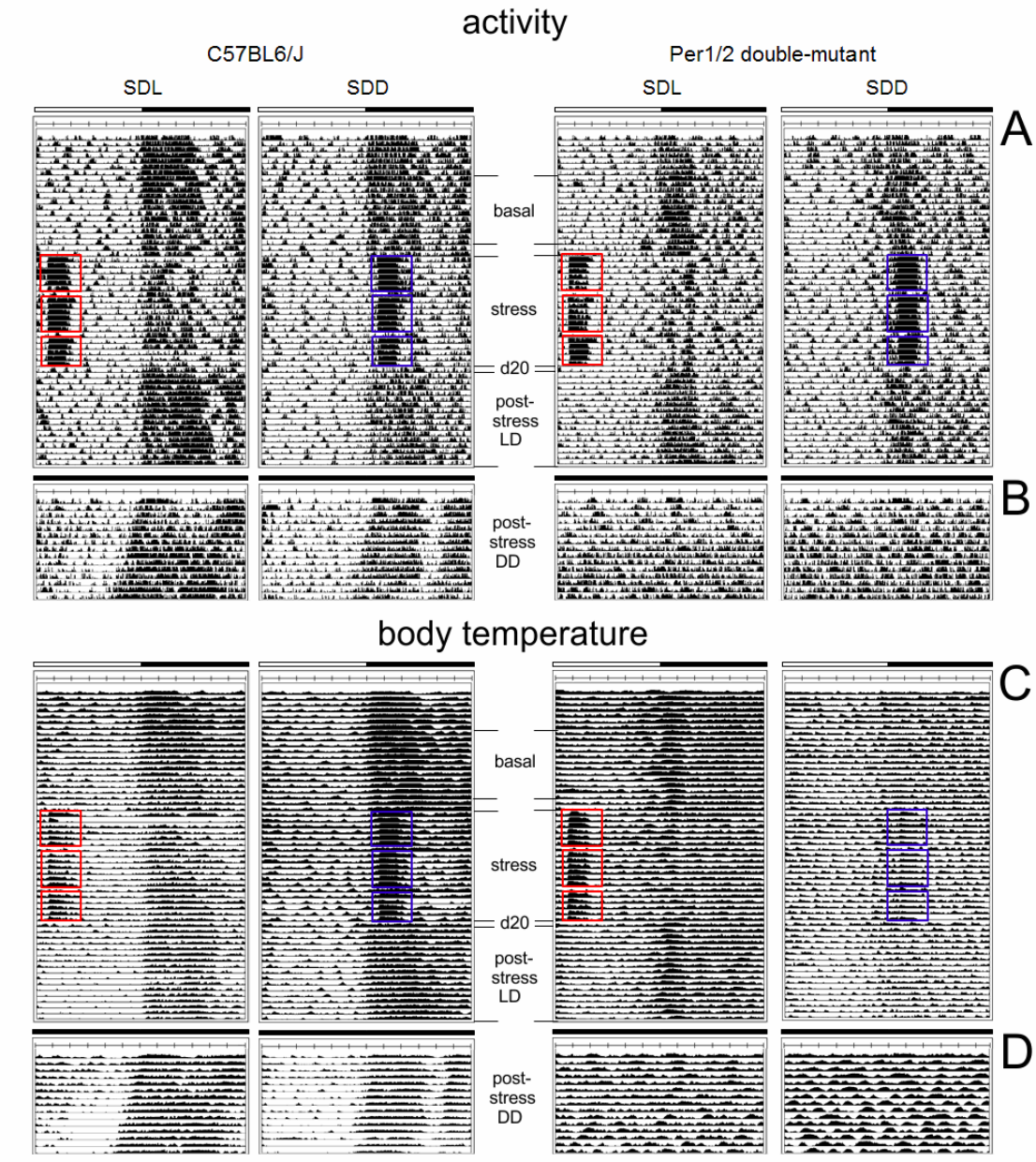

activity
C57BL6/J
Per1/2 double-mutant
SDL
SDD
SDL
SDD
A
basal
stress
d20
post-
stress
LD
B
post-
stress
DD
body temperature
C
basal
stress
d20
post-
stress
LD
D
post-
stress
DD

Figure 2

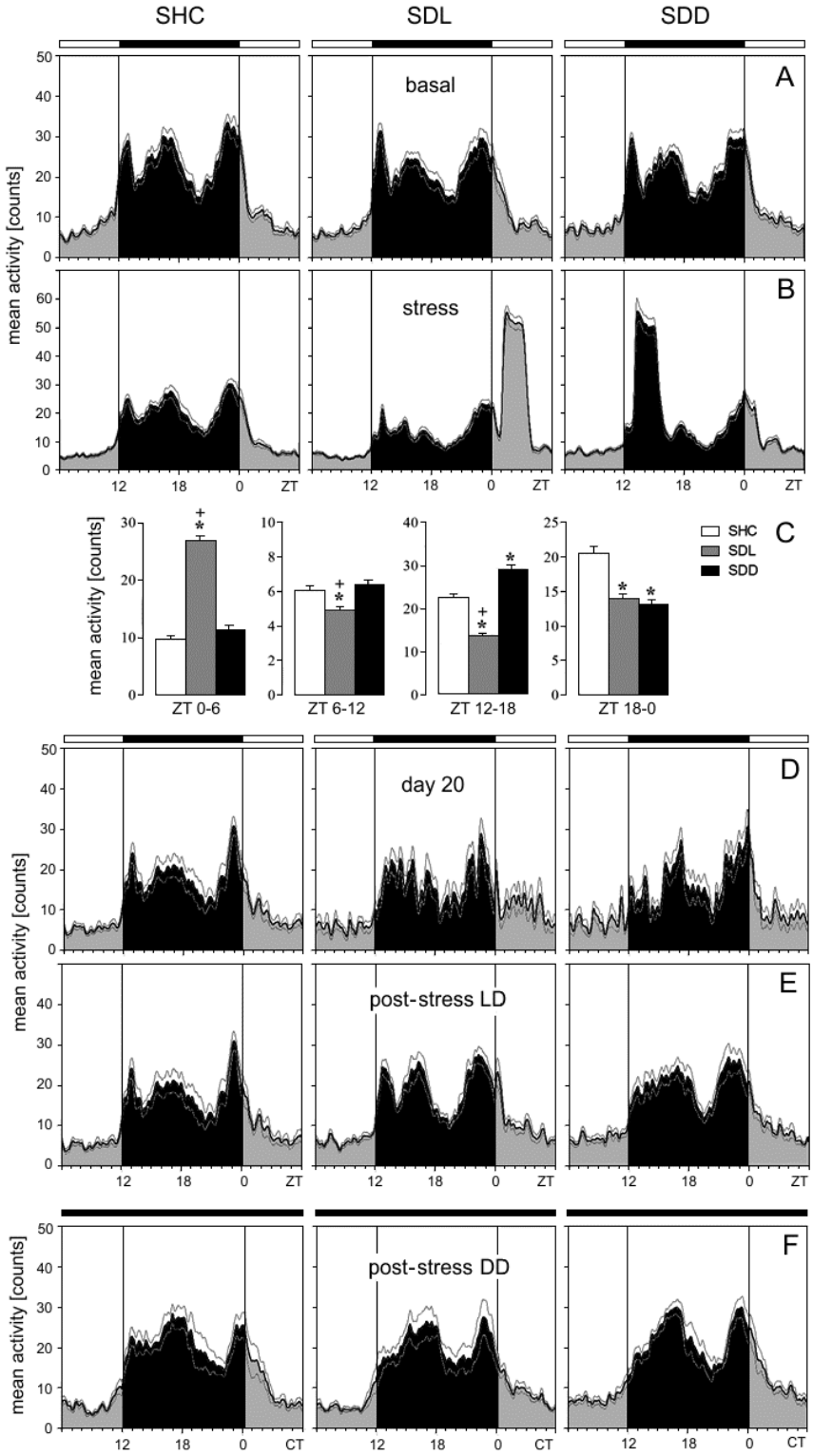

SHC
SDL
SDD
basal
A
stress
B
mean activity [counts]
ZT
C
SHC
SDL
SDD
ZT 0-6
ZT 6-12
ZT 12-18
ZT 18-0
day 20
D
post-stress LD
E
post-stress DD
F
CT

Figure 3

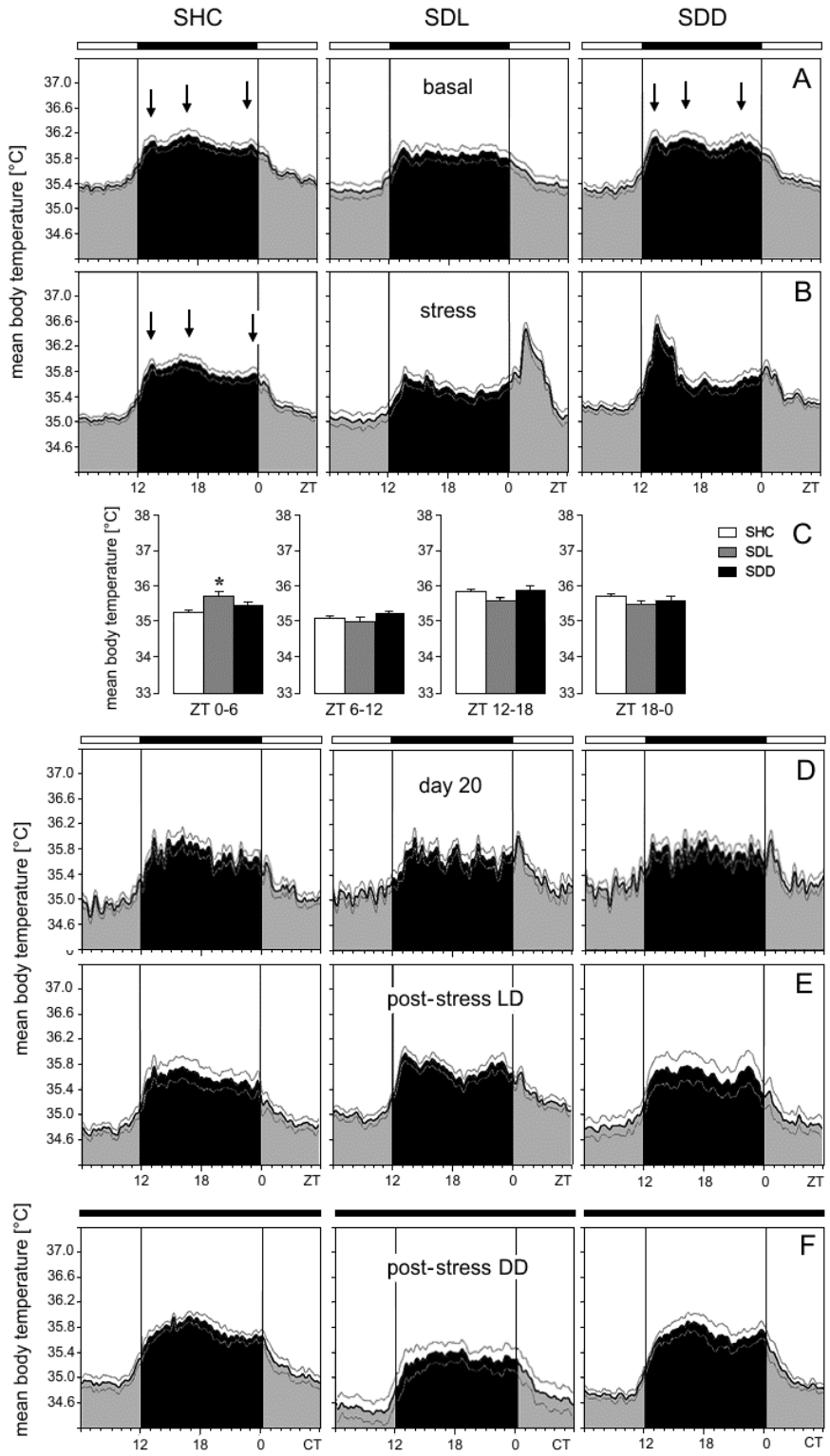

SHC
SDL
SDD
basal
A
stress
B
mean body temperature [°C]
37.0
36.6
36.2
35.8
35.4
35.0
34.6
12
18
0
ZT
C
SHC
SDL
SDD
ZT 0-6
ZT 6-12
ZT 12-18
ZT 18-0
38
37
36
35
34
33
*
day 20
D
post-stress LD
E
post-stress DD
F
CT

Figure 4

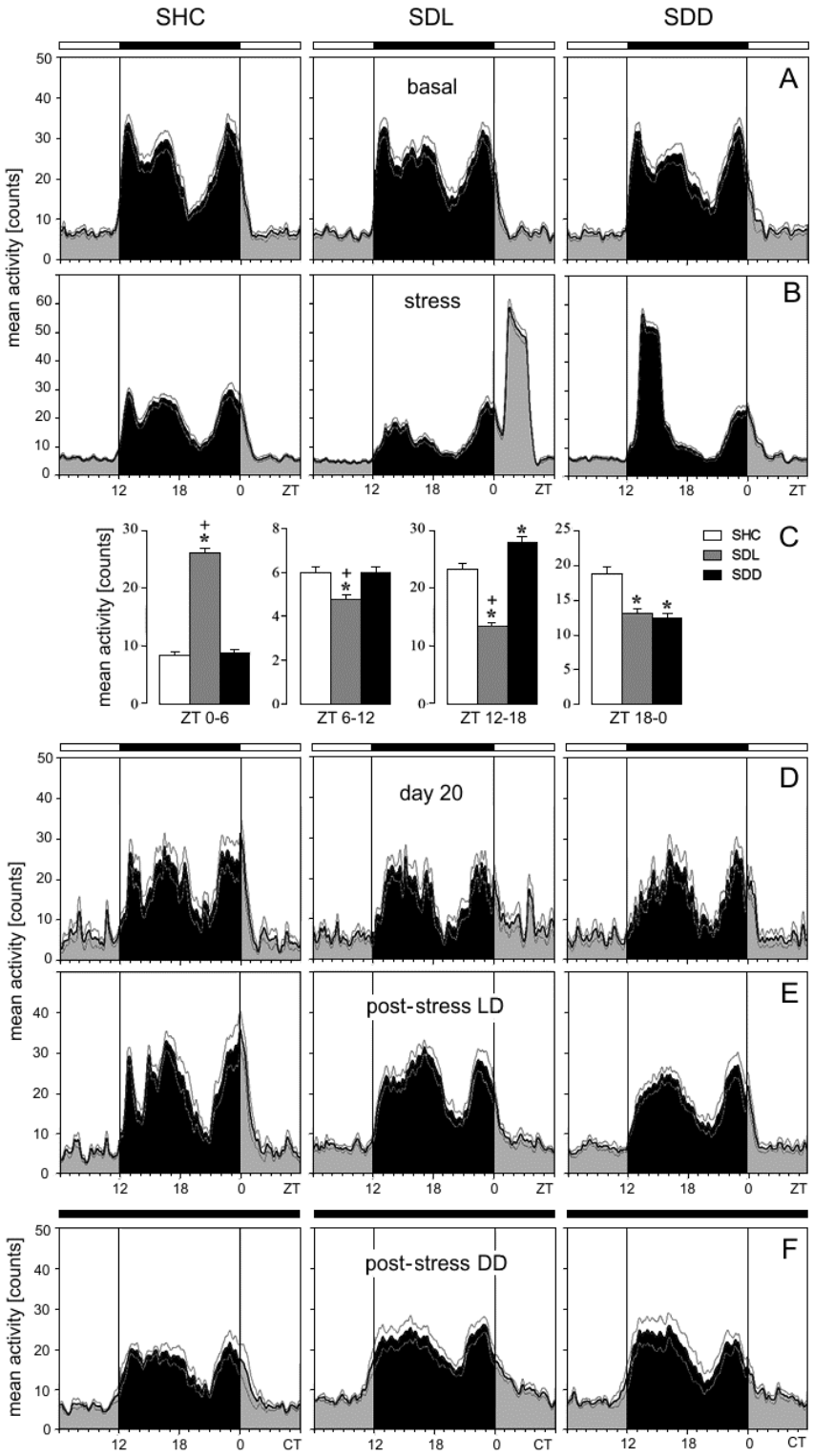

SHC
SDL
SDD
basal
A
stress
B
mean activity [counts]
ZT
C
SHC
SDL
SDD
ZT 0-6
ZT 6-12
ZT 12-18
ZT 18-0
day 20
D
post-stress LD
E
post-stress DD
F
CT

Figure 5

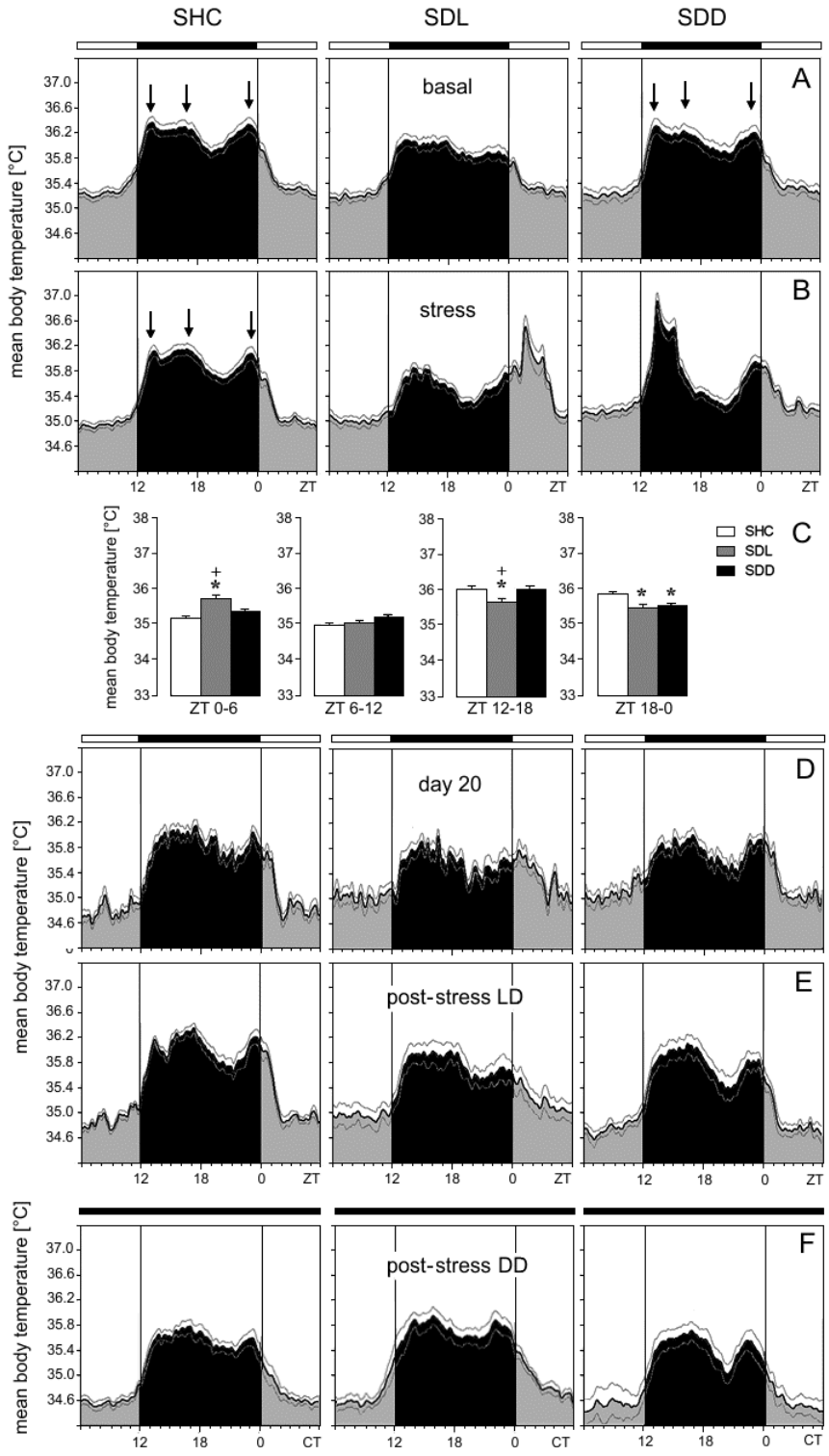

SHC
SDL
SDD
basal
A
stress
B
mean body temperature [°C]
37.0
36.6
36.2
35.8
35.4
35.0
34.6
12
18
0
ZT
C
SHC
SDL
SDD
ZT 0-6
ZT 6-12
ZT 12-18
ZT 18-0
day 20
D
post-stress LD
E
post-stress DD
F
CT

Figure 6

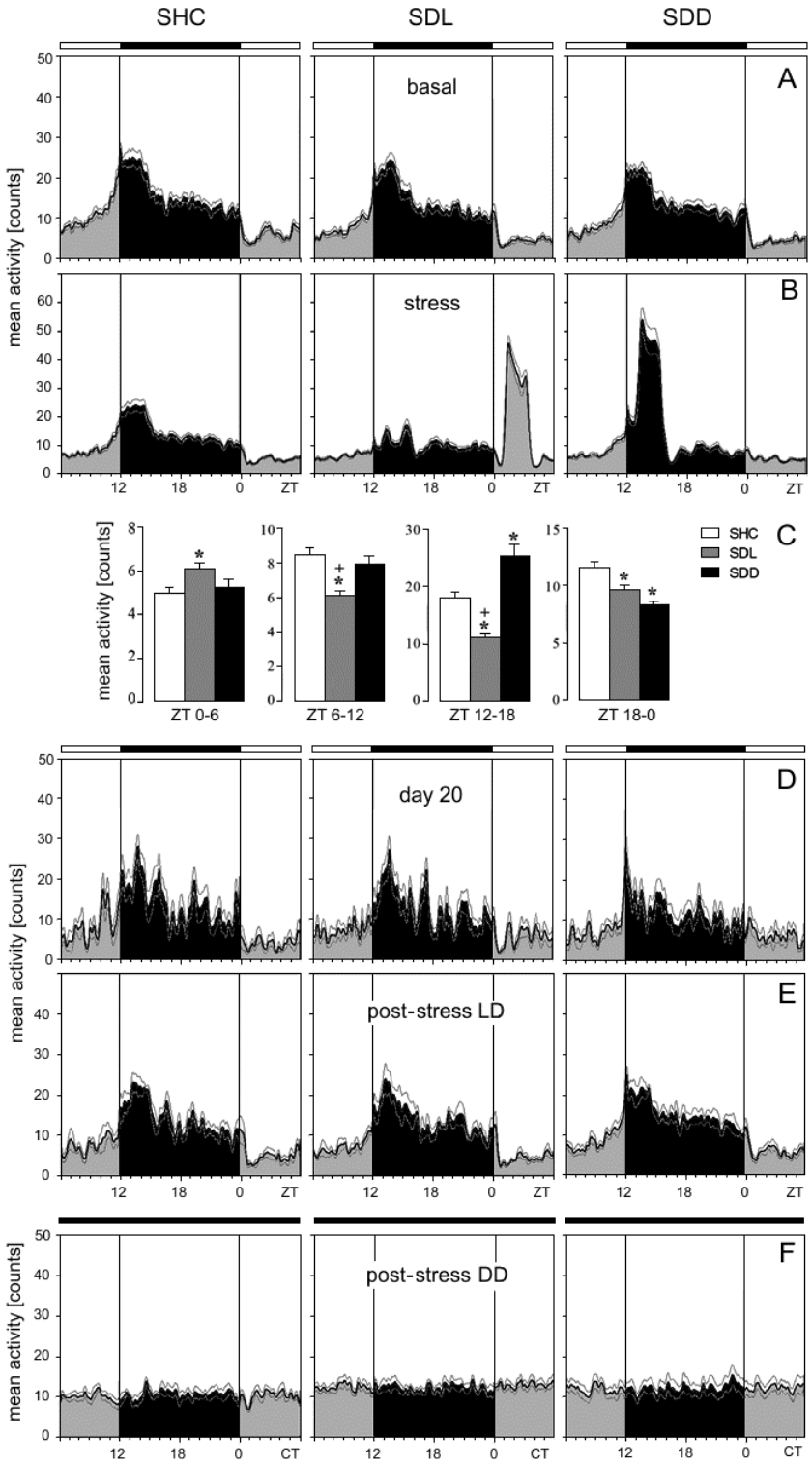

SHC
SDL
SDD
basal
A
stress
B
mean activity [counts]
ZT
C
SHC
SDL
SDD
ZT 0-6
ZT 6-12
ZT 12-18
ZT 18-0
day 20
D
post-stress LD
E
post-stress DD
F
CT

Figure 7

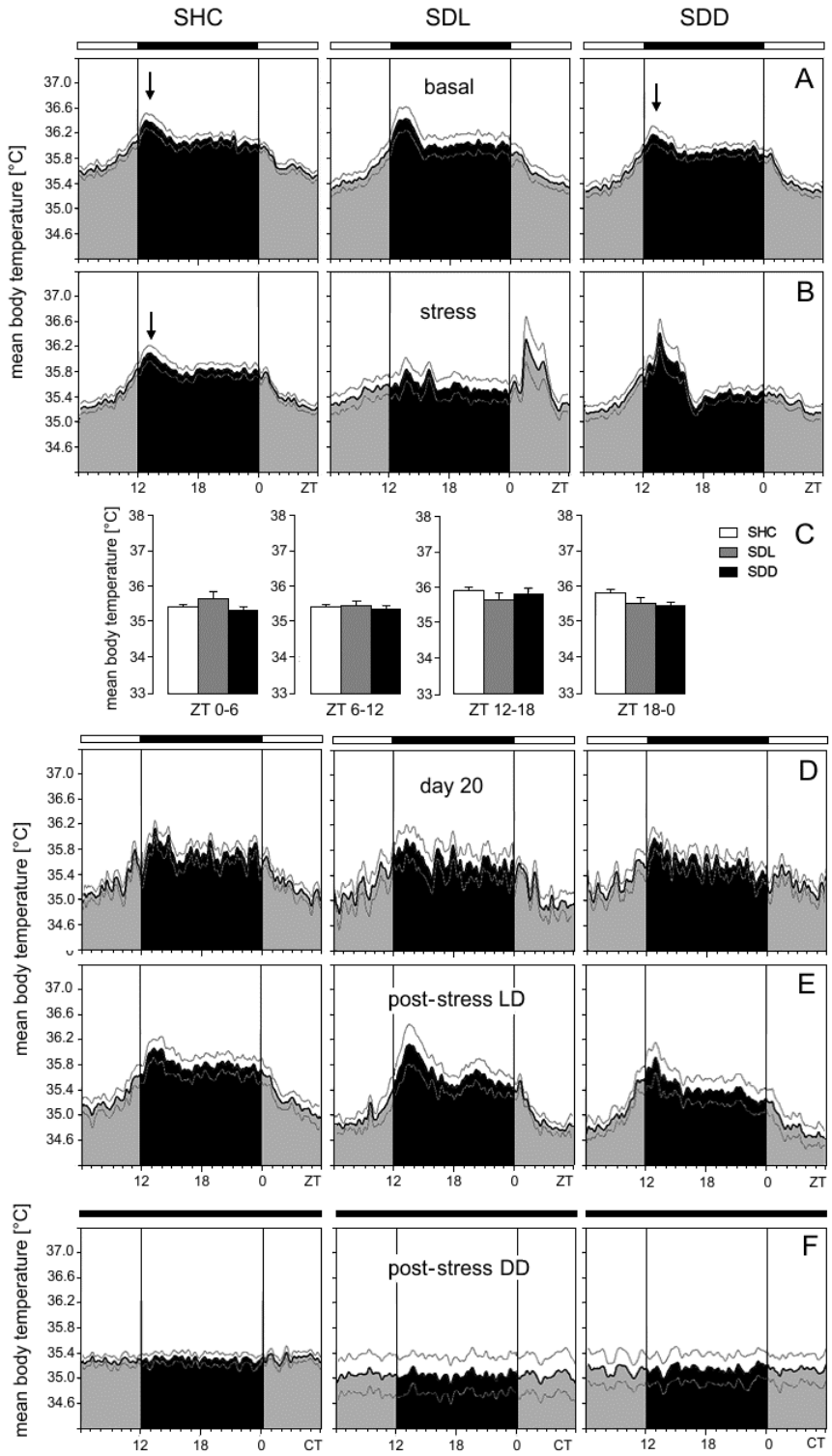
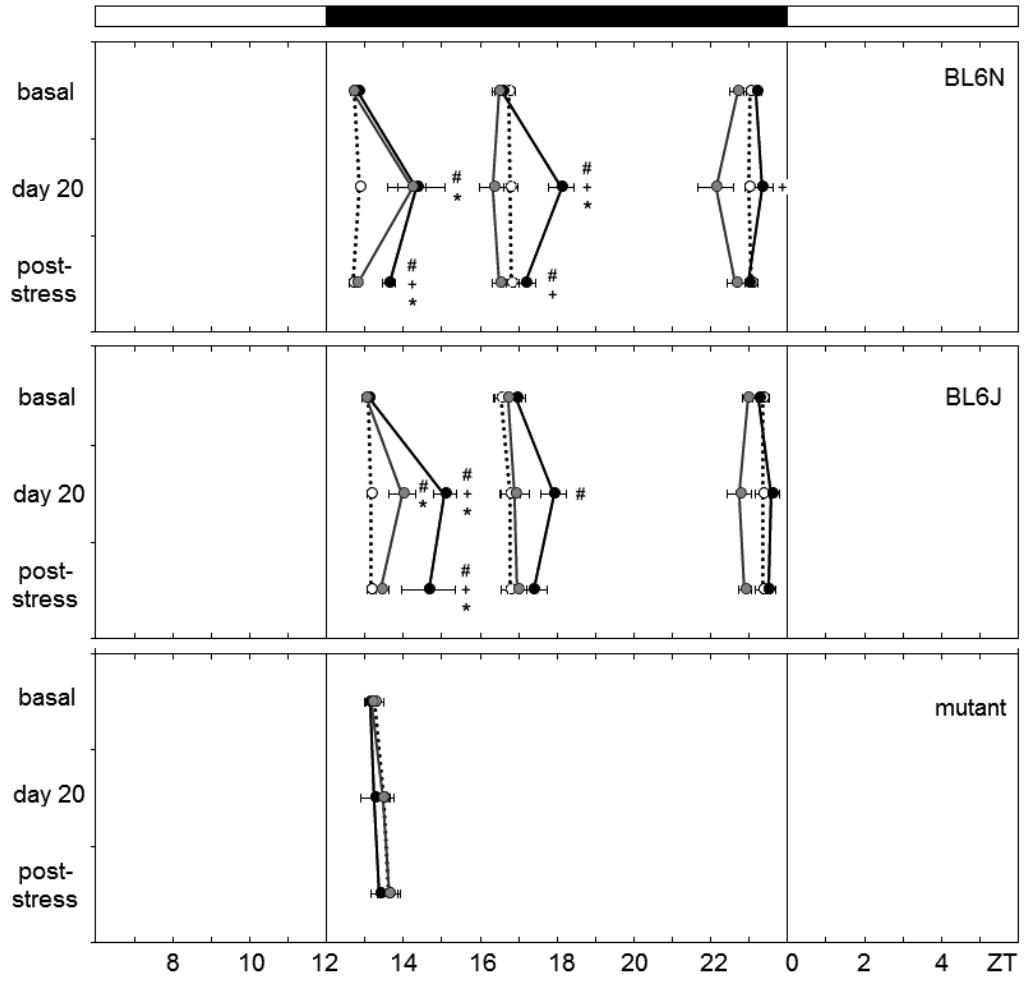

SHC
SDL
SDD
basal
A
stress
B
mean body temperature [°C]
37.0
36.6
36.2
35.8
35.4
35.0
34.6
12
18
0
ZT
C
SHC
SDL
SDD
38
37
36
35
34
33
ZT 0-6
ZT 6-12
ZT 12-18
ZT 18-0
day 20
D
post-stress LD
E
post-stress DD
F
CT

Figure 8


basal
day 20
post-
stress
BL6N
basal
day 20
post-
stress
BL6J
basal
day 20
post-
stress
mutant
8
10
12
14
16
18
20
22
0
2
4
ZT

APPENDIX

ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
ANS	Autonomic nervous system
APS	Ammonium Persulfate
ARC	Arcuate nucleus
AVP	Arginine-Vasopressin
AVP-R1b	Arginine-Vasopressin type 1b receptor
BCA	Bicinchoninic Acid
BDNF	Brain-derived neurotrophic factor
Bmal1/BMAL1	Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (italic: gene, capital: protein)
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid binding globulin
CCGs	Clock-controlled genes
Clock/CLOCK	Circadian locomotor output cycles kaput (italic: gene, capital: protein)
CNS	Central nervous system
CORT	Corticosterone
CRE	Cyclic adenosine monophosphate responsive elements
CREB	Cyclic adenosine monophosphate responsive elements binding protein
CRH	Corticotropin-releasing hormone
CRH-R1	Corticotropin-releasing hormone type 1 receptor
Cry/CRY	Cryptochrome (italic: gene, capital: protein)

DD	Constant darkness
DMEM	Dulbecco's modified eagle's medium
DMH	Dorsomedial hypothalamus
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GABA	γ-aminobutyric acid
GC	Glucocorticoid
GHT	Geniculohypothalamic tract
GR	Glucocorticoid receptor
GRE	Glucocorticoid-responsive element
h	Hours
HMGCR	Hydroxymethylglutaryl coenzyme A reductase
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
HSL	Hormone sensitive lipase
IFN-γ	Interferon-γ
IGL	Intergeniculate leaflets
IHC	Immunohistochemistry
IML	Intermediolateral column
ISH	In situ hybridization
LC	Locus coeruleus
LD	Light-dark
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LSD	Least significant difference

Mc2r	Melanocortin-2 receptor
MDD	Major depressive disorder
min	Minutes
MP	Milk powder
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NE	Norepinephrine
PACAP	Pituitary adenylate cyclase-activating protein
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline/0.1 % Tween-20
Per/PER	Period (italic: gene, capital: protein)
PER2::LUC	PERIOD2::LUCIFERASE
PFA	Paraformaldehyde
PVN	Paraventricular nucleus of the hypothalamus
REV-ERB	Reverse erythroblastosis virus
RHT	Retinohypothalamic tract
rm ANOVA	Analysis of variance for repeated measures
Ror/ROR	Retinoic acid receptor-related orphan receptor (italic: gene, capital: protein)
RT	Room temperature
SCN	Suprachiasmatic nucleus
SD	Social defeat
SDD	Social defeat dark
SDL	Social defeat light
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean

SHC	Single-housed control
SNS	Sympathetic nervous system
SON	Supraoptic nucleus
SR-B1	Scavenger receptor class B type 1
StAR	Steroidogenic acute regulatory protein
subPVZ	Sub-paraventricular zone
TBS	Tris buffered saline
TBS-T	Tris buffered saline/0.1 % Tween-20
TEMED	Tetramethylethylenediamine
TrkB	Tropomyosin-related kinase B
VBS	Visible burrow system
VIP	Vasoactive intestinal peptide
vs	Versus
WT	Wildtype
ZT	*Zeitgeber* time

ACKNOWLEDGEMENTS

The present thesis has been conducted between January 2011 and October 2014 at the Department of Neurobiology and Genetics at the Julius-Maximilians-Universität Würzburg. This doctoral thesis would not have come to a successful completion without the support, suggestions, and the patience of many people, whom I would like to thank in the following.

Foremost, I would like to thank Prof. Dr. Charlotte Förster for being my thesis supervisor, for enabling me to conduct this interesting thesis project, and for her input and support during the past three years. On the other hand she gave me freedom to plan and perform my experiments without any pressure, which I appreciated a lot.

I also would like to thank Prof. Dr. Inga Neumann for giving me the opportunity to do the experimental part of this PhD thesis at her institute and for her input and scientific guidance.

I am very grateful to PD Dr. Angelika Schmitt for being the second supervisor of this doctoral thesis and for fruitful discussions at our meetings as well as to PD Dr. Gabriella Lundkvist for being my external supervisor, who welcomed me in her kind group at the Karolinska Institutet in Stockholm, where I could stay several awesome months. When I became too serious, her humor and friendly irony allowed me to laugh and lightened my perspective (swedish style!). Tack för ditt akademiska stöd och din personliga hjälp, Hurz.

I especially want to thank my advisor on the spot, Prof. Dr. Stefan Reber, who took care of me from the very beginning. He has actively been interested in my work and

has always been available to advise me. I am very grateful for his motivation, enthusiasm, and vast knowledge in the stress field that, taken together, made him a great mentor.

I would like to express my gratitude to Prof. Dr. Henrik Oster for the productive scientific cooperation and valuable discussions.

Thanks to PD Dr. David Slattery who has been supportive with many scientific advices and insightful discussions.

Special thanks to my former colleagues Dr. Andrea Füchsl, Dr. Clara Perani, Dr. Sebastian Peters, Dr. Nicole Uschold-Schmidt, Dr. Daniela Beiderbeck, Daniel Peterlik, and Dominik Langgartner for their support on various technical issues, especially during late night or whole night experiments ☺

I would like to thank the technicians Rodrigue Maloumbi, Gabriele Schindler, Martina Fuchs, Andrea Havasi, and Nicole Ritter for their great technical assistance.

Further, I would like to acknowledge PD Dr. Oliver Bosch, Stefan Buchhauser, and Nils Menninger for their constant help with electronics and software.

I also thank Angelika Kühn for valuable assistance in setting up the transponder room.

Great thanks to all the coauthors for their contributions to the publications included in this doctoral thesis; and to all the Bachelor/Master students for excellent experimental assistance, namely Lisa Lucke, Marc Stieglitz, Lukas Onderka, Kerstin Altmann, and Johanna Zöller.

I would like to thank all the people at the Department of Behavioral and Molecular Neuroendocrinology at the University of Regensburg for the pleasant working

atmosphere and the support in every aspect, especially Dr. Iulia Zoicas, Dr. Clara Perani, Dr. Sebastian Peters, Doris Bayerl, and Stefanie Klampfl.

I am also grateful to my ex-colleagues in Würzburg. Thanks to Dr. Christiane Hermann-Luibl for providing accommodation and answering all my questions related to thesis submission, Dr. Dirk Rieger for handling my ordering, and Irina Wenzel for competent assistance in any issue.

To Dr. Ingrid Wanninger I would like to express my thanks for giving me the opportunity to start working before my PhD defense and for proofreading; I also thank Gerda Klinger for proofreading and the whole FoKo team for their understanding and consideration in the last past months.

I also thank my dear friends for listening, offering me advice, and always supporting me, especially Stefanie Windschüttl, Michaela Voss, and Elisabeth Eichlseder.

Warm acknowledgements go to my parents and my sister Simone for their unfailing support at any time and for always being there for me; and to my partner and best friend, Andreas, for his patience, support, and encouragement, especially in the last exhausting months. He has taken care of whatever needed to be done without complaining, just that I could focus on completing my dissertation. I am very grateful to have you in my life.

Finally, I would like to thank the Deutsche Forschungsgemeinschaft (DFG) for three-year financial support.

CURRICULUM VITAE

DECLARATIONS

"Dissertation Based on Several Published Manuscripts"

Statement of individual author contributions and of legal second publication rights

Publication (complete reference): **Manuela Slavica Bartlang**, Inga Doris Neumann, David Anthony Slattery, Nicole Uschold-Schmidt, Dominik Kraus, Charlotte Helfrich-Förster, Stefan Oskar Reber. (2012) Time matters: pathological effects of repeated psychosocial stress during the active, but not inactive, phase of male mice. J Endocrinol 215(3): 425-437					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	S. O. R.	D. A. S.	C. H-F.	I. D. N.	**M. S. B.**
Data Collection	**M. S. B.**	D. K.	N. U-S.		
Data Analysis and Interpretation	**M. S. B.**	S. O. R.	D. A. S.	C. H-F.	
Manuscript Writing	**M. S. B.**	S. O. R.	D. A. S.		

Publication (complete reference): **Manuela Slavica Bartlang**, Sergey Alexandrovich Savelyev, Anne-Sofie Johansson, Stefan Oskar Reber, Charlotte Helfrich-Förster, Gabriella Birgitta Lundkvist. (2014) Repeated psychosocial stress at night, but not day, affects the central molecular clock. Chronobiol Int 31(9): 996-1007					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	G. B. L.	**M. S. B.**	C. H-F.	S. O. R.	
Data Collection	**M. S. B.**	S. A. S.	G. B. L.		
Data Analysis and Interpretation	**M. S. B.**	S. A. S.	G. B. L.	C. H-F.	S. O. R.
Manuscript Writing	**M. S. B.**	G. B. L.	S. O. R.		

Explanations (if applicable): Equal contribution first author: M. S. B. and S. A. S.; equal contribution last author: C. H-F. and G. B. L.

Publication (complete reference): **Manuela Slavica Bartlang**, Henrik Oster, Charlotte Helfrich-Förster. (2014) Repeated psychosocial stress at night, but not day, delays the entrained activity rhythm of male mice, *submitted*					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	**M. S. B.**	C. H-F.	H. O.		
Data Collection	**M. S. B.**				
Data Analysis and Interpretation	**M. S. B.**	C. H-F.			
Manuscript Writing	**M. S. B.**	C. H-F.			

Explanations (if applicable): Manuscript was submitted to Chronobiology International.

I confirm that I have obtained permission from both the publishers and the coauthors for legal second publication.
I also confirm my primary supervisor's acceptance.

Manuela Bartlang 18. Oct 2014 Regensburg

_____

Doctoral Researcher's Name Date Place Signature

Affidavit

I hereby confirm that my thesis entitled “Timing is everything: The interaction of psychosocial stress and the circadian clock in male C57BL/6 mice” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Regensburg, 18. October 2014

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation “Auf das richtige Timing kommt es an: Die Interaktion zwischen psychosozialem Stress und der inneren Uhr in männlichen C57BL/6 Mäusen“ eigenhändig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegeben Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Regensburg, 18. Oktober 2014

Ort, Datum Unterschrift